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Crop Production for Agricultural Improvement

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Chapter 1
Crop Improvement Through Different Means: Challenges and Prospects

Muhammad Ashraf, Muhammad Sajid Aqeel Ahmad, Münir Öztürk, and Ahmad Aksoy

Contents

Abstract In the recent years, the looming food scarcity problem has transformed plant sciences as an emerging discipline committed to devise new strategies for enhanced crop productivity. The major factors causing food scarcity are biotic and abiotic stresses such as plant pathogens, salinity, drought, flooding, temperature extremes, nutrient deficiency or excess, etc. which substantially limit crop productivity world-wide. In this scenario, such strategies should be adopted which may be

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employed to achieve maximum productivity and economic crop returns under such adversaries. Major strategies include pathogen/pest management practices, breeding of new crop varieties, screening and selection of existing crop gene pool, production of genetically modified (GM) crops, exogenous use of osmoprotectants and plant hormones, agronomic and soil reclamation practices, sustainable use of available water supplies, etc. In this book, we have mainly focused on physiological, biochemical, molecular and genetic tools for crop improvement under environmental adversaries. In addition, the adverse effects of different biotic (diseases, pathogens, etc.) and abiotic (salinity, drought, high and low temperatures, metals, etc.) stresses on crop development and the potential strategies to enhance crop productivity under such stressful environments have been critically discussed. Moreover, the role of nutrient, water and soil management in improving crop efficiency is also a part of this book.

Keywords Crop production • Food security • Crop improvement • Stress tolerance • Disease resistance

1 Introduction

The rapidly increasing human population is causing a number of challenges to sustain life on earth. For example, we are losing biodiversity, degrading environment, facing food scarcity, over-exploiting natural resources and performing activities that lead to increased levels of abiotic stresses in our environment. Among these, food scarcity is surely the largest issue that directly or indirectly relates to environmental issues. In this situation, it is imperative to keep updated ourselves with advances in plant production science to meet these scientific challenges and thus overcome the increasing food scarcity and sustain life on earth. For this purpose, we are in need to develop new high yielding and stress tolerant varieties, through modern biotechnological, molecular and genetic tools. We should have enhanced knowledge of stress tolerance mechanisms and should develop methodologies to overcome the stresses. We need to understand our environment and ecosystems in the changing environment and develop methodologies to conserve it. For this purpose, we invited a number of scientists worldwide to review the current scenario of the problems, current development and future prospects of the challenges and their solutions. Their contributions are compiled in this book that is a valuable contribution towards our struggle for improved crop production to meet the demands of the growing human population.

2 Human Population Growth

With the announcement of the United Nations on October 31, 2011 that the World's population has crossed 7 billion, the hard question raised in our minds is "will the current population growth rate be supported by carrying capacity of the earth?" It is a fact that currently, approximately 2.5 babies are being added to the world population per second (US Census Bureau 2010). The current growth rate of world's population is 1.8%.

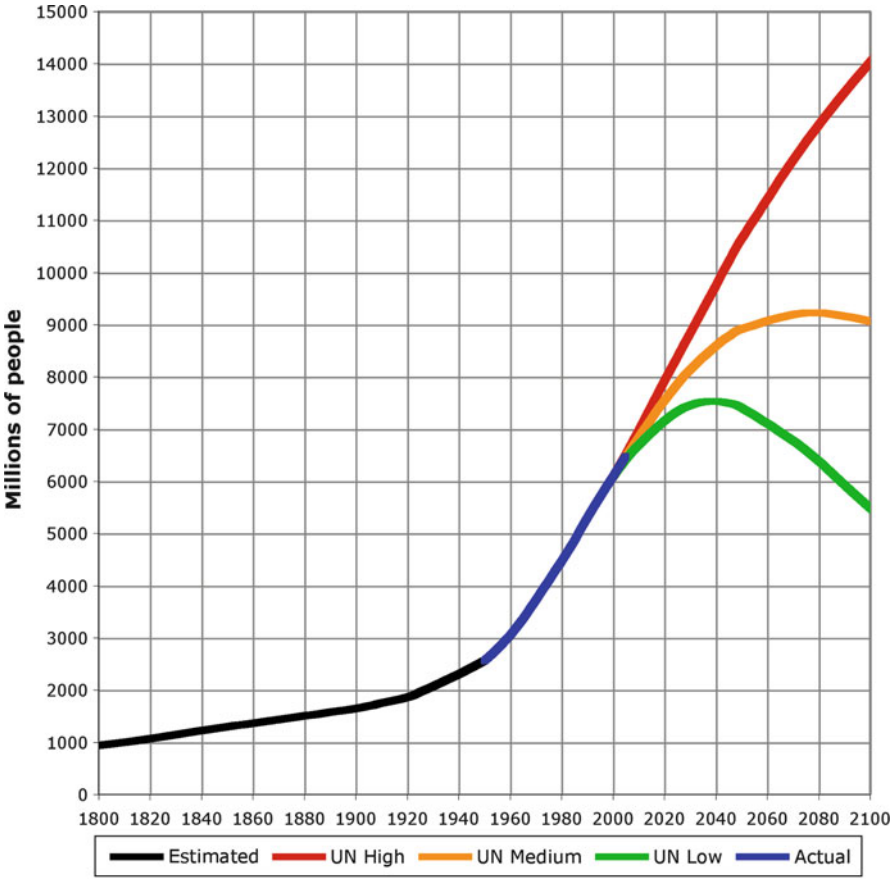

Fig. 1.1 The estimates of world population from years 1800 to 2100. The estimates are based on UN-2004 projections (*red*, *orange*, *green*) and US Census Bureau historical estimates (*black*). If the current growth rate continues, the world population will cross 14 billion at the end of twenty-second century that is almost double the current world population (7 billion) (Sources: UN 2004; U.S. Census Bureau 2010)

It is estimated that more than 120 million people will be added to the planet during the year 2011 while the deaths will be only about 70 million. So, there will be the addition of 50 million people to the planet this year (Population Institute 2011). The UN estimated that at the end of 2025 the human population will cross 8 billion while at the end of 2050 it could be over 10 billion. At the end of twenty-second century, the human population is projected to cross 14 billion (Bongaarts 1997; United Nations 2004). Where the most rapid growth would be? It is estimated to be in Asia and Africa, which will be the most crowded continents on earth. It is estimated that by the year 2025, out of 8 billion world's population, 6 billion will be living only in Asia and Africa. In comparison, the developed countries will be experiencing near zero population growth. Thus, at the end of year 2025, approximately 80% of world population will be living in under-developed countries of Asia and Africa (Fig. 1.1).

3 Global Demands for Food Supply

It is claimed that the food production on the globe is enough to support the world's current population. However, the fact is that a large proportion of the population is still starved. Since the start of twenty-first century, the proportion of malnourished people has been reported to be almost halved in the past 40 years. Nevertheless, recent estimates indicate that the proportion of malnourished/starved people is once again steadily increasing. For example, 843 million people under- or mal-nourished in 1990–1992 increased to 923 million in 2007 (FAO, 2010). In 2009, this figure further increased to 1023, while a little decrease in this figure was reported in 2010 as 925 million under- or mal-nourished people in the world. This shows that the share of malnourished/starved people in the world has steadily increased during the past two decades. If the current trend continues, one can easily estimate the situation of food supply in near future particularly in the developing countries (Figs. 1.2 and 1.3).

Although the statistics presented by FAO indicates that the proportion of hungry people has decreased significantly at the global scale, it is a fact that every day, almost 16,000 children (one child every 5 s) die from nutrition-related causes. According to another estimate, nearly 9 million children died before they reached their fifth birthday only in the year 2008. One third of these deaths were due directly or indirectly to hunger and malnutrition. Most of these deaths occurred in Asia Pacific and African countries including Chad, Congo, Ethopia, Niger and India (Fig. 1.4).

4 Global Food Production

Although, there are 250,000–300,000 known plant species on planet earth, only 150–200 of these are used by humans for dietary purposes. About 75% of the world's food is generated from only 12 plants and 5 animal species (FAO 1999a). Among these, only three crops (rice, maize and wheat) contribute ~60% of calories and proteins obtained by humans from plants, while animals provide about 30% of human requirements for food and agriculture (FAO 1999b). The food production is steadily increasing with the demand. For example, during the year 2011 a record production of cereal grains (2,325 million tonnes) has been estimated by FAO that is 3.7% more than that in the year 2010. Thus, about 507 million tonnes cereal crops have been estimated to be in stock in 2011 by FAO. Overall, there is an increase of 6.0% in wheat, 2.6% growth in the coarse grains and a 3.4% rise for rice production has been estimated by FAO during the year 2011 (FAO 2011a). Nevertheless, a question arises as to whether the people are still hungry worldwide? This is due to the reason that most of the food production is in developed countries, while developing countries experience less increase in food production, resulting in food scarcity and hunger related issues in these regions. Secondly, the World Bank estimates that the increase in global food prices in 2008, accompanied by a global economic

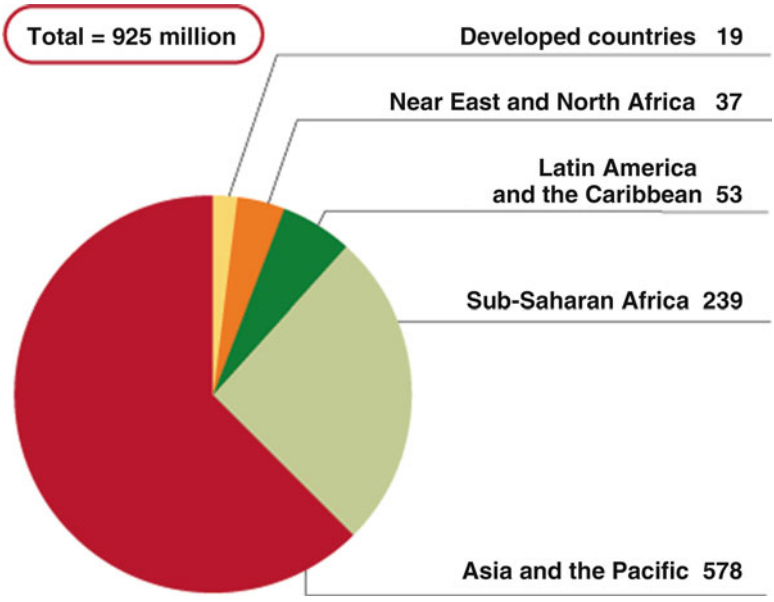

Fig. 1.2 The distribution of hungry people-world wide during 2001. Note that out of 925 million people worldwide, the largest proportion of malnourished people is in the Asia and the Pacific (578 million) followed by Sub-Saharan Africa. Here, the cause of hunger in Asia and the Pacific is population explosion while in the Sub-Saharan Africa is environmental extremes. The least proportion is in developed countries (19 million) that experience almost zero-population growth (Source: World Hunger Education Service 2011; FAO 2010)

depression in 2009 and 2010 has pushed an additional 100–150 million people into poverty worldwide leading to increase in global hunger (Mitchell 2008; Bread for the World 2011) (Fig. 1.5).

5 Is the Population Explosion a Real Problem?

The rapid growth in human population raises a serious question about environmental health and food security issues. The biggest question in our minds is that will the earth be able to support 14 billion people in the year 2100, the population double to the present day (7 billion), with the same limited resources of the present day? Will our requirements of food, health and education and residence be met? Shall we be able to sustain our renewable and non-renewable resources? What would be the situation of biodiversity and croplands? Will our future generations be supplied with clean water and air? Indeed, the policy-makers, economists and ecologists are worried by this situation as these things seem hard to sustain in future, particularly in the developing countries of Asia and Africa.

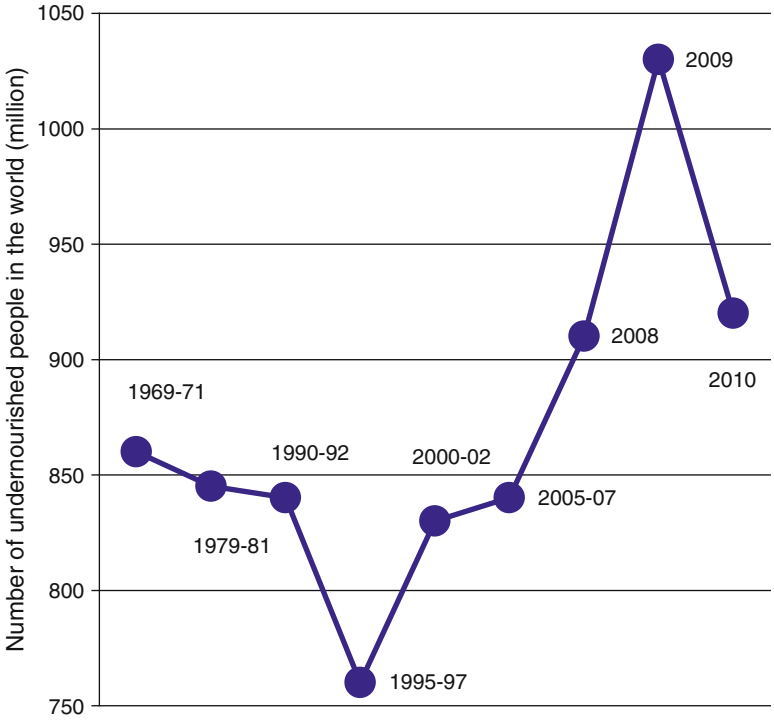

Fig. 1.3 The extent of hungry people from 1969 to 2010. There was a slight decrease in global hunger from 1969 to 1997. However, afterwards, a rapid increase is reported, although there is again a decline in global hunger in 2010 (Sources: World Hunger Education Service 2011; FAO 2010)

The fact is that currently, approximately 434 million people live in areas of either extreme water stress or scarcity. It is estimated that depending on future trends of human population growth, in the year 2025 approximately 2.6–3.1 billion people will be living in areas of water-scarcity (Valerio 2008; US Department of State 2006). Similarly, approximately 600–986 million people will be living in regions where cultivated land will become critically scarce in 2025. Despite the improvement in crop production after Green Revolution aided by technological advances, agricultural experts are worried. The debate is how long crop production will be enough to feed increasing human population. In future, the crops will be produced mostly from today's cropland. Therefore, our current croplands must remain fertile to sustain food production. The minimum amount of land needed to provide the vegetarian diet for one person without the input of any artificial chemical fertilizer and loss of soil nutrients is 0.07 ha. Currently, 415 million people already live in countries having land less than required for a person for this purpose (Population Action International 2011).

Our forested lands are also becoming critically scarce. It is estimated that currently, more than 1.8 billion people live in 36 countries where the forested land is less than

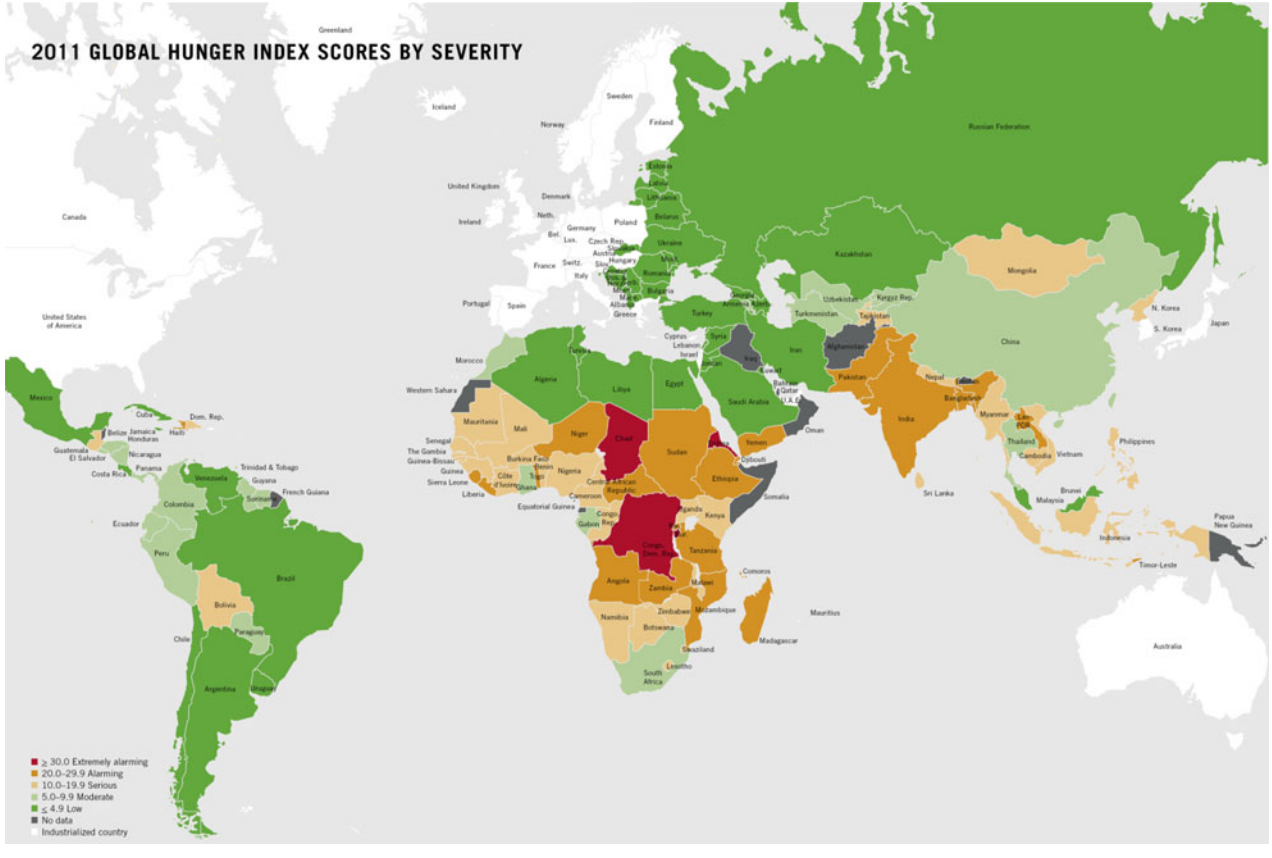

Fig. 1.4 The distribution of global hunger by country (Source: International Food Policy Research Institute 2010; http://www.ifpri.org/ifprimaps/index.php/ghi/v2010)

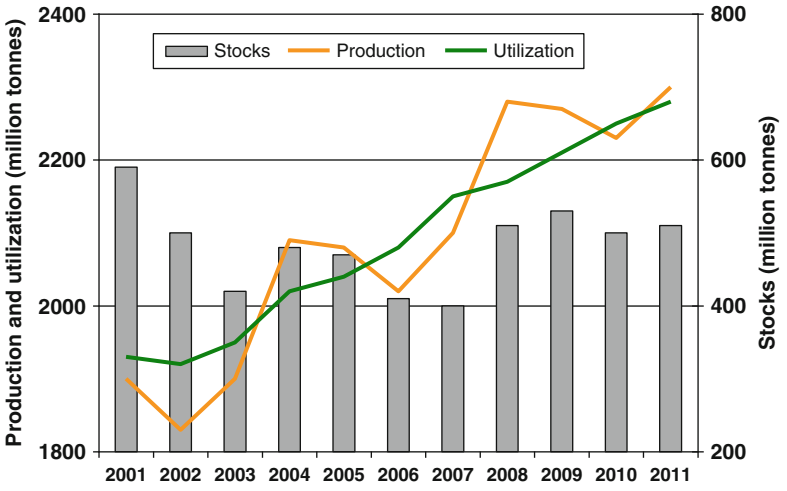

Fig. 1.5 World cereal production and utilization during 2001–2011 (Source: FAO 2011a; http://www.fao.org/worldfoodsituation/wfs-home/csdb/en/)

0.1 ha per capita. This indicates a critically low level of forest cover in these regions. Based on the current deforestation trends and medium population projection, approximately 3 billion people (double to present day) will be living in countries having critically scarce forest land in 2025. At present, more than 1.1 billion people live in biodiversity hot-spots. These regions comprise about 12% of the earth dry-land wherein about 20% of human population is currently living. The alarming fact is that in comparison to the annual growth rate of world's population (1.3%), the growth rate in these biodiversity hotspots is 1.8%, pushing the regions under pressure. Thus, these regions are under severe threat by human activities (Population Action International 2011).

Despite an increase in global crop production is claimed by the FAO, the prices of food commodities have reached to a historical high limit in the year 2011. The average Food Price Index (FPI), a measure in the inflation of food prices, was approximately 100 during 2002–2004. With a consistent increase in the later years, it is now estimated to be more than 200 in the year 2011 indicating that the global food prices have bloomed almost double within only 6 years (FAO 2011b). This has no doubt pushed more people in poverty and made the nutrition related issues more severe (Fig. 1.6).

6 Challenges for Sustainable Crop Production

Currently, the crop production world-wide is facing a number of challenges. These, include, environmental constraints, diseases and pathogens, loss of genetic diversity, and global climate change. Among the abiotic stresses, drought is the most important and most common limitating factor of crop production in arid and semi-arid regions

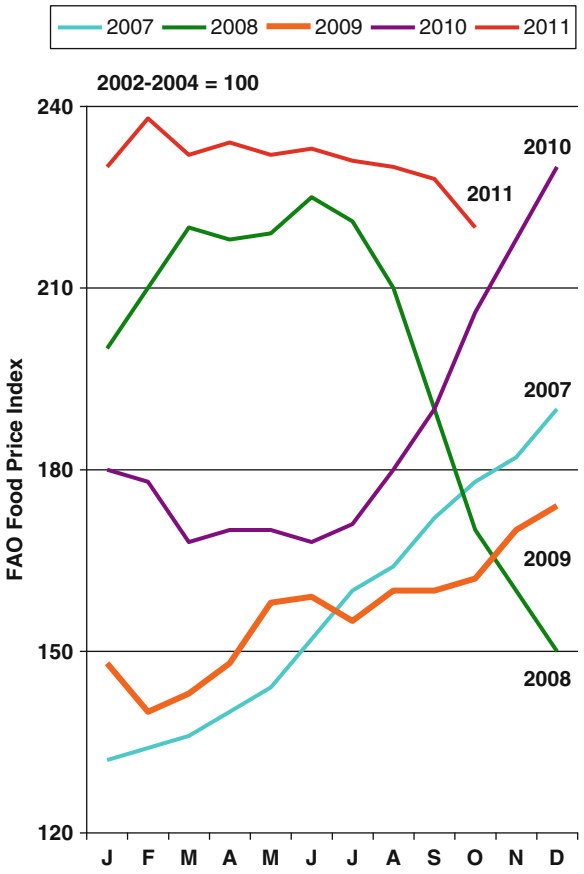

Fig. 1.6 FAO Food Price Index (FPI) from 2002 to 2011. The highest FPI can be seen for the year 2011 (Source: FAO 2011b)

of the world (Saranga et al. 2001). It is estimated that more than 1/4 of total land area is dry and about 1/3 of the world's cultivable land is under water shortage conditions (Kirigwi et al. 2004). The crop quality and production is also seriously influenced by global climatic changes which enhance the frequency and intensity of water shortage thereby making the situation more serious (Hongbo et al. 2005).

Salt stress is the second most prevalent abiotic stress in the world that adversely impacts plant growth (Pessarakli 1991). It is estimated that over 800 million hectares are salt affected in the world either by salinity (397 Mha) or sodicity (434 Mha) which is over 6% of the total land area in the world (FAO 2005). Most of the salinity and all of the sodicity is natural; however, a significant proportion of recently cultivated land has become saline because of land clearing and irrigation. The United Nations Environment Programme (UNEP) and Food and Agriculture Organization (FAO) have estimated that approximately 45 Mha out of 230 Mha of irrigated land in the world are salt affected (FAO 2005). Approximately, 10 Mha of the irrigated land is forced out of cultivation every year due to high salinity (Szabolcs 1989) and one third to half of the irrigated land may be heading towards this fate (Nelson et al. 1998).

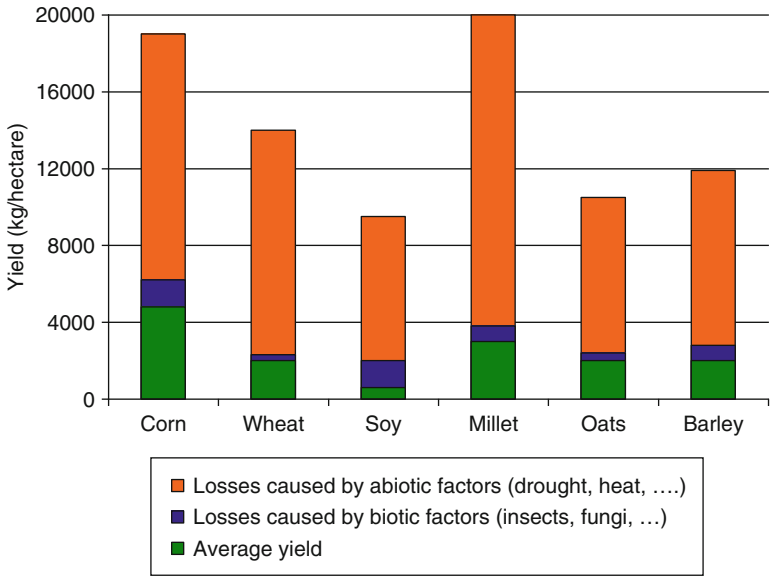

Fig. 1.7 Estimated crop losses due to biotic and abiotic stresses (Bayer Crop Science 2008, http://www.seedquest.com/News/releases/2008/october/23973.htm)

High temperature stress is another major factor that significantly affects plant productivity particularly in arid zones (Bray et al. 2000). Heat stress or heat shock, caused by rise in ambient temperature beyond a threshold level, is a major threat to crop production worldwide (Hall 2001). In general, heat stress is considered when temperature elevates 10–15°C above ambient temperature. However, the probability of its occurrence depends on period of high temperatures occurring during the day and/or the night. Elevated temperatures may lead to alteration in geographical distribution as well as also result in altered growing season of agricultural crops, allowing crop maturity to reach earlier by causing threshold temperature for the start of the season (Porter 2005). Intergovernmental Panel on Climatic Change (IPCC) has estimated that global mean temperature will rise 0.3°C per decade (Jones et al. 1999) and this will reach to 1°C and 3°C by years 2025 and 2100, respectively. The situation becomes worse when heat stress usually combines with drought and salinity stresses, further impeding crop production worldwide.

Other problems of relatively less intensity that hinder crop production include, environmental pollutants such as heavy metals, pesticides, fertilizers, petroleum products, and other organic and inorganic chemicals. Soil mismanagement and loss of soil fertility due to excessive cultivation of crop is also threatening the crop production worldwide. In addition to all these abiotic stresses, biotic stresses such as diseases, pests and pathogens also contribute significantly towards crop losses worldwide, though their contribution is significantly less than that of abiotic stresses (Fig. 1.7).

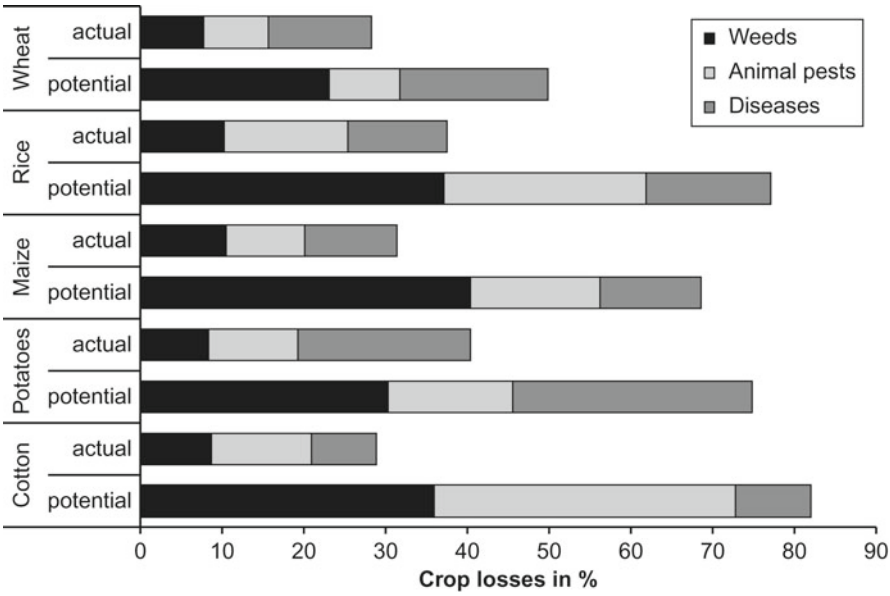

Fig. 1.8 Estimated global crop losses in major crops due to pests and pathogens (Qaim 2011)

7 Crop Losses Due to Biotic and Abiotic Factors

It is estimated that abiotic and biotic stresses collectively contribute more than 50% crop losses worldwide. A survey conducted by Bayer in 2008 indicated that crop losses caused by stresses were significantly greater than the average yield of economically important crops (corn, wheat, soy, millet, oats and barley). They also showed that the abiotic stresses caused significantly higher crop losses than did the biotic ones. For example, the highest crop losses were shown for millet, a crop of the arid regions, where average yield was 2,000 kg/ha and crop losses were 3,800 and 20,000 kg/ha due to biotic and abiotic stresses, respectively. Similarly, the average yield of corn in 2008 was 4,500 kg/ha while the crop losses due to biotic and abiotic stresses were 6,000 and 19,000 kg/ha, respectively. The third highest crop losses were recorded for wheat, another economically crucial crop of third world countries. The average yield of wheat was approximately 1,500 kg/ha, while crop losses were 2,000 kg/ha due to biotic stresses and 14,500 kg/ha due to abiotic stresses. Almost a similar extent of crop losses due to abiotic and biotic factors was reported for barley, oats and soya crops. All these data indicate that crop losses due to abiotic stresses were more severe than those by the biotic ones (Fig. 1.7).

In another report, Qaim (2011) compared the crop losses due to various biotic agents such as disease, weeds and animal pests in five economically important crops, i.e. wheat, rice, maize, potatoes and cotton (Fig. 1.8). He showed that these biotic agents

collectively caused approximately 28–40% harvest loss in these economically important crops. Here, the highest crop losses were shown in potatoes (40%) followed by rice (38%) and maize (30%). The harvest losses in wheat and cotton were 28%. Among the biological agents, the highest contribution towards crop losses was by diseases, followed by animal pests and the least was due to weed competition (Fig. 1.8).

8 Strategies for Crop Improvement

In view of the situation prevailing for food security worldwide, it is amply clear that we need to devise concrete methodologies to increase average crop yield. At the first instance, we need to control haphazardly increasing human population so that pressure on our croplands for crop production could be reduced. Secondly, we need to combat environmental adversaries, a major reason of crop losses worldwide, by developing conventional and advanced methodologies. This can be achieved by water management, soil manipulation, nutrient management, screening and selection of the existing gene pool, conventional and molecular breeding, tissue culture, genetic transformations and molecular enhancements. Additionally, we have to manage crop losses arising from biotic agents through disease and pest management.

As discussed earlier, the impact of abiotic stresses on yield losses is more severe than that by the biotic ones. Therefore, we have to combat abiotic stresses in the first instance so as to fulfil our desire to increase crop productivity worldwide. Normally, it is achieved through conventional breeding and selection strategies to select tolerant varieties/lines. Although, such efforts have enduring impact, their development is usually slow and requires a considerable time to succeed (Witcombe et al. 2008). In the recent past, use of various molecular enhancements has shown a promising means to induce short-term resistance to abiotic stresses and have been summarized in various reviews (Ashraf and Foolad 2007; Alcázar et al. 2010; Ashraf 2009; Ashraf et al. 2011). More recently, genetic transformations have also been shown to be another effective and long lasting means to improve crop productivity under stress conditions (Cushman and Bohner 2000; Zhang et al. 2000; Vinocur and Altman 2005; Mittler and Blumwald 2010; Roy et al. 2011). All these reports indicate that there is still a potential to improve crop production under stress conditions in future to overcome the problem of food security of growing human population.

It is a fact that biotic stresses, although have comparatively less damaging impact on harvest losses, most of the genetic modifications to enhance crop productivity have been performed to confer resistance against biotic stresses. For example, Huang et al. (2002) compared the genetic modifications in crop plants against various stresses. They concluded that majority of genetic transformations have been performed for insect resistance (37%), herbicide resistance (29%), stalked traits

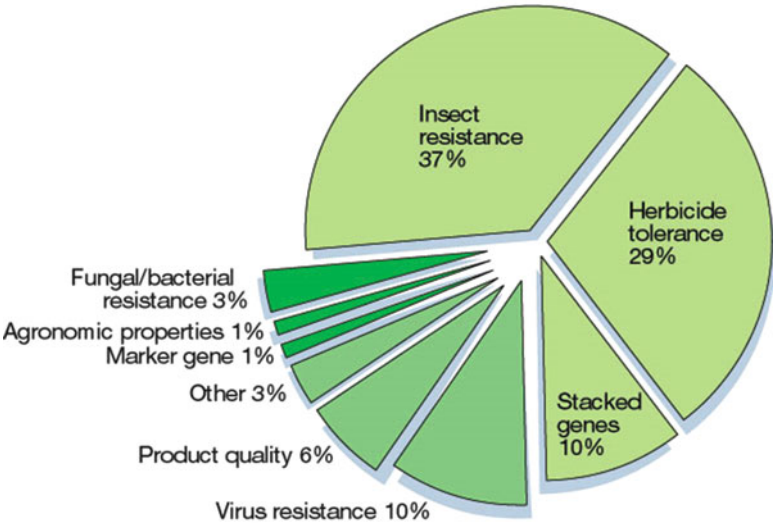

Fig. 1.9 Genetically modified crop traits tested in developed countries from 1987 to 2000 (After Huang et al. 2002)

(10%) and virus resistance (10%). In comparison, a little attention has been paid to agronomic properties (1%), marker genes (1%) and resistance against abiotic stresses that constitutes only 3% of all GM crops tested under field conditions (Fig. 1.9). This shows that we need to focus our efforts to develop GM crops that can perform better under field conditions against abiotic stresses, a major problem for crop production worldwide.

9 Conclusion

It is amply clear from the above discussion that we will be facing food security issues in near future particularly in the developing countries where most of human population will be living. Additionally, the increasing crop losses due to environmental adversaries will amplify food security issues. Majority of crops losses are due to abiotic stresses that cause more than 50% harvest loss. Although, scientists are working hard to increase the average yield of various economically important crop plants, a limited success is achieved due to the increasing extent of abiotic and biotic stresses. Therefore, there is a dire need to devise methodologies to enhance crop production particularly in the stressed-regions of the world.

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Part I
Breeding for Crop Improvement

Chapter 2
Bridging Genomic and Classical Breeding Approaches for Improving Crop Productivity

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Contents

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Abstract Numerous genomic tools have been used vigorously for studying the inherent genetic polymorphisms which were instrumental in resolving the phylogenies of many crop species, developing genetic maps, initiating marker assisted selection and incorporating genes from distantly related taxa-introduction of Bt genes in cotton, corn etc., these together set a stage for developing crop varieties with improved genetic potential to multiple stresses. Wider adaptation of genomic based breeding in crop improvement programs is impeded due to the narrow genetic base resulting from selection pressures applied during the domestication of many plant taxa, which also can confer genetic vulnerability to crop gene pools. Genomic based breeding may contribute to increasing crop genetic diversity by introgressing novel alleles from feral and or alien species. Association mapping approaches coupled with identifying single nucleotide polymorphisms the most elemental form of polymorphism in the genomes, may facilitate breeding by design. In this article, efforts to advance genomic-based breeding for improving crop species, providing food, feed, fuel and fiber to the world community, will be discussed.

Keywords RFLP • SSRs • SNPs • QTLs • Marker-assisted selection • GM cotton • GM rice • GM wheat • GM soybean • GM maize • GM sorghum

1 Introduction

Molecular markers have been proved vital tools for bridging the genomic tools with the classical breeding procedures for improving the genetic potential of multiple crop species (Rahman et al. 2009). In conventional breeding schemes, various traits of interest are combined in one genotype by hybridizing two genotypes (Beckmann and Soller 1986). Various studies elucidate that pyramiding of complex traits conferred by numerous genes which contribute directly or indirectly to the development of same trait, is really a mammoth task to achieve by deploying classical breeding methods (Beckmann and Soller 1986). Marker-assisted selection (MAS) is an approach which utilizes the tightly linked DNA markers for diagnosing plants having that particular trait of interest (Ribaut and Hoisington 1998). MAS can expedite the process of improved breeding by reducing time for selection of true to type genotype, increasing efficiency in selection procedure and efficient utility of the available resources. In other words MAS is a procedure to merge genomic and conventional resources in a better way (Moose and Mumm 2008). Second strategy for merging the conventional and genomic resources is bringing the deployment of transgenic technology for developing genetically modified (GM) crops. Transgenic crops overcome the limitations of utilizing genetic resources among different species (Qaim and Subramanian 2010). To meet the demands of new era breeding with desired characteristics is unavoidable which is only possible with GM technology (Qaim and Subramanian 2010). In this review economically important crops will be discussed in the context of utilizing the aforementioned technologies to improve their genetic potential of crop plants.

2 Wheat

Among the biotic factors, substantially depressing wheat production, are the rust diseases like leaf rust (Singh et al. 1998), stripe rust (Helguera et al. 2003), and stem rust (Mago et al. 2009). A fungus *Puccinia recondite* causes leaf rust. Two genes *Lr34* and *Lr46* which causes slow rusting have been found effective to combat many disease causing fungi (Singh et al. 1998). All combinations of other Lr genes and *Lr34* genes (Kloppers and Pretorius 1997) have explained the hypersensitive resistance responses. DNA markers have been identified which are linked with the other leaf rust genes (Huang and Gill 2001) and *Lr34* (Suenaga et al. 2003) which have further utility for probing the F_2 wheat plants, and also in succeeding generations, containing the gene(s) which can potentially cause resistance to the disease. In another study resistant genes for leaf rust *Lr47, Lr24*, *Lr1*, *Lr9*, were introgressed into bread wheat gentypes (Nocente et al. 2007) using MAS. Similarly, translocation lines 6VS/6AL derived from a cross *Triticum aestivum/Haynaldia villosa which* harbors a gene *Yr26* located on chromosome 1B show resistance to the majority of races of *Puccinia striiformis* f. sp. *tritici* (Pst) causing yellow or stripe rust. DNA markers *Xwe173* and *Xbarc181* were utilized for monitoring the introgression in cultivated wheat varieties (Wang et al. 2008). Another gene *Yr15*, imparts resistance to stripe rust, tagged with two SSR markers *Xgwm413 and Xbarc8*, further these markers served the purpose of diagnosis in all genetic backgrounds except in one (Murphy et al. 2009). Commercialization of the first wheat variety "Patwin" was done by the University of California at Davis (http://www.plantsciences.ucdavis.edu; Helguera et al. 2003) is a master piece example that was developed with the help of diagnostic DNA markers which assisted in introgression of *Yr17* and *Lr37* genes for resistance against stripe rust and leaf rust respectively into one genotype.

T. timopheevii ssp. *Armeniacum,* confers resistance against a recently appeared strain of stem rust (Ug99). The resistant gene *Sr40* was tagged with a closely linked marker *Xwmc344* (0.7 cM), and later two flanking markers *Xgwm374 and Xwmc474* (~2.5 cM) were identified, together can be used in marker-assisted incorporation and pyramiding of *Sr40* to develop superior lines (Wu et al. 2009b). Another gene *Sr39* was introgressed along with *Lr35* gene for resistance against leaf rust into wheat from *Aegilops speltoides.* Mago and Co-workers (2009) induced homoeologous recombinations between the *Ae. Speltoides and* wheat chromosome and developed a set of recombinant lines with reduced *A. speltoides* parts. For the resultant resistant and susceptible genotypes, DNA markers were utilized for conveniently pyramiding of other stem rust resistant genes with enhanced sources of Sr39 which effectively combat the Pgt pathotype TTKSK and its other strains in wheat. Two genes from *Thinopyrum ponticum* (*Sr25* and *Sr26*) were introduced into wheat and proved to be useful against new strains of TTKSK (syn. Ug99) and its types. Co-dominant markers for *Sr25* and *Sr26* were identified which can be potentially used in MAS (Liu et al. 2010).

Powdery mildew is another threat to wheat production. SSR markers linked with genes *Pm4a* and *Pm5e* have been detected (Huang et al. 2003; Ma et al. 2004).

Markers linked with another gene *Pm4b* (STS_241, *Xgwm382,* Me8/Em7_220) were identified which can improve resistance to the powdery mildew disease in wheat (Yi et al. 2008).

A locus Glu-1 has some impact on the wheat's quality of bread making. Coding and promoter regions of this locus were scrutinized for polymorphisms (Radovanovic and Cloutier 2003; Ma et al. 2003). Two specific PCR based markers were confirmed and utilized for alleles identification at *Glu-B1x* locus for its further utility in introgression of cultivated wheat varieties (Xu et al. 2008).

A linkage map of all 14 chromosomes was developed containing 280 SSRs, and also for detection of tan spot resistance associated QTLs. A tetraploid wheat doubled haploid (DH) population was derived by crossing a *T. turgidum* var Lebsock and *T. turgidum* subsp. *carthlicum* (accession PI 94749). A total of five QTLs for tan spot resistance were identified on chromosome arms, 3BL, 7BL, 5AL and 3AS. The out come of this study can facilitate genetic dissection of agronomic traits and marker identification for MAS (Chu et al. 2010).

2.1 GM Wheat

Transgenic studies in wheat have been focused mainly on improvement of grain quality characteristics and effect of expression of endogenous genes on dough quality (Francki 2009). It has been experimentally proven that expression of endogenous gene have positive or negative impact on grain quality and dough characteristics. Impact of genes 1Ax1, 1Dx5, LMW-GS, HMW-GS and pinA have been experimentally determined (Alvarez et al. 2000; Blechl et al. 2007; He et al. 2005; Tosi et al. 2004, 2005; Masci et al. 2002; Martin et al. 2006).

Fusarium graminearum, causes Fusarium head blight which is a challenging disease of wheat globally. Wheat has low resistance against this disease due to narrow genetic diversity in the existing pool. GM wheat containing barley class II chitinase gene was found effective against *F. graminearum* when experimentally tested (Shin et al. 2008).

RNA interference is a sequence specific gene silencing mechanism which can be utilized in determining gene functions. The application of RNAi in wheat has confirmed the function of VRN1, VRN2, SBE11a, SBE11b, EIN2, PDS, GPC and 1Dx5 (Francki 2009).

It has been studied recently that ferulic acid esterase which is derived from *Aspergillus niger* or endo-xylanase (from *Bacillus subtilis*) when expressed under the control of endosperm-specific *1DX5* glutenin promoter have an impact on wheat baking quality (Harholt et al. 2010).

National Institute for Biotechnology and Genetic Engineering has evaluated Arabidopsis AVP1 gene by introducing into tobacco for assessing its role for developing resistance against salinity and drought which are major limiting factors for crop productivity. Arabidopsis AVP1 gene encodes a vacuolar pyrophosphatases that function as proton pump and generates an electrochemical gradient in vacuole

activating vacuolar membrane-antiporters including Na^+/H^+ antiporter, which helps in sequestration of Na+ into vacuole as well as overexpression of AVP1 gene promotes vegetative growth by enhancing root development under the influence of auxins. Results of this study elucidate the significance of this gene in salinity and drought tolerance. This gene can further be utilized in economically important crops like wheat (Ibrahim et al. 2009).

3 Rice

All over the world yield of rice is being depressed by a fungal disease called Bacterial Blight (BB). Three genes *xa*5, *xa13* and *Xa21* causing resistance to BB were incorporated in susceptible rice cultivars and were tracked using STS markers flanking these genes (Chunwongse et al. 1993; Huang et al. 1997; Singh et al. 2001). Basmati rice is also highly vulnerable to BB. In another study, pyramiding of two genes *Xa7* and *Xa21* was carried out using MAS for improved resistance for BB in hybrid rice (Zhang et al. 2006). Foreground selection was integrated with background analysis using mapped SSR markers to detect the genes *xa13* and *Xa21* which show resistance against BB and superior quality features while these genes were non-Basmati resource derived. In India an improved Pusa Basmati 1 line, developed through MAS, has been commercialized (Gopalakrishnan et al. 2008). SSR markers were utilized to introgress three major genes for resistance *xa5, xa13 and Xa21* in a superior indica rice variety (Sundaram et al. 2008).

Magnaporthae grisea (fungus) causes a disease blast which is another destructive disease of rice. Three vital genes (*Pi*1, *Piz*-5 and *Pi*ta) control this disease. Utilization of tightly linked RFLP markers has facilitated the pyramiding of these genes and also mapping of these genes on respective chromosomes 11, 6 and 12 (Hittalmani et al. 2000). There are many concerns about a race-specific resistance in many crop plants which can be overcome by non-race-specific resistance that was effectively used in breeding against fungal diseases. Some strains of *Japonica* rice contain a resistant *pi21* allele, is able to improve resistance to the blast disease in rice (Fukuoka et al. 2009).

Tightly linked SSR and RFLP markers with a Waxy gene allele were employed to improve the grain quality of a rice cultivar Zhenshan-97A (Zhou et al. 2003).

Among abiotic stresses, limited water condition is the most detrimental factor for causing substantial reduction in yield. Root traits remained a major focus for tackling this menace. Root length was increased by 12–27% in IR64 by introgressing four QTLs for penetrating roots from Azucena (*japonica* variety) (Shen et al. 2001). Another QTL involved in osmotic adjustment (OA) under drought condition was mapped on chr-8 (Robin et al. 2003) would be helpful in future rice improvement program. Synteny between rice and maize was found for a QTL for OA mapped on chr-3 of rice and chr-1 of maize. This QTL accounts for numerous agronomic and physiological traits contributing tolerance to drought (Zhang et al. 2001). Conservativeness among these regions can pave the way for translating information

generated on a well-studied crop species to less-studied crop species. In this case, diagnostic DNA markers have utility in probing individual plants harboring QTLs for useful allele (Nguyen et al. 2004).

Plant type in rice is controlled primarily by a gene *Spk*(t), which was targeted through map-based cloning method (Komori et al. 2009). Both *Spk*(t) and its recessive allele *spk*(t) encode the same 259-aa proteins. However, a SNP was detected in the untranslated part of the gene sequence in 3′-splicing site which is *Spk*(t) allele specific. These findings will be useful in rice breeding program.

At low temperature unavailability of viable pollen causes spikelet sterility in rice. A SNP in alternative oxidase gene (OsAOX1a) and two closely linked QTLs (*Ctb*1 and *Ctb*2) contributing towards tolerance to low temperature in anthers were found tightly linked (Abe et al. 2002). A physical mapping study has revealed that seven putative genes were found for *Ctb*1. the utility of the identified SNP in marker assisted selection for the diagnosis of plants harboring QTLs which contribute for tolerance to cold has been proven (Saito et al. 2004).

MAS was utilized in assembling two major QTLs, one for shoot Na^+ reduction (qSNC-7) located on chr-7 and second for shoot K^+ accumulation (qSKC-1) located on chr-1 were found in three F3 lines developed by crossing Azucena and IR64 which is moderately tolerant (Lin et al. 2004).

3.1 GM Rice

Fifteen years ago first GM rice was developed and since then numerous traits related with yield, nutritional value and quality improvement have been dealt with. These efforts were complemented by sequencing its genome followed by characterization of multiple genes of interest. (Barry 2001; Sasaki and Burr 2000; Goff et al. 2002; Feng et al. 2002; Yu et al. 2002; Sasaki et al. 2002; Delseny 2003; The Rice Chromosome 10 Sequencing Consortium 2003; Sasaki et al. 2005).

Golden rice was developed by introducing daffodil and bacterial genes. The *psy* (phytoene synthase) and *crt1* genes that carry out the four steps required for the production of beta-carotene (Vitamin A in rice endosperm). However this rice variety could not get much attention due to many concerns (Toenniessen 2000).

Biotic factors cause loss to global production of rice 52% annually. Of this insect pests cause 21% of loss. The stable transgenic lines, expressing ASAL (*Allium sativum* leaf agglutinin) gene, were resistant against the sap-sucking pests (Yarasi et al. 2008). Genetic engineering for developing insect resistant rice is a very environment friendly and cost effective technique. *Bt (Cry 1 ac and Cry 2ab)* genes, in *Bacillus thuringiensis* species provide a variety of genes to develop transgenic plants, including rice (de Maagd et al. 2001; Tabashnik et al. 2003).

Three different studies were conducted in Centre of Excellence Molecular Biology regarding genetic engineering of rice. Through particle bombardment GM rice indica varieties Basmati 370 and M 7 were generated which express the novel *cry2A* (Bt) insecticidal gene. This novel *Bt* transgene was analysed for stable

integration. Cry 2A protein was found effective against two major rice pests of Indian subcontinent the yellow stem borer and the rice leaf folder.

In a second study through particle bombardment three genes related with insect resistance (the Bt genes *cry1Ac* and *cry2A*, and the snowdrop lectin gene *gna*) were introduced simultaneously into *indica* rice varieties M7 and Basmati 370. The transgene showed stable transmission and expression and significant defense against the most important insect pests yellow stem borer (*Scirpophaga incertulas*), brown plant hopper (*Nilaparvata lugens*) and rice leaf folder (*Cnaphalocrocis medinalis*). This approach led to multi mechanism defense.

In a third study three plasmids containing four genes were used to cotransform Indica rice (*Oryza sativa* L. cvs. Basmati 370 and M7). Two separate vectors were used to transform the Bt genes *cry1Ac* and *cry2A*, while the *hpt* (hygromycin phosphotransferase) and *gna* (snowdrop lectin) genes were transformed through a single, co-integrate vector. It was reported that the introduction of multiple agronomically favourable genes into the rice genome by co-transformation is a feasible approach for engineering elite rice varieties (Maqbool and Christou 1999; Maqbool et al. 1998, 2001).

Transgenic *Bt* hybrid rice has been evaluated in the farmer field in China and was confirmed to be resistant against rice leaf folder (*Cnaphalocrocis medinalis*, RLF) and yellow stem borer (Tu et al. 2000). Numerous transgenic varieties have been produced and field tested for yellow stem borer resistance in Pakistan, India and Mediterranean (Bashir et al. 2005; Ramesh et al. 2004a, b; Breitler et al. 2001).

Transgenic insect resistant plants have been very successful through the years and it has become challenging for opponents of genetic engineering to oppose (Bhattacharya et al. 2006). GM rice against yellow stem borer, leaf folder and sheath blight are really effective (Bhattacharya et al. 2006)

In multiple investigations, genes derived from various plant species have used for introducing in major crops of interest. For example, insecticidal genes such as, protease inhibitors or ribosome inactivating proteins and lectins (Sharma et al. 2004) were introduced in rice. Plant lectins are protective against many organisms. The mannose-specific lectin gene *gna* has been extensively used to develop transgenic rice having resistance against numerous economically important insects (Foissac et al. 2002; Tang et al. 2001; Wu et al. 2002; Nagadhara et al. 2003, 2004) plants. Protease inhibitors which are antimetabolites can protect against a wide range of insect pests. GM rice plants expressing protease inhibitors have been produced already. These are Oryzacystatin, cowpea trypsin inhibitors, bean trypsin inhibitor (Mochizuki et al. 1999), barley trypsin inhibitors (Alfonso-Rubi et al. 2003) and soybean trypsin inhibitors, potato protease inhibitors II (Xu et al. 1996; Sharma et al. 2004). Cowpea trypsin inhibitor (CpTi) transgene in transgenic rice is effective against stem borer (Bentur 2006; Brar and Khush 2007).

Ribosome inactivating proteins have been reported to have antifungal activity in plants. Fungus resistant transgenic maize has been produced using ribosome inactivating proteins (Kim et al. 2003).

4 Cotton

Cotton is one of the most significant natural textile fiber crop worldwide (Rahman et al. 2008). It has been reported that improvement in lint production can be achieved by supplementing the conventional breeding procedures with modern genomic tools for initiating knowledge based breeding program in cotton (Rahman et al. 2009). In this regard, multiple non-conventional research efforts like various cotton databases, sequencing data etc. can help in accelerating the breeding progress (Chen et al. 2007). For initiating MAS in cotton there are many useful DNA markers associated with fiber quality traits (Zhang et al. 2003; Asif 2009). Zhang et al. (2003) identified several QTLs for fiber strength. Two QTLs for fiber strength tagged three SSRs and six RAPDs markers were grouped into one linkage group. One of the RAPDs was converted into sequence characterized amplified regions (SCAR4311920), and was applied for diagnosing the cotton plants containing the main QTL associated with fiber strength (Guo et al. 2003). Recently, this QTL was fine mapped on Chr-24 (D8) (Chen et al. 2009). In another experiment, RFLPs linked with fiber quality traits were found by surveying a population derived from an interspecific cross *G. hirsutum/G. barbadense* (Chee et al. 2005a,b; Draye et al. 2005). Similarly, SSRs originating from *G. barbadense* were utilized to assess the introgression of genomic regions obtained from *G. barbadense* into *G. hirsutum*. It results in increment of 12–20% in fiber length in *G. hirsutum* (Mumtaz 2007). Jixiang and Co-workers (2007) identified AFLP markers linked with fiber and agronomic traits which can be utilized for MAS. Recently, these QTLs linked with agronomic traits and fiber were located on At as well as Dt genomes of tetraploid cotton (Wu et al. 2009a)

Like other crop plants, cotton is also vulnerable to limited water conditions. Globally efforts are underway to identifying QTLs conferring traits help in drought, for using in MAS under water stress condition (Saranga et al. 2001; Paterson et al. 2003). Ullah (2009) detected collectively nine putative QTLs for drought tolerance in cotton. Babar et al. (2009) also detected some QTLs associated with drought conditions.

Fusarium wilt (FW), a disease of fungal origin, causes ultimate death in cotton after yellowing, wilting, defoliation and vascular tissue damage. A number of DNA markers were identified by surveying an F2 population derived from an intraspecific cross (*G. hirsutum*). One of the SSR markers (JESPR304) tightly linked with a FW resistant gene (*FWR*) was mapped on chromosome D3 (c17). Four QTLs were identified of which one QTL was found near marker JESPR304 at proximity of 0.06–0.2 cM. This QTL explained 52.5–60.9% phenotypic variance. This QTL can be further used to develop FW resistant cultivars using MAS (Wang et al. 2009).

Cotton leaf curl a viral disease, has substantially depressed cotton production in Pakistan, and also in neighboring countries like India and China. DNA markers (4 RAPDs and 2 SSRs) were found linked with resistance to the disease (Rahman 2002; Rahman et al. 2006). Two cotton lines NIBGE-2 (Rahman and Zafar 2007b) and NIBGE-115 (Rahman and Zafar 2007a) were developed by utilizing these markers.

4.1 GM Cotton

GM cotton containing Cry 1Ac gene has been commercialized in most of the cotton growing countries, covering ~50% of the total cotton growing area, for imparting resistance to lepidopteron insect pests. The gene was isolated from a soil bacterium species *Bacillus thuringiensis*. Numerous varieties of cotton bio-engineered to produce an insecticidal protein are called Bollgard cotton. It has been commercialized in 1996 by a private seed company in the USA. This product is an effective alternative to chemical insecticides to control the attack of cotton bollworm, *Heliothis virescens*, tobacco budworm and pink bollworm which causes reduction in cotton production costs and insecticide use (Perlak et al. 2001).

To further broaden the spectrum of insects to which the plant is tolerant and to provide an insect resistance management tool to hamper the onset of resistance Bollgard II cotton event 15985 was produced in which the Cry1Ac and Cry2Ab2 proteins has been introduced by genetic modification (Hamilton et al. 2004).

Research efforts are underway for identifying new gene(s) for enhancing the spectrum of insect pests resistance. Recently, a gene was derived from spider venom-toxin that confers resistance to herbivorous insects. The gene has been characterized in tobacco and efforts are on the way to transfer it to cotton (Khan et al. 2006).

The nitrilase gene which confers resistance to herbicide bromoxynil in cotton has been obtained from a bacterial species *klebsiella.* Genes for acetolactate synthase conferring resistance to the imidazolinone and sulfonylurea classes of herbicide were isolated from both the genomes of higher plants and bacteria (Stewart 1994). Herbicide resistant cotton has provided the farmers weed control without damage to cotton which has ultimately increased the income of the farming community (Brookes and Barfoot 2006, 2008).

Virus-resistant and salinity-tolerant GM cotton is at the stage of field evaluation (Zafar 2007). Genetic transformation of cotton with biolistic gene gun was carried out; however, the results were not so successful (Haq et al. 2005). Later, for cotton (*Gossypium hirsutum* L. cv. Coker-312) a silicon carbide whisker-mediated gene transfer system was developed which make recovery of fertile and stable transformants possible (Asad et al. 2008). Antisense RNA technology has also been used to develop transgenic plants resistant to cotton leaf curl disease. The mechanism of resistance was found to be post transcriptional gene silencing (Asad 2004). Transgenic cotton was produced using antisense movement protein gene AV2 in an Indian variety (F864) along with the npt II gene. Transgenic plants were tested for the integration of gene (Sanjaya et al. 2005).

Recently, *Arabidopsis* vacuolar H^+ pyrophosphatase gene (AVP1) was transformed in cotton exhibiting resistance against drought and salt stresses. Also, these transgenic cotton lines have shown improvement in staple length compared to the control. These findings are manifesting the utility of this gene to combat drought and salt stress in cotton (Pasapula et al. 2011)

5 Maize

Maize streak virus (MSV) disease causes 100% yield losses in tropical Africa. QTLs accounting for resistance to MSV in maize have been mapped. Microsatellite markers were utilized to evaluate resistance field trials. Linkage group consisted of 13 microsatellite markers and 3 QTLs were detected associated with resistance to MSV (Lagat et al. 2008).

MAS has been deployed in maize (*Zea mays*) for introgressing genes from wild sources and or novel sources (Ragot et al. 1995), for identifying plants having opaque2 gene (Dreher et al. 2003) and for simple (Ho et al. 2002; Morris et al. 2003) or complex traits enhancement (Bouchez et al. 2002; Willcox et al. 2002). Drought causes delayed silking, causing ~15% yield losses annually, which causes long anthesis-silking interval (ASI). There is a positive correlation between reduced ASI and better yields under drought stress. Genomic regions associated with both yield and ASI (Ribaut et al. 1997) were identified. Out of these, one genomic region showed allelic contribution for short ASI with low grain yield while the others provided alleles for short ASI accounting for high grain yield.

In another study, five genomic regions conferring drought tolerance were introduced in a maize inbred line CML247 from a drought tolerant donor line Ac7643. Performance of some genotypes was 2–4 times better than the control genotype, further development of new cultivars can be based on these genotypes (Ribaut et al. 2004). In another investigation, seedling emergence was increased by monitoring the introduction of QTL, has also shown positive impact of grain yield (Yousef and Juvik 2002). QTLs for grain yield and flowering time were identified in maize (Blanc et al. 2008).

High-quality SNP markers were identified by screening maize inbred lines. It has been demonstrated that during cultivar development genetic diversity has not been utilized extensively. SNPs are potent markers to be utilized in MAS and study of diversity. Two SNPs were identified from a gene putatively linked with diversity within two Chinese heterotic groups. Allelic frequency change at two SNPs and absence of their allele in Brazilian germplasm indicated linkage disequilibrium block of 142 kb (Lu et al. 2009).

5.1 GM Maize

In 1990s, conventional breeding procedures were supplemented by integrating transformation technology for improve the genetic base of corn germplasm. GM-maize was commercialized in mid 1990s containing genes conferring resistant to European corn borer and lepidopteran pests. GM-corn containing insect protection and herbicide resistance was commercialized not only in North America but also in other maize growing areas. In 2008, GM-corn containing at least one biotech trait covered more than 80% of total corn growing area in USA (Mullet 2009).

In the second generation biotechnology era, GM maize having three stacked transgenes or multiple traits for corn rootworm control, herbicide tolerance for more efficient weed control and lepidopteran insect control has been commercialized. It has been cultivated on more than 30 million hectares in 16 different countries by 2007. Transgenic maize hybrids not only increase yield but also contribute to reduction of green house gases, and pesticide use. Although first generation transgenic maize has contributed a lot, moreover next generation under development is even more promising. These traits are designed to enhance maize yield, growth under drought conditions, more efficient nitrogen use, enhancement of protection against insects and pests and improvement of grain quality for food, animal feed and biofuels. Improved farm practices along with maize breeding and biotechnology has remained instrumental in escalating maize productivity worldwide (Mullet 2009).

Transgenic corn containing maize hemoglobin and soybean ferritin were characterized to set a strategy for increasing the iron sink strength in grains. Transgenic kernels were compared to non-transgenic sibling kernels for accumulation of mRNA encoding seed storage proteins and proteins involved in mineral metabolism, as well as mineral content. These experiments help to set stratifies for improving nutritional quality in maize grains (Scott 2010).

In a recent study impact of fall armyworm on GM corn having Cry1Ab and Cry1F insecticidal crystal protein was examined. Field study and bioassays have elucidated Cry1F reduced foliar injury significantly by lowering survivorship of armyworm when compared to that on non-Bt corn tissues; whereas Cry1Ab has limited effects on fall armyworm (Hardke et al. 2011).

6 Sorghum

Sorghum provides at the same time fiber, biofuel, food and feed in the semi arid tropical regions of the world. It is a C_4 grass, and its genome has been sequenced which comprise 730 Mbp. Genetic information generated on sorghum can be utilized in the closely related species like maize and wheat etc. (Paterson et al. 2009).

Shoot fly causes substantial damage at seedling stage of sorghum. A microsatellite marker-based linkage map was constructed using RILs of a cross 296B (susceptible)/IS18551 (resistant) (Satish et al. 2009). Also a total of 29 QTLs conferring multiple traits of interest were identified. These are, seven for oviposition, six for dead hearts, six for abaxial trichome density, four each for leaf glossiness and seedling vigor, and two for adaxial trichome density. Resistance alleles were from IS18551 for most QTLs; however, 296B also contributed alleles to the six QTLs involved in conferring resistance. QTLs identified in this study will offer a firm foundation for initiating marker assisted selection (MAS) for improving resistance to shoot fly in sorghum.

Among diseases, resistance to Anthracnose disease was linked with a RAPD marker OPJ011437 which was converted into SCAR (SCJ01). Correspondence of this region was detected with contig-3966 of the sorghum genome (chr-8) which is useful in resistant plants diagnosis (Singh et al. 2006).

Stay-green mechanism is very important trait for improving drought tolerance in sorghum. Identification of four QTLs (*Stg1*, *Stg2*, *Stg3* and *Stg4*) was carried out utilising different populations (Haussman et al. 2002; Sanchez et al. 2002; Harris et al. 2007). Marker assisted breeding was utilized to develop NILs for *Stg2* (Sanchez et al. 2002). Further, 18 different NILs that were containing introgressed regions of the four important stay-green loci, *Stg1-Stg4* were developed through MAS (Harris et al. 2007).

Several QTLs linked with SSRs for early season cold tolerance were identified. These SSRs were further utilized to initiate MAS for early-season cold tolerance under various environments (Knoll and Ejeta, 2008).

6.1 GM Sorghum

To develop resistance against stalk rot (*Fusarium thapsinum*) the agronomically important gene chi II, which encodes rice chitinase under the constitutive CaMV 35 S promoter, was transferred to sorghum. (Zhu et al. 1998; Krishnaveni et al. 2001).

To improve tolerance to water and NaCl stress, a sorghum cultivar SPV462 was engineered with a *mtlD* gene (derived from *E. coli*) encoding for mannitol-1-phosphate dehydrogenase. The transgenic lines have shown that manipulating mannitol biosynthetic pathway can cause enhanced tolerance to salinity and water deficit in sorghum. The product has not yet commercialized (Maheshwari et al. 2010).

A mutated gene was introduced in sorghum for improving the nutritional quality (Tadesse et al. 2003). This gene encodes a key regulatory enzyme of the lysine pathway (dihydropicolinate synthase). Over expression of this gene produces sorghum lines with elevated lysine content.

7 Barley

Two closely linked QTLs involved in tolerance to low temperature co-occurred with a number of QTLs involved in regulation of cold-regulated (*COR*) genes functions (Vagujfalvi et al. 2003) were detected on barley chr-5 (Francia et al. 2004; Toth et al. 2004).

A gene (*Run8*) contributes in developing resistance against loose smut disease. Marker Assisted Breeding (MAB) was utilized to introgress this gene into a hulless barley cultivar using (double haploid) DH and a line (HB390) was produced and evaluated prior to release commercially in Canada. Another gene *Ruhq* causing resistance to covered smut disease was transferred into hulless barley using the same process (Grewal et al. 2008).

Findings of another study suggested that chr-3, chr-6 and chr-7 are harboring QTLs for malt quality traits and grain yield. However, QTLs located on chr-7 were most useful (Igartua et al. 2000). RFLP markers were used for monitoring the introgression of QTLs associated with yield for developing high yielding NILs. In multi-location trials, one line has shown high yielding property of one parent (Baronesse) while retaining a feature of better malting property of the other parent (Harrington), was recognized (Schmierer et al. 2005).

A new fingerprinting assay "temperature-switch PCR" (TSP) was tested for assessing its efficacy for marker development, its reliability and genotyping accuracy in barley. For assessing gene diversity 87 TSP markers were surveyed. This method has been designed for genotyping codominant SNP on agarose gels. This method has proven to be handy for SNP detection (Hayden et al. 2009).

7.1 GM Barley

Horvath et al. (2001) tested transgenic barley cv. 'Golden Promise', containing β-(1,3–1,4)-glucanase gene, over a period of 3 years in the field trial,. Their study indicated a relatively poor performance of the transgenic barley as compared to the control which might be due to the occurrence of somaclonal variations during regeneration of the transformants. In another study, occurrence of reduced fitness in a field grown transgenic barley has been attributed to somaclonal variations (Manoharan et al. 2006).

Backcrossing transgenic lines having expressing a β-(1,3–1,4)-glucanase, with the original wild type (wt) cv. 'Conlon', to get stable transformants led to plants carrying the transgene and having a wt-like growth habit (Manoharan et al. 2006). In another study, crossing of transgenic lines to elite cultivars resulted in homozygous lines that express the transgene and give a higher 1,000-grain weight and yield than the untransformed cultivar, which elucidated that it is possible to enhance the agronomic features of transgenic barley by cross-breeding with elite cultivars (Horvath et al. 2001; Goedeke et al. 2007).

8 Soybean

Five backcross populations (BC2F4, 468 lines) were derived from a cross *G. max* cv. A2008/*G. soja* acc. 468916 after verification at two different locations for 2 years. One QTL for lodging, four QTLs for yield, four QTLs for maturity, and for plant height five QTLs were identified. Alleles from *G. max* cultivar were showing higher yield potential than alleles from *G. soja* (Wang et al. 2004). In the same study, an SSR marker Sat-107 was found closely linked with the four-seeded pod (4SP) locus which can be effectively utilized for plant selection having this trait (Zhu and Sun 2006).

Marker Assisted Breeding was utilized in six genetic backgrounds to introgress an important QTL conferring high yielding potential (Concibido et al. 2003). The final seed phytoestrogen level is largely controlled by a set of ~6–12 loci (Kassem et al. 2006) making direct selection difficult. Marker assisted selection, enhanced the phytoestrogen content level well above in new soybean genotypes than the level of elite cultivars (Lightfoot 2008).

Charlson and co-workers (2009) identified four QTLs for canopy wilting in three drought environments using RILs population. QTLs for seed yield mechanism (Palomeque et al. 2009a, b), gene *Rps8* conferring resistance to stem and root rot (caused by *Phytophthora sojae*), (Sandhu et al. 2005), *Rsv*4 gene conferring soybean mosaic virus resistance (Hwang et al. 2006), *Rag1* gene imparting resistance to soybean aphid (Li et al. 2007) can effectively be utilized in soybean.

8.1 GM Soybean

Transgenic fertile soybean plants were generated capsid polyprotein (pCP) which were resistant to *Bean pod mottle virus* (BPMV). The progeny of the homozygous transgenic line exhibited systemic resistance; these lines could potentially be useful in generating commercial cultivars showing resistance to BPMV (Reddy et al. 2001).

An attempt was made for developing GM soybean that has nutritional properties similar to fish oils (Damude and Kinney 2008). Enriching poultry meat with LC n-3 PUFA is a source of increasing intakes. Studies have shown that the use of oil from transgenic soya in which the fatty acid metabolic pathways have been modified can increase the n-3 VLC-PUFAs of chicken meat. Feeding broilers a soya oil rich in SDA produced meat with approximately 150–200 mg LC n-3 PUFA/100 g meat (Rymer and Givens 2009).

Transgenic soybean lines have been produced which contain the decarboxylase gene (*oxdc*) obtained from a Flammulina spp. using the biolistic process. Further analysis revealed that expression of the *oxdc* gene results in resistance to *S. sclerotiorum* (Cunha et al. 2010).

9 Conclusion

The blend of both classical and genomic breeding approaches has been proven to be very valuable for crop improvement. Still there are many aspects which need attention and have a capacity to progress. Whole genome sequencing and study of syntenic regions have proven the utility of sequence information from one crop to another. In the present world scenario there is a great need of better yield and vigor of vital crops to fulfill the needs of the growing population. Transgenic crops having superb and valuable properties are the most needed entity today. Marker assisted

selection and transgenic crops are opening new horizons in the field of genomics. In the coming future further advancement in the field of biotechnology will lead to overcome the present dilemmas.

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Chapter 3
Breeding for Improved Drought Tolerance

Abazar Rajabi and Eric Schmieder Ober

Contents

Abstract The combination of a changing global climate and an increasing world population requires that crops for food, fibre and fuel need to be more resilient to hotter and drier conditions than they have been in the past. Given the years required to develop and release new varieties, breeders have been working hard to achieve this goal. Various strategies being used to counteract drought stress and some examples of successes will be reviewed. These include improvements in maize yield stability, due in part to improved drought tolerance; new varieties of wheat with increased water use efficiency; upland rice with deeper roots, and pearl millet with better yields in arid areas. While most of the selections are based simply on yield, greater emphasis is being placed on morpho-physiological traits associated with greater stress tolerance. Molecular markers based on QTLs linked to these traits also have

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been used, although less than the number of papers published in this area would suggest. There is promise in candidate genes that have been highlighted by 'omic studies, and great hope has been placed on transgenic approaches, but few of these have yet borne fruit in the field. The rapidly advancing genomic technologies and ready access to cheap sequence data are accelerating the breeding process. However, the ability to obtain relevant, high quality phenotypic information is the rate-limiting process; new innovations in 'high-throughput' phenotyping may provide solutions. Improved characterisation of test environments in multi-location trials combined with advanced statistical dissection of G x E interactions is helping breeders to improve target varieties. The use of models for predicting gene effects, particularly when combining multiple traits, will find greater application as these tools are developed further. Teams of breeders, physiologists, molecular geneticists, agronomists, pathologists, modellers and statisticians, who can communicate across disciplines, are required to tackle the challenge of producing enough food in a world where production is often constrained by insufficient water resources.

Keywords Drought tolerance • Breeding wheat • Sugar beet • Yield • Stress

1 Introduction

Drought decreases the yield of crops in many production systems throughout the world, especially in arid and semi-arid regions. As water resources for agronomic uses become more limiting, the development of drought tolerant varieties gains greater importance (Barker et al. 2004). For plant breeders and physiologists, drought tolerance is a complex trait. The difficulty of dealing with such complexity has motivated many studies to unravel the genetic and physiological basis of drought tolerance in field crops (Ludlow and Muchow 1990). For any research to have an impact on the genetic improvement of drought tolerance, the effort must be established as a long-term commitment and have clear objectives relevant to field conditions with a strong focus on yield. Historically, most improvements in crop drought tolerance have come from incremental improvements achieved by long-term breeding efforts. It is unlikely that this will change in the foreseeable future. However, recent food security issues, combined with the threats resulting from climate change, impose even greater demands on the breeder to improve yields and yield stability. Increased productivity requires a two-pronged approach: at the top end, strive to increase yield potential with each cycle, and at the other, strive to lift the low yields obtained under poor conditions.

To deal with the latter, this chapter examines certain aspects of how yields can be improved through breeding when water is limiting, using predominantly sugar beet and wheat as examples. This is not a comprehensive review of the literature on the subject, as many good reviews have been written recently (Richards 2006; Reynolds and Tuberosa 2008; Praba et al. 2009; Fleury et al. 2010; Passioura and Angus 2010; Richards et al. 2010; Tardieu and Tuberosa 2010; Ashraf 2010; Blum 2011).

2 Mechanisms of Drought Tolerance

The term 'drought tolerance' is used frequently, but is often ill-defined. It has meaning to molecular biologists, crop physiologists, farmers, and global change modellers, but the context changes with scale from cell to landscape, and therefore, it is interpreted and used differently (Passioura 2007). 'Drought tolerance' in this chapter is the ability to capture the greatest proportion of yield potential (the full expression of genetic potential in the absence of environmental limitations) when water is limiting, and includes any plant process that contributes to this result. Note as well that here 'drought' does not mean Sahara-like conditions, but merely any environment where the supply of moisture is insufficient to support crop growth and development without inhibition. Strictly speaking, drought escape (completion of reproduction before drought), and avoidance (maintenance of high tissue water potentials despite shortage of rainfall) can be differentiated from tolerance, which is the ability of tissues to function despite decreased tissue water potentials induced by water deficit. Mechanisms of drought tolerance include: the accumulation of solutes that help maintain turgor through osmotic adjustment (Boyer et al. 2008); accumulation of compatible solutes such as proline and glycine betaine that also help protect the integrity of cellular machinery (Ashraf and Foolad 2007; Chen and Murata 2011), changes in membrane composition via alteration of constituent lipid and steroid species (Olsson 1995); changes in cell wall properties to allow growth at decreased turgor (Sharp et al. 2004); repair mechanisms that replace damaged macromolecules and detoxify excess reactive species such as superoxide O_2 (Miller et al. 2010). Dehydration tolerance describes the ability of plants to continue metabolism at very low water potentials and to repair and recover after release from desiccation; except for seeds, such extreme conditions are rarely met in agricultural settings.

Plants can produce harvestable yields in water-limited environments through combinations of drought escape, avoidance and tolerance. Thus, many different traits have been proposed as selection targets for indirect selection and genetic analysis (Ludlow and Muchow 1990; Edmeades et al. 2000; Morison et al. 2008; Praba et al. 2009; Reynolds et al. 2009). However, genotype-by-environment interactions commonly confound interpretation of experimental results, and realized genetic gain is often lower than predicted. Thus, there have been few attempts to select for improved drought tolerance via indirect selection based on putative drought tolerance traits, or such efforts often have not contributed to successful commercial improvements of drought tolerance in the varieties grown by farmers. There is a clear urgency for a step-change in the productivity of crops in dry conditions, and improved varieties are a key component for achieving this. In this chapter we highlight where there have been successes, and possible avenues for progress.

In order for the cultivation of a variety to be profitable in water-limited areas, it should possess both improved yield potential and drought tolerance so that it is competitive across a range of environments, in addition to the requisite disease resistance and quality traits expected by the market. Thus, breeding for drought tolerance in elite commercial germplasm focuses on adding novel and improved

levels of drought tolerance to genotypes that already demonstrate commercially viable levels of yield in the best environments. To achieve further improvements in drought tolerance, molecular technologies in combination with novel pedigree breeding methods are required.

3 Empirical Breeding

Improving drought tolerance and productivity is one of the most difficult tasks for plant breeders. The difficulty arises from the diverse strategies adopted by plants themselves to combat drought stress depending on the timing, severity of stress and stage of crop growth (Hussain 2006). Breeding for tolerance has been hampered by interactions between genotype and environment resulting from variation and intensity of rainfall from year to year.

There are three main breeding approaches for drought tolerance. The first is to breed for high yield under non-stress conditions (Mitra 2001). In one hand, the maximum genetic potential of yield is expected to be realized under non-stress conditions when expression of genetic variation is maximal (Rosielle and Hamblin 1981). On the other hand, a high positive correlation generally exists between performance in stress and non-stress conditions (Cattivelli et al. 2008; Ober et al. 2010). Therefore, a genotype superior under non-stress conditions will also yield relatively well under drought conditions. However, there is no general agreement on the concept of expression of maximum genetic potential in non-stress conditions (Kumar et al. 2007), as genotype by environment interactions may restrict the high yielding genotype from performing well under drought. The second approach is to breed under actual drought conditions (Hurd 1971; Mitra 2001). The problem of this approach is the high variability in drought intensity from year to year and as a consequence, selection pressure on breeding materials is inconsistent across generations as it is highly dependent on environmental conditions. This problem along with low heritability of yield (Blum 2011) makes the breeding programme complicated and slow.

The third approach is to incorporate morpho-physiological traits related to drought resistance into high-yielding genotypes (Mitra 2001). However, this approach is complicated because the physiological and genetic basis of adaptation to drought conditions has not been well understood. An alternative approach is to improve the yield potential of already resistant material provided that there is genetic variation within such material (Bidinger et al. 2005; Mitra 2001). In order to develop high yielding, drought-tolerant genotypes, simultaneous selection in non-stress environment for yield and in drought condition for stability may be done. As such, the breeding methodology to be applied for drought tolerance is the same as that applied for other purposes.

In general, self-pollinated crops could be improved by pedigree and bulk selection methods and cross-pollinated crops by recurrent selection. Back cross is the appropriate methodology for transferring drought tolerance related traits to

a high-yielding genotype. On the other hand, family selection methods such as half-sib and full-sib selections maintain a broad genetic base and make it possible to develop the desired drought tolerant genotype (Yunus and Paroda 1982; Mitra 2001).

There is considerable hope being placed on biotechnology to supply the drought tolerant varieties of the future using transgenic methods. Innovations in this area most likely will come from large multinational seed companies that have the resources to develop, test and licence this material. However, transferring discoveries from the lab bench to farmers' fields is taking longer than expected, and in the meantime, CGIAR centres and the private sector continue to release improved conventional varieties (Gilbert 2010). For the less lucrative or non-hybrid crops, publicly financed breeding will continue to carry the effort.

4 Breeding for Harsh Environments

There is a low probability for simultaneous improvement of yield in both unstressed and severe stress environments (Rosielle and Hamblin 1981). When selection for yield under severe drought stress is performed this produces a genetic shift towards expression of constitutive traits that moderate water use. Such traits include reduced leaf area and leaf area index (Blum and Sullivan 1986; Fukai et al. 1999), constitutively low leaf transpiration rate (Kholov et al. 2010), early flowering (e.g., Blum and Pnuel 1990; Yadav and Bhatnagar 2001) and reduced tillering in cereals.

Low constitutive (potential) total plant dry matter was associated with better water status and yield under drought stress in rice (Fukai et al. 1999). Pearl millet hybrids selected for drought resistance were relatively higher yielding in a series of stress environments (Bidinger et al. 2005; Yadav et al. 2011). However, this gain under stress was achieved at the cost of a lower yield in the non-stressed environments. Stress adaptation was consistent with early flowering, limited tillering and low biomass, traits which are not compatible with a high yield potential phenotype. Secondly, high grain yield cultivars have a large sink, which means a high rate of assimilate demand from the source (Blum 2011). A large sink demand from the source can lead to earlier leaf senescence under stress, compared with a plant with a smaller sink (Khanna-Chopra and Sinha 1988). Genotypes with large yield potential may use water at a faster rate than less productive genotypes, which could be detrimental in situations such as terminal drought in Mediterranean climates where early season soil moisture needs to be conserved for later stages such as grain filling. Thus, genotypes bred for high yield potential may have resource demands that can not be met in severe stress environments.

Where drought stress is severe, a cultivar adapted to the region becomes more specific and less common to other regions. This is well supported by other studies showing that wide adaptation was achieved at the cost of poorer performance in specific stress environments. Thus, a variety with uniform superiority over all environments is a rare occurrence (Blum 2011).

5 Germplasm Resources

Breeders strive to create genetic variation within a pool of breeding germplasm prior to making selections. While there is some concern that in general the genetic base of crop species is narrowing (Tanksley and McCouch 1997), there exists substantial genetic variation for many agronomic, morphological and physiological traits within, for example, sugar beet (Ober et al. 2005) and winter wheat (Ober et al. 2010). Novel sources of pest and disease resistance and tolerance to abiotic stresses are also sought from exotic germplasm such as wild relatives of crop species. One effort to introduce this type of genetic background into elite breeding material is the creation of synthetic wheats (Reynolds et al. 2007). This highlights the importance of maintaining germplasm collections, vital work that is often under-funded in national programmes. There are many examples of how germplasm collections have been used in practice. Venuprasad et al. (2007) found that selection for rice yield under severe drought stress was effective if the population carried high levels of drought tolerance derived from a tolerant donor. This strategy works if the relative drought susceptibility of parental lines is known before making any crosses. In this case, screening founder germplasm for drought tolerance early in the breeding process makes sense. Without this information, evaluating crosses at later stages in the breeding process might be more effective.

In India, pearl millet populations containing various proportions of landrace materials from the dry regions performed better than high yielding cultivars under limited water supply (Yadav and Weltzien 2000). Landrace materials expressed better tillering and stable flowering under drought stress as compared with high yielding cultivars. Landraces were more productive under severe drought than elite materials and expressed less delay in flowering (Yadav 2008). When these drought resistant landraces were top-crossed onto elite materials of large panicles it was possible to achieve greater yield and drought resistance in the hybrids.

A rice breeding program in China (He et al. 2010) used backcross breeding with several donors of drought resistance. A two-round-selection for yield under severe drought together with a two-round-selection for yield under irrigation resulted in 113 BC2F8 introgression lines which included superior yielding lines under both irrigated and drought stress conditions.

6 Yield Stability

For any long-term breeding effort to be successful it is critical to strike the appropriate balance between defining clear breeding objectives and ensuring flexibility within the breeding program. Given the many proposed breeding strategies for improving drought tolerance, the breeder needs a clearly defined set of check hybrids as internal references to decide whether the chosen breeding strategy is achieving adequate progress. This is complicated by the fact that individual check varieties remain static while yields should progress with each breeding cycle. Thus, each introduction of a

new check variety should also reflect improvements made in drought tolerance. To improve drought tolerance, the breeder can either target specific combinations of putative drought tolerance traits or simply select for yield in large populations of genotypes in environments that reflect conditions in key market areas. In the former case there is scope to determine which trait combinations have contributed to improved performance and subsequently apply this knowledge to define breeding targets for testing (Podlich et al. 2004; Hammer et al. 2006). Most likely, a combination of both approaches is required. Here we use sugar beet as an example of breeding for drought tolerance, although many of the principles can apply to other hybrid crops and indeed inbreeding crops such as wheat and other cereals.

Improving sugar beet yields in Iran can be used as an example of how breeding for drought tolerance is applied in practice. In Iran, spring sugar beet production is wholly reliant on irrigation as the average annual rainfall in the country is about 250 mm, which mainly occurs during the winter and is not useful for a spring crop such as sugar beet. The average water requirement of the crop to realize the yield potential is approx. 9,000 m^3 ha^{-1}, which is almost entirely provided by irrigation. Due to a high evaporative demand and competition for water, the water table has been greatly lowered and this has increased the pumping charges. Therefore, continued irrigation at this rate does not seem to be a viable long-term solution for supporting future yield increases. One promising method is to develop drought tolerant varieties so that water resources are used more effectively.

The Sugar Beet Seed Institute (SBSI) is one of the leading research institutes working on drought stress in sugar beet, with projects on deficit irrigation, altered sowing pattern, the effects of drought stress on morpho-physiological traits, yield and technological quality, etc. Evaluation of sugar beet hybrids and their parents in stressed and non-stress conditions in Iran indicated that there is heterosis for yield under drought, which could be exploited by development of hybrids (Sadeghian et al. unpublished). This might be the case for other main crops such as maize, sorghum and pearl millet where hybrids are developed towards rainfed and water-limited environments (Blum 2011). There is strong evidence that heterosis in certain tropical maize materials is expressed in effective use of water and improved plant water status, probably as a result of larger root system in the hybrids (Araus et al. 2010). On the other hand, the parental effect is crucial for hybrid performance under drought stress (Blum 2011). This indicates that having one or both drought tolerant parents is the main condition for achieving a drought resistant hybrid. In some cases (e.g. Castiglioni et al. 2008; Araus et al. 2010; Yadav 2010), a recombination of parental drought resistance with high rate of heterosis for yield (namely high yield potential) might be possible.

Family line breeding and progeny selection over 8 years has resulted in improved drought tolerance in sugar beet for Iranian conditions. However, traditional breeding by using yield as a selection index and performing multi-environment yield trials has been costly and slow due to the multigenic nature of the trait and large genotype by environment interactions (Blum 2011). This necessitates using more efficient selection methods such a recurrent selection for further improvement of the level of drought tolerance. In addition, selection for secondary physiological traits plus yield in stress conditions have been shown to be more effective than direct selection for

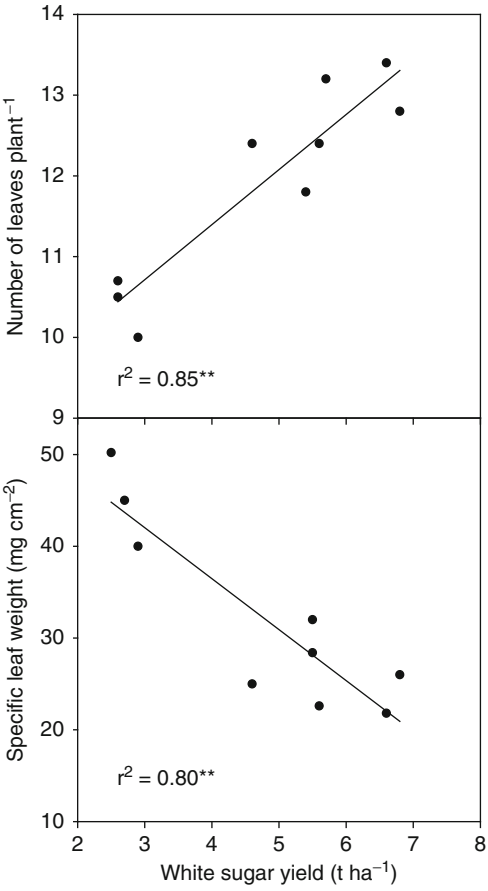

Fig. 3.1 Relationship of number of leaves and specific leaf weight with white sugar yield in three sugar beet populations grown under drought stress condition (Abdollahian Noghabi and Mohammadian 2005)

yield *per se* (Bänziger and Lafitte 1997; Bruce et al. 2002). One of the reasons for using physiological traits in selection is that they may have higher heritability than yield (Cruickshank et al. 2004). However, as each single trait that shows significant genetic correlation with crop performance in water-limited conditions can explain only a fraction of the total variance in yield, therefore one has to consider a combination of traits as selection criteria for improving yield in drought-prone environments.

Abdollahian Noghabi and Mohammadian (2005) studied the effects of varying degrees of drought stress on morpho-physiological traits of three sugar beet populations. They found that among the root and top traits measured, leaf number and specific leaf weight (leaf dry mass per area) had the greatest positive and negative correlations with white sugar yield, respectively (Fig. 3.1). Development and maintenance of the canopy for the capture and efficient use of light is critical for the accumulation of biomass under water-limited conditions as well as irrigated conditions. Rajabi et al. (2009) examined sugar beet genotypes of different genetic backgrounds and established that genetic variation in water use efficiency (dry matter produced per unit water used) of sugar beet could be indirectly exploited by measuring the stable carbon isotope discrimination ratio (Delta) in leaf and root tissue. Rajabi et al. (2008) found

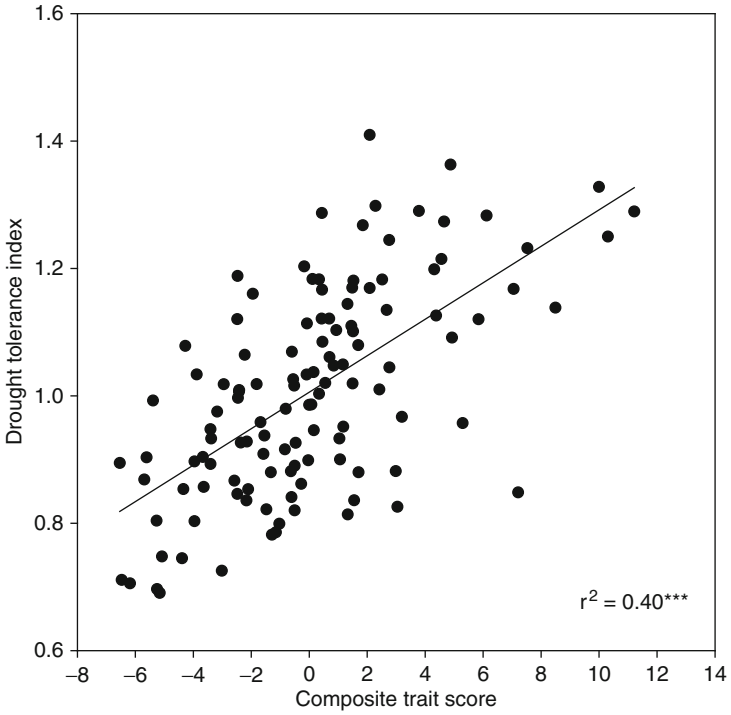

Fig. 3.2 The composite trait score explained 40% of the variation in drought tolerance amongst 120 wheat lines grown under managed drought conditions in the UK in 2008 (Fig. 3.3). Individual traits can explain only a small proportion of the variance in complex traits such as drought tolerance. Therefore, a composite trait score that combines the influence of several key traits was a better descriptor of drought tolerance. This score sums (with equal weighting and according to the sign of the correlation coefficient) standardised values for: green canopy cover, photosynthetic efficiency, flag leaf size, maintenance of ear dry mass at anthesis, leaf porosity, wax score, flag leaf senescence

that Delta, water use efficiency and specific leaf weight were mainly governed by additive gene effects, indicating that a greater genetic gain is expected for these traits whatever type of selection is applied. However, narrow-sense heritability was greater for Delta than specific leaf weight (Rajabi et al. 2008). Despite genetic variability for a range of other traits such as relative water content, osmotic adjustment, cell membrane stability, stomatal density, canopy temperature, ash content, etc. there was poor correlation with yield under drought stress conditions (Ober et al. 2005; Rajabi 2006). There are various ways that multivariate data sets can be handled to produce useful information. Multiple regression techniques and biplot analyses are two approaches that can highlight which combination of morpho-physiological traits best explain the variation in a complex target trait such as yield. The combination of traits describes a phenotypic ideal, or ideotype, that breeders could aim for in selections. Such a set of traits can be described numerically by computing a composite trait score that sums standardised values for each trait. The trait values can be given equal weighting, or weighted according to genetic correlations or heritabilities (Fig. 3.2).

Fig. 3.3 Large polytunnel rainout shelters used to apply managed drought conditions in the field. Shown are plots of 66 wheat genotypes grown at Broom's Barn, Suffolk, UK. The cost-effective facility allows control over the timing and severity of the water deficit with minimal impact on crop microclimate (Ober et al. 2010)

Every breeding strategy for drought-prone environments also has to consider that the timing and intensity of the stress events vary significantly from year to year and plants designed to cope with a specific type of drought may under-perform when the stress conditions are different or absent (Cattivelli et al. 2008). This is particularly true for cereals with sensitive stages of reproduction, but less of a concern for vegetative crops such as sugar beet. Selection under favourable environments also has positive effects when plants are grown in stressed environments (Cattivelli et al. 2008). In a typical Mediterranean environment, selection based on the absolute performance of the genotypes across environments is more successful than selecting for the minimum yield decrease under stress with respect to favourable conditions. An experiment including 46 sugar beet genotypes representing different genetic backgrounds grown in drought and irrigated conditions indicated that sugar beet genotypes with high yielding capacity when irrigated also tended to perform well under drought (correlation coefficient $r = 0.64^{***}$), while the genotypes with minimum yield loss under stress did not belong to the group of high yielding genotypes in either irrigated or drought conditions (Ober et al. 2004). Similarly, there was a positive correlation between irrigated and droughted yields across a wide range of wheat genotypes when grown under managed drought conditions in the field (Ober et al. 2010; Fig. 3.3).

7 Conclusion

Increases in yields and yield stability can be achieved with selection and improvement of germplasm on the basis of performance under water-limited conditions that reflect the targeted environment. Selection response may be relatively slow, but should lead to more stable production across environments. Breeding progress depends on the availability of reliable selection tools and facilities where stress can be carefully controlled by managing the level and timing of water deficit. The scope for genetic gains in drought tolerance is remarkable. This can be achieved through judicious choice of selection environments and breeding strategy, rather than through a conventional approach of testing in a random sample of environments, which are often biased towards optimal conditions and less representative of actual farm conditions. Although we have given some specific examples of breeding approaches, each crop species and target environment may have different challenges, and therefore different solutions. These solutions do not have to be complex. While understanding the mechanisms of drought tolerance, the multifarious physiological effects of various allelic combinations and their genetic or epigenetic control can be a complicated phenomenon, this should not daunt the plant breeder. The successful yield gains for stressed environments made so far must continue, and indeed must increase to meet the challenges of a changing climate and growing world population.

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Chapter 4
Breeding for Biotic Stress Resistance/Tolerance in Plants

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Abstract The long-term goal of crop improvement for biotic stress tolerance in plants is a traditional objective of breeders. Plants must continuously defend themselves against attacks from bacteria, viruses, fungi, invertebrates, and even other plants. This chapter will therefore summarize the benefits and drawbacks of resistance versus chemical protection. Attempts will be made to provide a description on the effective genetic and molecular mechanisms that plants have developed to recognize and respond to infection by a number of pathogens and pests, such as non-host resistance, constitutive barriers and race-specific resistance, including recent advances in elucidating the structure and molecular mechanisms used by plants to cope with pathogens and pest attacks. This chapter also covers the most relevant problems in breeding for resistance to parasites and will include aspects related to specificity of defense mechanisms, specificity of parasitic ability, inheritance of resistance, gene-for-gene interaction, and durability of resistance. Major considerations in breeding for resistance to parasites, conventional sources of resistance and possible alternatives, namely mutation breeding, genetic manipulations, tissue cultures, and molecular interventions to develop plants resistant to pests and pathogens will also be dealt.

Keywords Defense mechanisms • Genetic basis of resistance • Signal transduction network • Pathogenesis related proteins • Transgenic plants

1 Introduction

Most of the problems facing agriculture in the twenty-first century relate to the growing world population, which is expected to stabilize at around 10–12 billion during the next 70 years (Heszky 2008). The almost doubled population will require a more than proportional increase in food production. During the last decade, world grain yield increased around 0.5% per year, which is three-fold lower than the population growth rate in the same period. The main task for breeders and agronomists will therefore be to increase yields while reducing the use of chemicals. The difficulties of this mission are due to: (i) the limited possibilities of expanding the cropped land area; (ii) the environmental legislation which limits the use of chemicals for disease control; (iii) climate change and the/predicted worsening of biotic and abiotic stresses; (iv) the reduced source of useful traits from crops wild relatives (Cook 2000).

Beacuse more than 42% of the potential world crop yield is lost owing to biotic stresses (15% attributable to insects, 13% to weeds, and 13% to other pathogens), a reduction in this incidence will be one of the more important possibilities for improving plant production (Pimentel 1997). Cook (2000) divided these possibilities as follows: (i) improvement of plant material (breeding for tolerance/resistance); (ii) improvement of root health (e.g. field rotation, soil tillage, soil-borne diseases control); (iii) improvement of irrigation practices (optimal water quality and availability); (iv) protection against airborne hazards (foliar diseases etc.). In this context, the development of tolerant plants to biotic stresses is therefore an important objective of plant breeding strategies with relevant implications for both farmers and the seed and agrochemical industries. In fact genetic resistance has several obvious advantages over the use of chemical pesticides or other methods for parasite control. These include nominal genetic permanency, negligible cost once cultivars are developed, and quite high efficiency. The major downside of genetic resistance to biotic stresses is the fact that selection pressure is placed on parasites populations to develop means of overcoming the resistance, thus practically limiting the time of effectiveness (Table 4.1). In this chapter the genetic, biochemical and molecular mechanisms by which plants defend themselves against attack from pathogens will be examined. In addition breeding approaches towards their improvement will be described.

Table 4.1 Overview of potential and actual losses attributable to fungal and bacterial pathogens, viruses, animals pests and weeds as well as the efficacy of the applied pest control operations in maize, wheat, rice, barley, potatoes, soybean, sugar beet, and cotton

	Pests and pathogens				
	Fungi and bacteria	Viruses	Animal pests	Weeds	Total
Loss potential (%)[a]	14.9	3.1	17.6	31.8	67.4
Actual losses (%)[a]	9.9	2.7	10.1	9.4	32.0
Efficacy (%)[b]	33.8	12.9	42.4	70.6	52.5

Source: Modified from Oerke and Dehne (2004)
[a]As percentage of attainable yields
[b]As percentage of loss potential prevented

2 Fungal and Bacterial Diseases

A plant pathogen is defined as an organism that for a part or all of its life cycle grows inside a plant; this has a detrimental effect on the plant growth and development and ultimately on yield. Several reviews have been published in this field to which the readers are referred for a more in-depth description (e.g. Hammond-Kosack and Jones 2000; Dickinson 2003). The main findings emerging from these studies indicate that pathogens have evolved specialist ways to invade plants: (i) some penetrate the plant surface directly using mechanical pressure or enzymatic attack; (ii) others pass through natural plant openings, for example, stomata or lenticels; (iii) many take advantage of existing wounds. Once inside the plant, three main colonization strategies are deployed by pathogens to use the host plant as substrate for their growth and development: (i) biotrophic organisms ensure the plant cell remains alive; (ii) hemibiotrophic organisms initially keep host plant cells alive, but then kill them at later stages of the infection; (iii) necrotrophic organisms first kill plant cells and then metabolize their contents. In this respect, pathogenesis is the term used to describe the sequence of processes from host and pathogen contact (infection, colonization and plant pathogen reproduction) to the development of the complete syndrome. A pathogen strain that causes disease is termed virulent and its success can be attributed to several factors such as : (i) rapid and high rate of reproduction during the main growing season for plants; (ii) a very efficient dispersal mechanism and long-term survival capacity; (iii) high capacity to generate genetic diversity through haploidy and subsequent sexual reproduction.

2.1 Plant Defense Mechanisms

An overview of forms of plant resistance, defined on the basis of innate and acquired resistance, and their mechanisms of response to pathogens is given in Table 4.2. According to Kiraly et al. (2007) innate resistance is exhibited by the plant in two forms: non-specific (nonhost or general) resistance, which is effective against several pathogenic species or several strains (races, biotypes, pathovars) of a single pathogen, and specific resistance. In the latter case, one plant cultivar (variety) can resist infection by one or a few pathogenic strains.

Although plants are in continual contact with potential pathogens, a successful infection is rare. The ability of a particular plant species to prevent successful colonization by a given pathogen species is referred to as nonhost resistance. The molecular basis of nonhost resistance is poorly understood, but presumably relies on both constitutive barriers and inducible responses that involve a large array of proteins and other organic molecules produced, respectively, prior to infection or during pathogen attack (cf. Jones and Dangl 2006; Ferreira et al. 2007). This is in contrast to the vertebrate immune system, in which specialized cells devoted to defense are rapidly mobilized to the infection site, where they either kill the invading organisms or limit their diffusion.

Table 4.2 Overview of forms of plant resistance

Resistance phenomenon	Mechanism
1. *Innate resistance*	
1.1. Non specific, general resistance	
Non-host resistance	HR; ROS; BAX inhibitor-1; PEN genes
Basal resistance against bacteria	Flagellin/FLS2 interaction; ROS; antimicrobial compounds
Race non-specific mlo resistance and quantitative resistance to fungi	Cell wall thickening; Antimicrobial compounds; ROS
Resistance to necrosis-inducing stresses	High antioxidant capacity
1.2. Specific resistance (cultivar/pathogenic race specificity)	
Extreme resistance-symptomless gene-for-gene resistance	Unknown
Rx-resistance against viruses without HR	
Symptomless reaction to rust pathogens, no visible HR	
Gene-for-gene resistance	ROS; Phytoalexins; Phenol oxidation; Stress proteins
R-gene ↔ Avr-gene interaction associated with the hypertensive response (HR)	
Resistance to pathogen toxins	Enzymatic detoxification; Lack of toxin recept
Gene silencing	Recognition and decomposition of foreign RNAs with ribonucleases
2. *Acquired resistance*	
After a primary infection and acquired resistance develops against a second infection "Stress memory"	Accumulation of SA; Stimulated antioxidants; Gene silencing; Rhizobacterial induction

Source: Modified from Kiraly et al. (2007)

The typical preformed, constitutive defenses are morphological, structural, and chemical barriers. An example of morphological barrier is the height of lips of stomatal guard cells (Hoch et al. 1987). Certain fungal rust pathogens possess specific detection mechanisms that sense the height of stomatal guard cell lips encountered on susceptible plants. Moreover, waxes, cutin, suberin, lignin, cellulose, callose, and cell wall proteins act as structural barriers that are rapidly reinforced upon the pathogen infection process (Punja 2001). Plants also constitutively produce a variety of secondary metabolites (e.g. phenolics, saponins, terpenoids, steroids and glucosinalates), and antifungal proteins, many of which act as antimicrobial compounds during defense (see Dangl and Jones 2001, for a review). These compounds may be present in their biologically active forms or stored as inactive precursors that are converted to their active forms by host enzymes in response to pathogen attack or tissue damage.

Plants employ two modes of their innate immune system to contrast pathogen infections (see Tsuda and Katagiri 2010, for a recent review). The first mode of immunity is referred to as pattern-triggered immunity (PTI) that is triggered by

molecular patterns common to many microbial types. The second mode is triggered by recognition of pathogen effectors and is called as effector-triggered immunity (ETI). At least some cases of PTI and ETI extensively share downstream signaling machinery, that is, PTI and ETI appear to be mediated by an integrated signaling network. However, activated immune responses in ETI are more prolonged and robust than those in PTI. Furthermore, the previous cited authors have reported that network analysis has also revealed that synergistic relationships among the signaling sectors are evident in PTI, which may amplify the signal, whereas compensatory relationships among the sectors dominate in ETI, explaining the robustness of ETI against genetic and pathogenic perturbations. Thus, plants seem to use a common signaling network that differs in PTI and ETI.

There is evidence that induced or acquired resistance includes the hypersensitive response (HR), a form of programmed plant cell death, cell-wall strengthening, and the expression of various defense-related *R* genes (R, resistance; Staskawicz et al. 1995) that mediated recognition of pathogen effectors. The *R* genes activate a series of defense signaling cascades and pathogenesis-related (*PR*) gene expression to generate systemic acquired resistance (SAR); this primes the plant for resistance against a broad spectrum of pathogens (Durrant and Dong 2004; Dangl and Jones 2001). This multicomponent response requires a substantial commitment of cellular resources, including extensive genetic reprogramming and metabolic re-allocation. Thus, defenses are kept under tight genetic control and are activated only if the plant detects a prospective invader.

2.2 *Genetic Basis of Resistance*

Genetic analysis of disease resistance in plants began over 100 years ago when Biffin (1905) reported that resistance in wheat to stripe rust (*Puccinia striiformis*) was inherited as a single recessive Mendelian trait. Since this initial work, many genes conferring resistance to pathogens in crop plants have been characterized, and the genetic basis of pathogenicity (virulence/avirulence) has been studied in many plant pathogens. This knowledge culminated in the development of the gene-for-gene hypothesis by Flor (1971) based on genetic studies of the interaction between flax and the flax rust pathogen, which has provided a framework for much if not all of the work on disease resistance in the years since. In genetic terms, resistance is generally defined by the mode of inheritance, with broad distinctions between oligogenic (controlled by one or few genes of major effect) and polygenic (controlled by many genes of low individual phenotypic effect) resistance.

2.2.1 Qualitative Resistance

Evidence made it clear that many cases of resistance were inherited in a simple way. Most characterized resistance genes are dominant in action; for example the *Hm1*

gene of maize conferring resistance to *Cochliobolus carbonum* race 1, a causal agent of northern leaf spot of maize, secretes an HST known as HC-toxin, which interferes with a histone deacetylase (HD) altering host gene expression (Brosch et al. 1995). Indeed, resistant (insensitive to HC-toxin) maize lines contain a dominant resistance gene *Hm1*, which encodes for a NADPH-dependent reductase whose function is to reduce (detoxify) the HC-toxin that the fungus produces to cause disease in susceptible maize (Johal and Briggs 1992). However, some recessive resistance genes have proven important sources of durable resistance – e.g. gene *Sr2* conferring resistance to stem rust in wheat (McIntosh et al. 1995); gene *mlo* for mildew resistance in barley (Jørgensen 1994). The barley *Mlo* gene has been cloned and encodes a transmembrane protein that is a negative regulator of cell death and powered mildew resistance (Büschges et al. 1997). Notably, further research showed that functional *Mlo* genes also exist in *Arabidopsis* (Consonni et al. 2006). Thus, *mlo*-mediated non-specific resistance to powdered mildew might be a more widespread phenomenon among plants than previously hypothesized. There are also many examples of resistance genes that display partial dominance (gene dosage dependence; e.g. the resistance gene *Lr9* in wheat to *Puccinia recondita*; Samborski 1963).

Dominance or recessiveness of resistance genes is, however, not absolute and can even be governed by the attribute used to measure the disease phenotype (Johnson 1992), genetic background, pathogen isolate or environment. Examples of oligogenic resistance are known in which additive and non-additive interaction occurs between genes at separate loci. The genes *Lr13* and *Lr34* in wheat interact in an additive manner to confer resistance to leaf rust, not only with each other, but also with other genes for resistance to leaf rust (Kolmer 1992). Non-additive gene interaction occurs when two genes in the host are only effective when present together. In such cases, the genes in the host are referred to as complementary. Baker (1966) demonstrated complementary action of the genes *Pc3* and *Pc4* conferring resistance to *Puccinia coronata* in the oat cultivar Bond.

Resistance to bacterial infections is not well developed as virus and fungal resistance, partly because bacterial diseases are a main problem only in crop plants like potato, rice, and some fruit trees. Similarly to fungal diseases the most effective type of protection is genetic resistance, which is based on single dominant or semidominant genes. Different classes of *R* genes cloned from various plant species were characterized and tested for their ability in conferring resistance against bacterial pathogens. For example, among these, a map-based cloned *Xa21* gene from rice, gave resistance to bacterial blight , a serious disease in rice caused by *Xantomonas oryzae. Xa21* specify a receptor- like kinase formed bi LRRs in the putative domain and a serine-threonine kinase in the putative intracellular domain pv. *oryzae,* after transferring this gene from a wild rice species to a cultivated indica variety (Wang et al. 1996). Moreover, the last cited authors found on the broad-spectrum resistance of transgenic rice with the *Xa21* gene against 29 diverse isolates, suggesting that a single cloned gene is sufficient to confer multi-isolate resistance. In the same way, the resistance gene *Bs2* from pepper was transferred to tomato, which then had resistance to bacterial disease (Tai et al. 1999). Infiltration of different maize lines

with a variety of bacterial pathogens of maize, rice and sorghum has permitted to identify a maize gene, *Rxo1*, which conditions a strong HR to the non-host bacterial pathogen *X. o.* pv. *Oryzicola* (Zhao et al. 2004). The same locus carries a gene (designated *Rba1*) controlling resistance to the maize and sorghum bacterial stripe pathogen *Burkholderia andropogonis*. It was surprising that the same locus controlled resistance to two of only four bacterial pathovars tested. This suggests that this locus may condition defense reactions to other bacterial pathogens.

2.2.2 Quantitative Resistance and QTLs

Quantitative resistance, in contrast to qualitative resistance, is generally considered as partial resistance in a particular cultivar (Young 1996). This type of disease resistance is controlled by multiple loci, referred to as polygenes or quantitative trait loci (QTLs), and does not comply with simple Mendelian inheritance. Examples of such polygenetically inherited resistance are the partial resistance in potato to *Phytopthora infestans*, in maize to *Puccinia sorghi*, and in barley to *Puccinia hordei* (Parlevliet and Zadoks 1977).

Although genetically complex forms of disease resistance are still poorly understood, an effective strategy for studying complex and polygenic forms of disease resistance is known as QTL mapping, which is based on the use of DNA markers (see Young 1996, for a review). With QTL mapping, the roles of specific loci in genetically complex traits can be described; this has also permitted insight to be gained into fundamental questions that have puzzled researchers in the field of plant pathology for decades. Although results of QTL mapping indicate that it is generally not the case, there are examples of several (>10) QTLs involved in quantitative resistance; however, it is much more common to find only three to five loci: frequently, 1 or 2 QTLs predominate.

QTL mapping may also help to determine whether individual QTLs are race-specific or not, and when there is an indication of specificity, the degree to which partial resistance differs between races. For example, quantitative resistance to *P. infestans* in potato was initially described as race-nonspecific (Van der Plank 1982). Dissecting the contributions of individual QTLs, it was clearly demonstrated that loci show distinctly different resistance effects against different pathogen races (Leonards-Schippers et al. 1994). Indeed, only 5 of the 11 statistically significant genomic regions showed no specificity against just two races tested, while the others were significant against just one. Moreover, genetic mapping with DNA markers makes it possible to ask whether homologous resistance genes exist in related plant taxa and may help to test the hypothesis that QTLs are simply variants of qualitative resistance loci that have been (partially) overcome by their respective pathogen. For instance, in rice blast, 3 of the QTLs mapped to the same marker intervals as previously identified qualitative blast resistance genes. It is conceivable that these QTLs represent allelic variants of the known qualitative resistance genes, though only more precise mapping and gene cloning can resolve this definitely. In potato late blight, 1 QTL coincided in location with a dominant, race-specific gene known as *R1*,

as well as *Rx2,* a gene for resistance to potato virus (Leonards-Schippers et al. 1994). Moreover, a second late blight QTL mapped to the same region as *Rx1,* a second major resistance gene for potato virus X. Further progress in QTL mapping technology will include the molecular cloning of the underlying genes, including those that confer partial resistance. For example, major genes such as resistance to *Pseudomonas syringae* in tomato and *Arabidopsis* have been isolated and cloned based on map position, while others have been isolated through transposon-tagging (cf. Young 1996). More recently, Hu et al. (2008), to isolate disease resistance QTLs, have established a candidate gene approach that integrates linkage map, expression profile and functional complementation analyses. This strategy has proven applicable in rice for identifying the genes underlying minor resistance QTLs in bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* and fungal blast caused by *Magnaporthe grisea* systems and it may also help to shed light on disease resistance QTLs of other cereals. The results also suggest that a single minor QTL can be used in rice improvement by modulating the expression of the gene underlying the QTL. Pyramiding 2 or 3 minor QTL genes, whose expression can be managed and that function in different defense signal transduction pathways, may allow the breeding of cultivars that are highly resistant to bacterial blight and blast.

2.3 Plant R Gene-Mediated Disease Resistance

As mentioned above, plants do not have the benefit of a circulating antibody system, so plant cells autonomously maintain constant vigilance against pathogens by expressing vast arrays of *R* genes. These genes have been genetically defined in interactions with all major classes of plant pathogens including fungi, bacteria, and viruses.

In the classic gene-for-gene model – also known as receptor-ligand model – of host pathogen interactions, *R* gene products recognize pathogen elicitor, encoded by avirulence (*Avr*) genes (Fig. 4.1). Resistance gene-mediated resistance is a host-specific defense and can only be activated when both *R* gene and corresponding *Avr* gene are present (Staskawicz et al. 1995); the absence of either component results in disease, which is typically associated with damage and a reduction in yield of the host plant.

Because gene-for-gene are operative in defense response to fungal, bacterial and virus pathogens, and because the host defense responses are similar irrespective of the type of pathogen, common recognition and signal recognition transduction mechanisms are postulated to underlie the gene-for-gene relationship. A single model of pathogen response will therefore be given herein.

Currently, there are two alternative mechanisms to explain this model: direct and indirect interaction (Fig. 4.2). Direct interaction suggests that the pathogen *Avr* effectors interact with plant R proteins directly to trigger *R* gene-mediated resistance signaling. For example, the rice *R* gene *Pi-ta* was initially shown to directly interact with *AVR-Pita* from *Magnaporthe grisea* but no interaction between

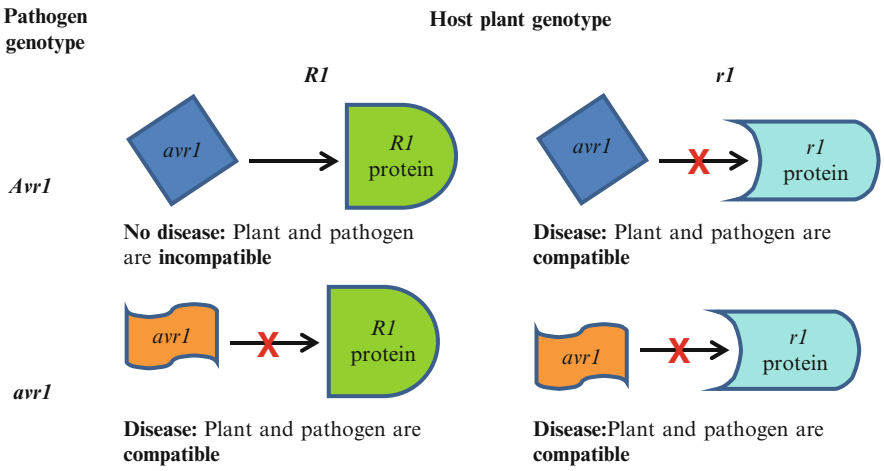

Fig. 4.1 Flor's gene-for-gene model. For resistance (incompatibility) to occur, complementary pairs of dominant genes, one in the host and one in the pathogen, are required. An alteration or loss of the plant resistance gene (*R* changing to *r*) or of the pathogen avirulence gene (*Avr* changing to *avr*) leads to disease (compatibility) (Modified from Hammond-Kosack and Jones 2000)

AVR-Pita and its susceptible allele *Pi-ta* was observed (Jia et al. 2000). In addition, a direct interaction was recently observed between the flax *L* alleles and corresponding flax rust *Avr* genes, which provides evidence for direct, allele-specific interaction between R proteins and diverse Avr proteins (Dodds et al. 2006). Conversely, most studied data prefer the indirect model also called "guard" hypothesis (Jones and Dangl 2006). In this model, R proteins act as "guardees" to monitor the variation/modification of host proteins after coupling with the corresponding Avr effectors. Moreover, evidence suggests that plants possibly employ alternate mechanisms to prevent pathogens from invading in different plant-pathogen interaction systems, which maintain a good balance between different R proteins and Avr effectors to coordinate the conflicts of interaction between different *R* genes and varied Avr effectors in host-pathogen co-evolution (Van der Hoorn et al. 2002). In these two models, R proteins may detect Avr effectors with conserved structure through direct interaction, or could indirectly recognize diverse unrelated pathogen factors after Avr proteins couple with their virulence targets (Chisholm et al. 2006). However, when and how R proteins detect diverse Avr effectors directly or indirectly is unclear and requires in-depth analysis.

More recently, Hann et al. (2010), by reviewing published results on bacterial virulence effectors and their activities, indicated that the major virulence strategy for plant pathogenic bacteria is a deployment of effector molecules within the host cytoplasm. As summarized by these authors (i) each bacterial strain possesses a set of 20–30 effectors which are redundant and interchangeable, and interact promiscuously with host targets, (ii) bacterial effectors have weak, somewhat nonspecific interactions with host molecules, targeting conserved protein domains or molecular

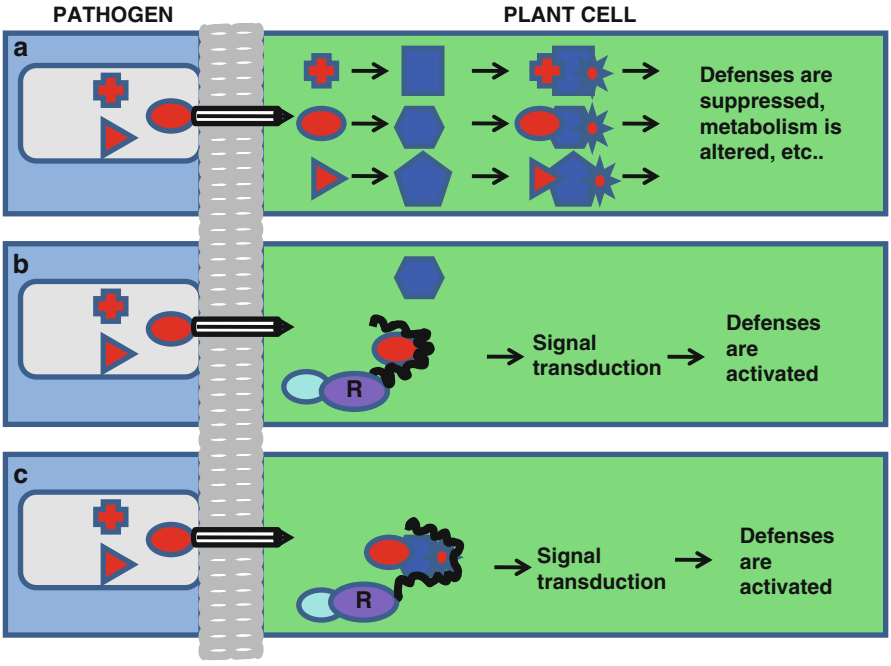

Fig. 4.2 Interactions between pathogen Avr proteins (*red*) and plant R proteins (*blue*). Once inside the plant cell, pathogen Avr proteins target host proteins that control defense responses, metabolism or other plant process that affect pathogen virulence. Panel (**a**): the plant cell does not express an R protein that is capable of recognizing any virulence protein, plant defenses are not activated, disease results from the collective action of the virulence proteins. Panel (**b**): classic receptor-elicitor hypothesis, in which an R protein (*purple*) directly binds a virulence protein, so a complex signal transduction network is activated, which in turn triggers plant defense responses. Panel (**c**): guard hypothesis in which an R protein (*guard*) detects a modified host protein (*guardee*, *red star*), eventually as a complex with the "attacking" virulence protein (Modified from McDowell and Woffenden 2003)

structures, (iii) these structures have been coopted by the plant defense machinery as accessory proteins or baits in NB-LRR complexes, and (iv) the link between pathogenicity and immunity apparently lies in the molecular (enzymatic) activities of each effector. Although, the direction of evolution of host immune components with respect to effectors is unclear, some authors have suggested a positive-negative selection model in which positive selection for effectors is balanced with strong negative selection for specific effectors by NB-LRR complexes. The clearest example for such a positive-negative selection scenario is presented by AvrPto and AvrPtoB, which suppress PRR receptor kinases and are recognised by the Prf NB-LRR complex in tomato (Zipfel and Rathjen 2008). Thus, the two levels of pathogen perception may interact to slow pathogen evolution, which is important when recognition specificities are innate and cannot be acquired.

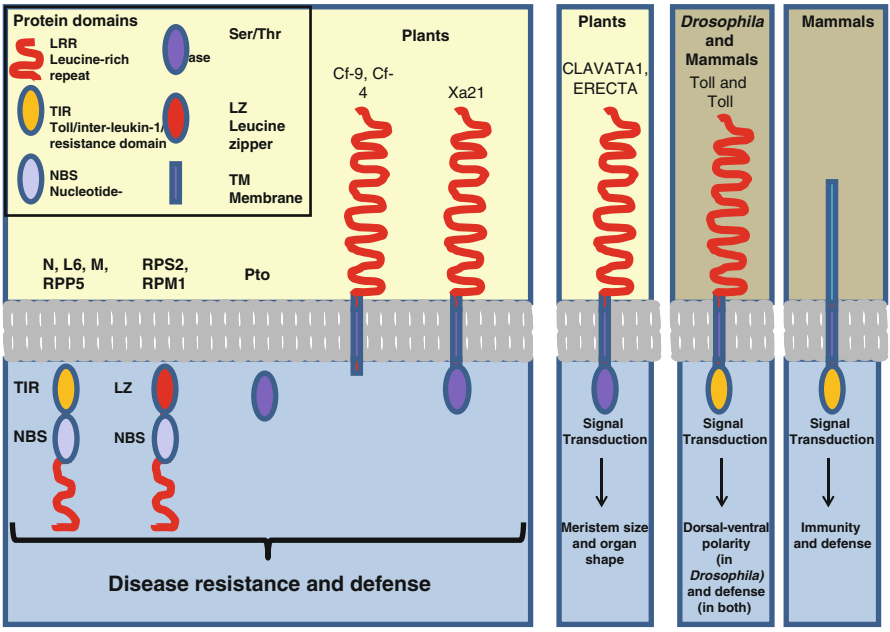

Fig. 4.3 Schematic diagram illustrating several plant resistance proteins and various plant LRR-containing proteins synthesized during the defense response. For comparison are included structurally related proteins that are involved in various aspects of plant development as well as other eukaryotic proteins that coordinate development and the induction of the immune response in animals (Modified from Hammond-Kosack and Jones 2000)

2.3.1 Structure of *R* Genes

The strong phenotypes and natural variability at *R* loci have been exploited by molecular geneticists to clone the *R* genes and investigate their molecular modes of action. To date, over 70 *R* genes have been cloned and some of them have been well characterized (for reviews see Jones and Dangl 2006; Shan et al. 2004; Toyoda et al. 2002). Notably, these studies have not only provided a large body of information on the structure, function and evolution of *R* genes that control resistance to diverse pathogens, but also have generated useful genetic materials to engineer novel resistant cultivars. In addition, some critical defense signaling components, such as NDR1, EDS1, RAR1, and SGT1, have been identified in *R* gene-mediated resistance signaling, which provide important clues to understanding the mechanism of *R* gene-mediated defense signaling (Lin et al. 2007; reviewed by McDowell and Woffenden 2003; Rathjen and Moffett 2003).

Examination of the predicted R protein sequences, based on protein domains that are described in Fig. 4.3, reveals the existence of shared sequence motifs that can be grouped into several superfamilies. The large majority of genes cloned so far belong to the nucleotide-binding site (NBS), leucine-rich repeat (LRR), a motif with homology

to the cytoplasmic domains of the *Drosophila* Toll protein and the mammalian interleukin-1 receptor (TIR), a coiled-coil (CC) or leucine zipper (LZ) structure, transmembrane domain (TM), and protein kinase domain (PK). According to these features, at least four principal classes are distinguished among most *R* genes as follows: NBS-LRR, Receptor-like kinase (RLK), LRR-TM and TM-CC (Fig. 4.3). The NBS-LRR genes represent the largest class of *R* genes, and encode proteins with a variable N-terminal domain of approximately 200 amino acids (aa), connected by a predicted NBS domain of approximately 300 aa and a more variable tandem array of approximately 10–40 short LRR motifs. The NBS-LRR genes are further categorized into three subgroups based on the motif within their N-terminus: TIR group, CC or LZ group and non-motif group. Furthermore, studies regarding the NB-LRR signaling pathway have been recently summarized by Eitas and Dangl (2010). The main findings reviewed by these workers indicate that (i) two NB-LRRs can function together to mediate disease resistance against pathogen isolates, (ii) the NB-LRR protein fragments that are sufficient to initiate defense signaling, and (iii) importantly, distinct fragments of different NB-LRRs are sufficient for function. Finally, the cited authors described that accessory proteins (e.g. Pto) and highly related Pto-like kinases have a significant role in regulating the function and down-streaming host genes in NB-LRR signaling.

As more *R* genes have been cloned in recent years, new motifs or structures have been uncovered in R proteins. *RRS1-R* from *Arabidopsis*, conferring resistance to *Ralstonia solanacearum* strain GMI1000 with a type III effector PopP2, which belongs to the YopJ/AvrRxv protein family encodes a typical TIR-NBS-LRR protein, but containing a transcriptional factor WRKY domain in its C-terminus (Deslandes et al. 2002). The WRKY domain is highly conserved and is composed of a region of about 60 aa containing a heptapeptide WRKYGQK in its N-terminus and a zinc-finger motif, which plays a crucial role in regulating plant defense responses (Journot-Catalino et al. 2006).

Recently, the plant resistance gene *Pi-d2*, conferring gene-for-gene resistance to the Chinese rice blast strain, ZB15, encodes a novel type of receptor-like kinase R protein with a predicted extracellular domain of a bulb-type mannose specific binding lectin (B-lectin) and an intracellular serine-threonine kinase domain (Chen et al. 2006). Among all isolated *R* genes, three novel genes do not belong to any of the four types: *Xa5* encoding a TFIIA transcription factor (Jiang et al. 2006), *Xa13* with homologous to nodulin MtN3, and *Xa27* without any hits in the available protein database, *R* genes encode putative receptors that respond to the products of '*Avr* genes' expressed by the pathogen during infection.

2.3.2 Evolution Mechanism of *R* Genes

In plants many *R* genes are located in clusters that comprise several copies of homologous *R* gene sequences arising from a single gene family (simple clusters) or colocalized *R* gene sequences derived from two or more unrelated families (complex clusters), and may also contain unrelated single genes interspersed between the

homologs (reviewed in Friedman and Baker 2007). *R* clusters range in size from two tandem paralogs to large complexes spanning several megabases. The largest *R* clusters characterized to date include the maize *Rp1* cluster (~1–52 homologs per haplotype; Smith et al. 2004), the lettuce *Dm3* (aka RGC2) cluster (~12–32 homologs per haplotype), and the potato major late blight resistance (MLB) cluster (~45 homologs per haplotype; Kuang et al. 2005).

Genic and intergenic sequence repeats within *R* clusters, generated by duplications and transposon insertions, provide a structural environment that permits mispairing during recombination, giving rise to unequal crossovers and interlocus gene conversions (McDowell and Simon 2006). Intergenic unequal crossover has the potential to place *R* genes in new structural contexts that may alter expression, whereas intragenic mispairing generates chimeric genes that may encode novel functions. Both types of unequal recombination will also result in altered gene copy number within the cluster (gene duplication on one chromosome and loss on the other) according to the number of genes present in the region between the mispaired recombination sites.

Sequence exchanges (unequal crossovers and/or gene conversions) have been reported in several *R* clusters (Kuang et al. 2005) and are associated with genic diversity, characterized by sequence shuffling and chimeric genes, and haplotypic diversity, characterized by a variable number of *R* homologs within the cluster and a general loss of syntenic/orthologous relationships between haplotypes. Furthermore, unequal recombination, at the *Rp1* cluster and at the *Cf4/9* cluster, has been shown to generate novel *R* haplotypes with resistance specificities that differ from either parent. Interestingly, similar clustering phenomena are seen at (a) virulence loci in multiple, evolutionarily distinct pathogen genomes (Dodds et al. 2006). This accumulated evidence indicates that *R* clusters facilitate rapid evolution via recombinatorial mispairings, generating novel *R* gene sequences that may encode altered specificities or have altered expression patterns.

2.3.3 Signal Transduction Network

The most relevant features of the defense condition indicate that the activation of defense responses in plants is initiated by host recognition of pathogen-encoded molecules called elicitors (e.g., microbial proteins, small peptides and oligosaccharides). A simplified model for signal transduction in plant defense provided by Yang et al. (1997) is given in Fig. 4.4. According to this model the interaction of pathogen elicitors with host receptors (many of which may be encoded by *R* genes) likely activates a signal transduction cascade that involves oxidative burst i.e. reactive oxygen species (ROS), calcium fluxes, ion channel fluxes, G-proteins, nitrogen oxide production, and dephosphorilation of unknown pathogens. Subsequent transcriptional and/or post-translational activation of transcription factors eventually leads to the induction of plant defense genes (Zhu et al. 1996). In addition to eliciting primary defense responses, pathogen signals may be amplified through the generation of secondary plant signal molecules such as salicylic acid (SA) (Durner et al. 1997).

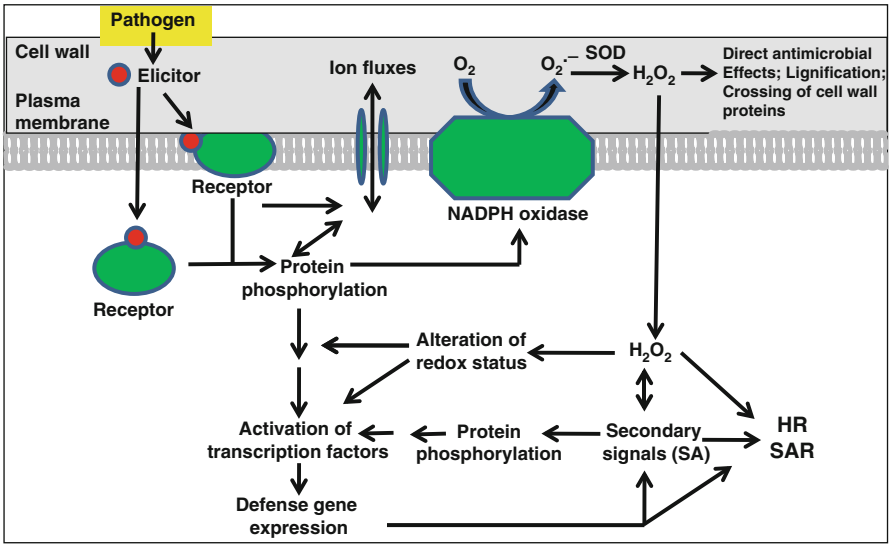

Fig. 4.4 Simplified model for signal transduction in plant defense responses. Host recognition of pathogen elicitors initiates early signaling events such as protein phosphorylation/dephosphorylation, ion fluxes and oxidative burst. Subsequent transcriptional and/or posttranslational activation of transcription factors leads to induction of plant defense genes and biosynthesis of endogenous secondary signals such as SA. Additionally, the activated NADPH oxidase complex generates reactive oxygen species (ROS) such as $O_2^{\cdot-}$ and H_2O_2 that alter the redox status of plant cells and affect defense signaling. SA, ROS, as well as defense genes, all contribute to the development of HR and SAR during plant-pathogen interactions. SOD, superoxide dismutase (Modified from Yang et al. 1997)

Moreover, advances in induced defense signaling research revealed regulators of induced systemic resistance and suggest a model in which jasmonic acid (JA)-related transcription factors play a central role in establishing the primed state for enhanced resistance (reviewed in Van der Ent et al. 2009).

Both primary pathogen elicitors and secondary endogenous signals may activate a diverse array of plant protectant and defense genes, whose products include glutathione S-transferases , peroxidases, cell wall proteins, proteinase inhibitors, hydrolytic enzymes (e.g., chitinases and β~-1,3-glucanases), pathogenesis-related (PR) proteins – PR proteins are host-encoded, abundant proteins induced by pathogens and many of them have antimicrobial activity *in vitro* or when overexpressed in transgenic plants, and phytoalexin – phytoalexins are low-molecular-weight, antimicrobial compounds (e.g., phenylpropanoids, terpenoids), whose synthesis is induced following pathogen infection, plus biosynthetic enzymes, such as phenylalanine ammonia lyase and chalcone synthase (reviewed in Yang et al. 1997). Notably, more recently Clay et al. (2009) have identified a metabolic pathway for glucosinates, previously identified as important in avoiding damage by herbivores, as a component of plant defense response against microbial pathogens.

Table 4.3 Families of pathogenesis-related proteins

Family	Type member	Biochemical properties	Molecular mass range (kDa)
PR-1	Tobacco PR-1a	Unknown	15–17
PR-2	Tobacco PR-2	B-1,3-glucanase	30–41
PR-3	Tobacco P,Q	Chitinase class I, II, IV, VI, VII	35–46
PR-4	Tobacco R	Chitin-binding proteins	13–14
PR-5	Tobacco S	Thaumatin-like	16–26
PR-6	Tomato inhibitor I	Proteinase inhibitor	8–22
PR-7	Tomato P69	Endoproteinase	69
PR-8	Cucumber chitinase	Chitinase class III	30–35
PR-9	Tobacco "lignin forming peroxidase"	Peroxidase (POC)	50–70
PR-10	Parsley "PR-1"	"Ribonuclease-like"	18–19
PR-11	Tobacco class V chitinase	Chitinase class V	40
PR-12	Radish Rs-AFP3	Defensins	5
PR-13	Arabidopsis THI-2.1	Thionons	5–7
PR-14	Barley LTP4	Lipid Transfer Proteins	9
PR-15	Barley OxOa (germin)	Oxalate oxidases	22–25
PR-16	Barley OxOLP	Oxalate oxidase-like protein	100 (hexamer)
PR-17	TobaccoPRp27	Unknown	Unknown

Source: Modified from Van Loon et al. (2006)

2.3.4 Pathogenesis Related Proteins (PR)

The concept of PR was introduced in 1980 to designate any protein encoded by the host plant but induced only in pathological or related situations (Antoniw et al. 1980), including viral, fungal or bacterial infections, parasitic attack by nematodes. The main criterion for inclusion among the PR is that the protein concerned is newly expressed upon infection, although not necessarily in all pathological conditions (Van Loon 1999). The term PR-like protein was proposed to accommodate proteins that are present in healthy plants, being induced essentially in a developmentally controlled, tissue-specific manner, and that are not synthesized in response to pathogen infection or related stresses. The distinction between PR proteins and PR-like proteins became blurred by the discovery of specific PR proteins in healthy tissues and the induction of PR-like proteins upon pathogen attack. Recently Van Loon et al. (2006) introduced the general term "inducible defense-related proteins" to include proteins that are mostly non-detectable in healthy tissues and for which induction at the protein level has been demonstrated after pathogen infection. The PR proteins encompass several different groups of structurally and functionally unrelated proteins, which have been grouped into protein families according to coding sequence similarities, serological relationships, and/or enzymatic or biological activities (Tarchevsky 2001; Ferreira et al. 2007). Seventeen classes are now considered, numbered in the order in which they were discovered, from PR-1 to PR-17 (Table 4.3; Van Loon et al. 2006); members of several of these families were demonstrated to have damaging actions on the structures of the parasite in *in vitro*

bioassays, thus exhibiting antifungal activity and supporting a possible role for these proteins in plant defense. PR-1 and PR-5 (thaumatin-like proteins and osmotins), are thought to create transmembrane pores and have therefore been termed permatins; PR-2 (β-1,3-glucanases) and PR-3, 4, 8 and 11 (chitinases), which attack β-1,3-glucans and chitin respectively, components of the cell walls in most higher fungi (Honeé 1999). PR-6 proteins (proteinase inhibitors) may target nematodes, whereas the PR-7 protein (an endoproteinase) may be involved in microbial cell wall dissolution (Jordá et al. 2000). The PR-9 family may act in cell wall reinforcement by catalyzing lignifications, leading to enhanced resistance against multiple pathogens (Passardi et al. 2004). Some members of the PR-10 family exhibit a weak ribonuclease activity, suggesting a role in defense against viruses (Park et al. 2004). Members of the PR-12 (defensins), PR-13 (thionins) and PR-14 (lipid transfer proteins) families display antibacterial and antifungal activities (Epple et al. 1997; García-Olmedo et al. 1995; Lay and Anderson 2005). PR-15 (oxalate oxidases) and PR-16 (oxalate oxidase-like proteins) proteins generate hydrogen peroxide that may be toxic to attackers or stimulate plant defense responses (Hu et al. 2003). PR-17 proteins, as yet uncharacterized, have been detected in infected tobacco, wheat and barley (Christensen et al. 2002).

2.4 Breeding Strategies

It is a common notion that if a new character is added to a breeding program, either gains in other characters will suffer (for example yield potential), or the program will have to be expanded by a factor which is dependent on the selection rate. The breeder therefore has to consider whether breeding for disease or pest resistance is economically sustainable. This decision depends mainly on the frequency and extent of disease in the area where the crop is to be grown and on the economic damage caused by the parasite. Moreover, the breeder should identify which type of defense mechanism is most suitable for introduction into the crop. He may choose major-gene resistance with complete expression. Advantages of this type of resistance are: (i) the simple inheritance, which is of course very desirable in a breeding program; (ii) the normally complete protection of the crop from the parasite. A risk in choosing complete major-gene resistance is that this type of resistance may turn out to be transitory. There are, however, cases where major-gene resistance has been durable.

The next step in a breeding program for resistance is the identification of an appropriate source of resistance. The genotypic variation within the genotypes of the crop and often also within related species should be investigated. Source for resistance may be found in taxonomic groups that are more or less distantly related to the crop, such as the cultivar itself, commercial cultivars, landraces, wild progenitors, related species and genera. The potential sources of resistance indicated here are listed in the order in which complications for the breeder increase. The main problems are: (i) failure to secure crosses between the crop and the donor species,

(ii) sterility of the interspecific or intergeneric hybrid, and (iii) poor intrachromosomal recombination (Harlan and De Wet 1971). Many generations of backcrossing are usually needed to remove undesirable traits introduced together with the resistance.

2.4.1 Conventional Methods

In many cases, a single *R* gene can provide complete resistance to one or more strains of a particular pathogen, when transferred to a previously susceptible plant of the same species. For this reason, *R* genes have been used in conventional resistance breeding programs for decades (Austin et al. 2002). *R* gene-mediated resistance has several attractive features for disease control. When induced in a timely manner, the concerted responses can efficiently block pathogen growth with light collateral damage to the plant. No input is required from the farmer and there are no adverse environmental effects. Unfortunately, *R* genes are often quickly defeated by co-evolving pathogens. In this context, it is worth noting that durable resistance is defined as "resistance that remains effective when a cultivar is grown widely in environments favoring disease development" (reviewed by Michelmore 2003). The concept of durable resistance makes no assumptions concerning the mechanisms or genetic control of resistance, and has proved a very useful concept in disease resistance breeding. Although it is now easier to identify and deploy useful *R* genes, the problem of durability remains. Many *R* genes lack durability because they can be defeated by a single loss-of-function mutation in the corresponding *Avr* gene (thereby rendering the pathogen 'invisible'). Because individual *Avr* genes often make only incremental contributions to virulence, pathogens can afford to alter or discard an *Avr* gene with little or no fitness penalty (Leach et al. 2001).

Traditional breeding strategies have used *R* genes 'one at a time' in crop monocultures. Such homogeneous host populations exert strong selection for mutation of the relevant *Avr* gene, and then become extremely vulnerable to the emergent pathogen. As an alternative to single-gene deployment, multiple *R* genes ('pyramids') can be bred into individual plant lines (Pink 2002). In principle, these pyramids require the pathogen to accumulate mutations in multiple *Avr* genes to escape detection. This is unlikely to occur if the mutations have a strong cumulative effect on virulence. Another approach is to grow a mixture of lines, each expressing a different *R* gene(s), in the same plot. A susceptible line can be included in the mixture, to reduce the selection pressure for mutations in *Avr* genes (Mundt 2002). A multiline protocol was tested in a study, with striking success (Zhu et al. 2000). Pyramiding and multiline deployment have not been widely used, owing to the time required for breeding assortments of *R* genes into elite cultivars. However, these strategies will become much more practical as the approaches described earlier are further developed. Furthermore, many *R* genes recognize only a limited number of pathogen strains and therefore do not provide broad-spectrum resistance. Furthermore, introgression of *R* genes into elite cultivars by conventional breeding is a lengthy process. However, recent molecular-level insights into the function of R proteins and downstream signal transduction pathways might provide strategies to remedy these deficiencies.

2.4.2 Applications of Marker-Assisted Breeding

Though polygenically inherited forms of resistance is nearly always durable (Parlevliet 1979) this type of inheritance is more difficult to handle in breeding programs. In particular, backcross programs to introduce polygenes from wild relatives of the crop are heavy or not easily managed. In many agricultural crops a low level of infection is acceptable, and partial resistance may be combined with other control measures, such as the application of pesticides. In the specific case of disease resistance, marker-assisted breeding may have a special role. In this respect, pyramiding several major resistance genes into a valuable genetic background is simplified via the use of marker-based selection (Song et al. 1995). Studies have indicated that pyramiding resistance QTLs can achieve the same level or even a higher level of resistance than that conferred by an *R* gene (Castro et al. 2003a, b; Richardson et al. 2006). This should be especially helpful when screening for one resistance gene interferes with the ability to screen for another, a frequent occurrence in disease resistance breeding. Rather than screen sequentially for the inheritance of single resistance (or simultaneously through progeny screens), individuals that have retained all of the genes of interest can be selected based on DNA marker genotype.

Similarly, gene deployment can be speeded-up via the use of marker assisted breeding. This approach, in which cultivars with complementary sets of resistance genes with differing race-specificities are grown by farmers, aims at achieving durable disease protection. In theory, the capacity to pyramid or deploy genes of interest is not restricted to major, single locus resistance genes. With QTL mapping, partial resistance loci can be treated as Mendelian factors and manipulated just like any major gene. This includes resistance alleles that apparently come from otherwise susceptible parents (Wang et al. 2006), providing the potential for selecting transgressively resistant genotypes. Consequently, QTLs from diverse donors can be quickly introduced into a desirable genetic background or deployed in a set of cultivars. Information about the race-specific (or race-nonspecific) nature of partial resistance loci can obviously play a key role in this process.

2.4.3 Alternative Possibilities for Resistance

It is worth noting that alternative possibilities of obtaining resistance have been exploited. These include mutation breeding and transgenic technologies.

Mutation Breeding

A mutagenic treatment may convert a susceptible genotype into a resistant one. If the mutation is a point mutation, the resistant mutant will be identical to the original cultivar, except for its resistance. Usually, however, there are undesirable side-effects of the mutagenic treatment. Several other genes may also have undergone changes,

or the mutation for resistance has undesirable pleiotropic effects. As a consequence, the selection of a resistant mutant should be followed by further breeding efforts (i.e. backcrossing) to produce a commercially acceptable cultivar.

Transgenic Prospects

Currently, there are no fungus-resistant transgenic crops on the market. However, a number of reports have shown promising results in field trials. One example is a potato line that is resistant to late blight (Song et al. 2003). Late blight, caused by the oomycete *Phytophthora infestans*, is infamous as the cause of the Irish potato famine in the nineteenth century and still today causes serious crop losses around the world. The gene that was introduced into the potato line was called *RB* and came from a wild Mexican potato species called *Solanum bulbocastanum*.

There are also prospects for transgenic use of single *R* genes that have previously been proven durable. For example, the pepper gene *Bs2* has provided long-standing resistance against bacterial spot disease, caused by the bacterium *Xanthomonas campestris*. *Bs2* has been cloned from pepper and shown to encode a NB-LRR protein (Tai et al. 1999). *X. campestris* is also a significant pathogen of tomato and a pepper *Bs2* transgene works effectively in tomato against *X. campestris*. Recently cloned *R* genes with potential use against fungal pathogens include the barley *Rpg1* gene and the tomato *Ve1* and *Ve2* genes (Kawchuk et al. 2001). *Rpg1* has provided remarkably durable resistance to stem rust for decades, while *Ve1* and *Ve2* target *Verticillium* species that cause wilt in many different crops. The *Ve* genes can provide resistance to different *Verticillium* species and are functional in potato when expressed as transgenes. The *Rpg1* and *Ve* genes are also interesting from a basic research standpoint because they have novel structural features that distinguish them from previously characterized *R* genes. Additionally, it will be particularly interesting to determine whether these genes can be used as prototypes to identify additional *R* genes by sequence similarity. Additional useful genes might be unearthed through investigations of so-called ‘non-host resistance’ (Heath 2000). This term refers to interactions in which all varieties of a plant species are resistant to all strains of a particular pathogen species (as opposed to intraspecific variability, which is observed for *R* gene-mediated resistance). Because non-host resistance is not genetically variable, this trait has not been amenable to classical genetic analyses. However, experimental tools now available in model plants (e.g. large-scale forward and reverse genetic screens) have made non-host resistance more accessible to genetic dissection. For example, *Arabidopsis* and tobacco are uniformly resistant to many microbes that plague crops (e.g. *P. infestans*, which caused the Irish potato famine) (Kamoun 2001). Moreover, it is worth noting that certain signal transduction components are used in *R* gene resistance and for some non-host resistances (Peart et al. 2002). Thus, it might be possible to identify effective resistance genes against crop pathogens from model species and transfer them to crops. It will be of great interest to determine whether non-host resistance results from natural pyramiding of *R* genes, and/or from use of *R* genes that recognize virulence factors that

are essential for the pathogen to cause disease. Note that non-host resistance might result from several mechanisms (Heath 2000) and it is possible that genetic dissection of non-host resistance will provide unanticipated tools for engineered resistance.

Efforts to transfer *R* genes from model species to crops, or between distantly related crops, could be hampered by a phenomenon termed 'restricted taxonomic functionality' (RTF) (Tai et al. 1999). For example, *Bs2* and several *R* genes from tomato can function as transgenes within related species from the same family (Hulbert et al. 2001) (e.g. tobacco, potato and pepper, which belong to the *Solanaceae*). However, *Bs2* does not function in *Arabidopsis*, nor does the *Arabidopsis RPS2* resistance gene function in tomato (Tai et al. 1999). The molecular basis of RTF is unknown but might reflect an inability of the R protein to interact with signal transduction components that have diverged in the heterologous host (Ellis et al. 2000). It remains to be seen whether RTF is a general attribute of *R* genes. A recent report suggests that it will indeed be possible to transfer certain *R* genes between distantly related species: the *Arabidopsis RPW8* gene provides broad-spectrum resistance to powdery mildew in *Arabidopsis* and in tobacco (Xiao et al. 2003). A solution to the RTF problem might be developed as we gain a deeper understanding of *R* gene signaling.

In bacterial the tomato disease resistance gene *Pto* gives race-specific resistance to *Pseudomonas syringa*e pv. Tomato carrying the *avrPto* gene, by overexpressing *Pto* race- non specifical resistance in transgenic tomatoes exhibited a superior tolerance (Tang et al. 1999). Similarly to fungal resistance, overexpression of PR proteins or transfer of PR protein genes, such as the barley lipid transfer protein (LTP2; Molina and García-Olmedo 1997), from other sources has led to increase resistance against bacterial infection. In several plant species, bifunctional enzymes with lysozyme activity have been detected which are hyphotised to be involved in defense bacteria. After transfer of the bacteriophage T4 lysozyme gene, transgenic potatoes had reduced susceptibility toward *Erwinia carotovora atroseptica* infection (Duering et al. 1993). Transfer of the human lysozyme gene resulted to increase resistance against both fungal and bacterial diseases (Nakajima et al. 1997)

In several studies, transgenic plants expressing cereal ribosomal inactivating proteins (RIPs) were used to test defense properties attributed to this group of proteins (reviewed in Balconi et al. 2010). Research in our laboratory showed that transgenic tobacco plants, expressing the maize RIP *b-32* gene driven by the *wun 1* promoter, had increased protection against infection from the soil-borne fungal pathogen *Rhizoctonia solani*. Similarly, other research with wheat transgenic lines, indicated that maize RIP b-32 protein was effective, as anti-fungal protein, in reducing Fusarium head blight (FHB) symptoms. To further explore the antifungal activity of the maize RIP b-32, transgenic maize plants have been developed containing the *b-32* coding sequence driven by a constitutive *35SCaMV* promoter. In this study four homozygous independent maize transgenic lines, with differential ectopic expression of RIP b-32, were evaluated, in comparison with plants expressing RIP *b-32* only in the endosperm, for response to *Fusarium verticillioides* colonization by leaf tissue bioassays. The identification of progenies with a differential RIP b-32

expression in the leaves was useful for setting up pathogenicity experiments. Transgenic progenies expressing RIP b-32 in leaf tissues resulted as less susceptible than the negative control when evaluated for response to *F. verticillioides* attack, showing significantly reduced colony diameter around the inoculated leaves; a good correlation between the RIP b-32 content in the leaves and the level of resistance to Fusarium attack was observed. Collectively, these results confirm that the incorporation of maize RIP *b-32* gene and the ectopical expression of RIP b-32 protein, appears to be an effective and reliable tool in crop disease management programs.

3 Viral Diseases

Viruses are among the most important kinds of plant pathogens causing severe economic losses in many crops. Genetic resistance is one of the different systems to protect crops from virus infection, including also the control of biotic vectors, the use of virus-free plant materials, and practices for avoiding the transmission. If available, the genetic resistances are still considered the most effective mean for avoiding the viral diseases. The study of virus resistance genes implies several questions regarding the molecular mechanisms involved in the plant-virus interaction. Resistance to viral diseases has been divided, similarly for other pathogens, into two principal families: non-host and host-resistance. Host resistance to plant viruses has been more investigated and considers the case where all genotypes within a plant species show resistance or fail to be infected by a particular virus. More than 80% of reported viral resistances is monogenic. The remainder shows quantitative inheritance. About half of monogenic resistances show dominant inheritance. In most but not all cases, dominance has been reported as complete (Fraser 1986). Furthermore, a third important family of host resistance has been identified, initially in studies involving TMV, i.e. SAR. This response can be activated in many plant species by diverse pathogens that cause necrotic cell death (Ross 1961), resulting in diminished susceptibility to later pathogen attack. As SAR has recently been reviewed (Durrant and Dong 2004), this topic is not discussed further here. Virus-induced gene silencing, another induced defense mechanism to virus disease, has also been reviewed recently (Baulcombe 2004).

3.1 Genetic Basis of Virus Resistance

Plants contain many (>200) resistance genes (*R*) that confer resistance to different strains of viruses. The largest class of *R* genes encodes a NB-LRR type of protein. So far all *R* genes that have been isolated conferring resistance to viruses belong to this class. It is tempting to assume that the *R* gene products directly or indirectly interact with other (host or virus-encoded) factors, but this still needs to be demonstrated. Approximately 30% of the *R virus* genes have been tagged with molecular genetic markers that can be exploited for indirect selection via genotype, for locating *R* genes in plant genomes, and for gene isolation. Relatively few QTL for plant viral resistance have been tagged or genetically mapped.

Considerable progresses were also made in the study of *R* gene structure and in the explanation of mechanisms of resistance and viral evolution. The advent of molecular methods has demonstrated that *R* genes may represent different loci with shared or independent evolutionary history, or different alleles at the same locus. There are cases where resistance alleles at two or more loci are required to observe the resistant response. A well-known example is the *bc-u* system in *Phaseolus vulgaris* for resistance to a wide array of BCMV pathotypes. Resistance is observed only when the *bc-u* locus is homozygous recessive and one or more pathotype-specific genes, *bc-1*, *bc-2*, and *bc-3*, are also homozygous at one or more of three additional loci (Drijfhout 1978). In *Capsicum*, for example, full resistance is observed to another potyvirus, *Pepper veinal mottle virus*, only when the resistance alleles *pvr12* (formerly *pvr22*) and *pvr6* are homozygous (Caranta et al. 1996).

One type of *R* gene cluster contains a set of genes, showing similar inheritance and resistance phenotypes that control very closely related viral genotypes. A notable example of this pattern occurs in *Pisum sativum* where recessive resistance has been mapped to two *R* gene clusters on linkage groups II and VI. This type of *R* gene cluster occurs widely in monocots and dicots. For example, the wheat *Bdv1* allele conferring resistance to *Barley yellow dwarf virus* (BYDV) is linked to fungal *R* genes *Lr34* and *Yr18* (Singh 1993). A comprehensive genome-wide analysis of *R* gene clusters and their distribution within a series of crop genomes linked by comparative genetic mapping has been published for the *Solanaceae* (Grube et al. 2000). This study clearly demonstrated that *R* gene clusters often occur at homologous positions in related genomic regions, even in genera that diverged tens of millions of years ago. These clusters may therefore consist of evolutionarily related sequences that diverged to control very different pathogen groups.

The typical R-gene-mediated responses include host-cell death (HR) like that occurring in fungi and bacteria and will not here repeated. The induction of this response is preceded by a specific recognition of the virus, and in many cases this is based on matching (dominant) gene products of the plant (produced from dominant resistance genes, *R* genes) and the virus (avirulence genes). Dominant resistance is frequently associated with the HR response (Fraser 1986), possibly due to the frequent use of HRas a diagnostic indicator for field resistance by plant breeders. HR, induced by specific recognition of the virus, localizes virus spread by rapid programmed cell death surrounding the infection site, which results in visible necrotic local lesions. HR-mediated resistance is a common resistance mechanism for viruses and for other plant pathogens. Because the extent of visible HR may be affected by gene dosage (Collmer et al. 2000), genetic background, environmental conditions such as temperature, and viral genotype, etc., schemes that classify or name virus *R* genes based on presence or absence of HR may obscure genetic relationships. Over the past 10 years significant advances have been made in the understanding of the molecular basis of the HR-mediated resistance. More than 40 plant *R* genes showing monogenic dominant inheritance have been cloned (reviewed in Kuang et al. 2005). Several of these confer resistance to plant viruses (Martin et al. 2003).

Few resistance genes have proved exceptionally durable. Genetic resistance often fails because a resistance-breaking (RB) pathogen genotype increases in frequency.

Based upon data obtained predominantly from plant resistance to fungi, polygenic resistance is often presumed to be more durable than monogenic resistance. The analysis of polygenic resistance traits tends to be much more complex than monogenic or oligogenic traits, so researchers often focus on monogenic resistance because it can be studied and utilized more readily.

3.2 Resistance Mechanisms

The natural resistance mechanisms underlying virus resistance in plants have been largely treated in several reviews (Goldbach et al. 2003) and will be here briefly summarized. The main finding emerging from these studies indicates that the genetic material of viruses may be either DNA or RNA, and may be single- or double-stranded. Approximately 77% of characterized plant viruses possess a single plus-(messenger) sense strand of RNA. Infection of plant tissue requires damage to the cell wall and/or plasma membrane which, for insect-borne viruses, is achieved by the penetration by the insect stylet during feeding. Once inside the cell, the virus particle is uncoated to release its nucleic acid, and for at least some plus-stranded RNA viruses, such as tobacco mosaic virus (TMV), uncoating is achieved by cytoplasmic ribosomes which also translate the RNA. Plant virus nucleic acids are not integrated into the host genome. Common translational products amongst most, if not all, viruses, include coat protein, one of more proteins involved in the replication process, and factors involved in the systemic transmission of the virus away from the site of infection. The genomes of cauliflower mosaic virus (CaMV) and tobacco mosaic virus (TMV) contain seven and five open reading frames (ORFs), respectively, which function in the replication and movement of the viral DNA, symptom development, and encapsidation. Genome replication for positive-strand RNA viruses occurs in the cytoplasm, apparently utilizing the translation apparatus of the host. Plant virus movement proteins (MPs), in association with various components of the cytoskeleton of the host cell, facilitate transport of nucleoprotein complexes or virus particles into adjacent cells by way of modified plasmodesmata, channels between plant cells. The processes controlling long-distance transport of virus particles or viral nucleic acids within the phloem are distinct from those controlling movement between mesophyll cells. Once inside the phloem, a rapid movement of virus particles has been documented; for some viruses (e.g. TMV), the coat protein (CP) is necessary for this process; however, for other viruses the CP protein may not be involved.

3.3 Breeding for Viral Diseases

3.3.1 Conventional Strategies

One of the most important durable successes of plant breeding for virus resistance was the development of sugar beet with the source of resistance to rhizomania (Fig. 4.5). The disease is caused by the virus BNYVV (Beet Necrotic Yellow Vein Virus)

Fig. 4.5 Section of sugar beet root infected by rhizomania

transmitted by the fungus *Polymyxa betae*. Rhizomania is widespread in many Europeans countries and available data indicate a spread on 60% of total sugar beet cultivated area. Damage to the sucrose production can cause up to a 80–100% yield loss (Biancardi et al. 2002). Three forms of virus have been classified (A, B and P) according to the structure of RNA.

The rhizomania symptoms are evident especially on the roots as: (i) excessive proliferation of the rootlets assuming a beard-like appearance around the tap root; (ii) constrictions of the root tip leading to a wineglass shape; (iii) necrotic rings in the root tip section. Diseased beets, if analyzed, show low sugar content, processing quality, etc. Immunoenzymatic tests (ELISA) performed on the roots can easily quantify the infection.

A and B types there are often associated, while the P is always alone and has been localized only near Phitivier (France). The use of ELISA test for the determination of virus content in the storage root has significantly contributed to the selection of resistant varieties. These genotypes have allowed survival of the sugar beet crop in many cultivated areas. After the discovery of the first resistant materials of

Italian origin, derived from sugar beet progenitor *Beta maritima*, with multigenic resistance, defined "type Alba", more efficient sources of monogenic resistance were introduced, also these derived from *Beta maritima*, allowing optimal productive performance also in infected soils. The sugar beet cultivars with the "Rizor" source of resistance, developed in 1985 by De Biaggi, was the first variety showing an optimum level resistance in heavily infected fields. Later, after some years, the source "Holly" has been isolated on materials of USDA's origin. These two sources of resistance have a good heritability and few cycles of selection are sufficient to improve the resistance trait. Resistance such as "Holly" is classified as monogenic, like that of type "Rizor". The two resistant traits have been mapped very close on several genetic maps. Other sources of resistance have been found recently in these wild beets also belonging to the *Beta vulgaris* L. ssp. *maritima* (L.) Arcang, that is the ancestor of the cultivated beets. Among these, the source named "WB42", developed at the USDA in Salinas (California, USA), is stirring a considerable interest. Studies are still in progress to determine the relationship between the two major sources of resistance (Holly and Rizor).

Genotypes carrying the monogenic sources of resistance frequently exhibit different levels of expression, probably due to the presence of minor genes interacting with the major allele in heterozygous individuals. The resistant varieties used today, when tested in severe disease conditions applied in greenhouses, display no more than 80% resistant plants. Improvement of this percentage should allow better sugar yield even in severely diseased fields. Since the resistance in commercial varieties is usually transmitted by the pollinators, this goal should be possible using varieties in which all plants carry the genes of resistance at least in heterozygous conditions. This result is becoming possible by: (i) using resistant pollinators and seed-bearers; (ii) analyzing with molecular markers for rhizomania resistance genes all pollinating and/or seed-bearing beets employed in seed production; and (iii) discarding the recessive and, when possible, the heterozygous plants. In addition, further sugar yield improvements should be possible combining in the same variety the different sources of resistance. This would be essential where the known sources of resistance appear to be overcome by suspected mutations of BNYVV, or in presence of the more pathogenic strains of the virus (Liu and Lewellen 2007; Panella and Lewellen 2007).

3.3.2 Transgenic Strategies

A knowledge of the molecular biology of aspects of virus function has led to the proposal of three general strategies for plant protection against viruses using genetic engineering techniques: (1) modified cross-protection; (2) the use of satellite nucleic acids; and (3) the use of anti-sense RNA.

Transgenic crop varieties have been successfully deployed to control viral diseases. One of the classical examples is the success with the genetically engineered papaya, which virtually rescued the papaya industry in Hawaii from the threat of the dreaded ring spot disease (Yeh et al. 1998). The transgenic approach would be more appropriate in

situations where sufficient levels of resistance to the virus are not available in the related germplasm or the resistance is difficult to transfer by normal crossing techniques because of either reproductive isolation or linkage with other undesirable traits. The production of virus-tolerant transgenic plants has been based on several approaches which follows into two categories: protein-mediated and RNA-mediated protection (reviewed by Prins et al. 2008). In most instances, a gene coding for the complete viral protein or part of a viral protein has been introduced into the crop by transformation.

One strategy used to obtain virus-resistant plants is to transfer genes from the pathogen itself into the plant (pathogen-derived resistance). The most widely used approach is to express the virus coat protein in transgenic plants. In theory, the expression of viral genes disrupts viral infection or symptom development. The first virus-resistant variety to be grown was papaya ringspot virus (PRSV)-resistant papaya (Ferreira et al. 2002; Gonsalves 1998). The GM variety contains a gene that encodes a PRSV coat protein, a strategy that mimics the phenomenon of cross protection. In true cross protection, infection by a mild strain of a virus induces resistance to subsequent infection by a more virulent strain (reviewed in Culver 2002). This approach has been extended to other plants, for example rice (Hayakawa et al. 1992), plum tree (Ravelonandro et al. 1997), tomato (Kaniewski et al. 1999), and peanut (Magbanua et al. 2000). Field trials have also been performed, in the USA, with coat protein-mediated virus-resistant wheat, soybean, sugarcane, sugar beet, cucumber, sweet potato, grapefruit, pineapple, and papaya (USDA 2002).

Another form of pathogen-derived resistance is the use of viral replicase genes (or RNA-dependent RNA polymerase genes), which presumably act by post-transcriptional gene silencing. This technique has been used to confer resistance to potato leafroll virus in potato, to barley yellow dwarf virus in oats, cucumber mosaic virus in tomato, rice tungro, spherical virus in rice, and wheat streak mosaic virus in wheat (Koev et al. 1998; Gal-On et al. 1998; Huet et al. 1999; Sivamani et al. 2000). Because different degrees of virus resistance have been obtained with coat protein-mediated resistance, attempts have been made to ameliorate resistance against cucumber mosaic virus via satellite RNA, especially in tomato (Stommel et al. 1998). This approach has caused controversy, however, because a single-point mutation in the satellite RNA can transform it into a harmful necrogenic form (Tepfer 1993). To protect plants against more than one virus, RIPs, have been expressed in transgenic plants. RIPs are strong inhibitors of protein synthesis and, depending on the plant species from which they originate, they have different levels of toxicity against different hosts. Poke weed antiviral protein (PAP) confers resistance to PVX and PVY in transgenic potatoes and PAPII confers resistance to TMV, PVX, and fungal infections in tobacco (Balconi et al. 2010).

On a more experimental scale are approaches to achieve virus resistance by using antibodies against the virus coat protein. Such antibodies can neutralize virus infection, presumably by interacting with newly synthesized coat protein and disrupting viral particle formation Xiao et al. 2000). Similar to RIPs, broad-spectrum antibodies might be used to protect plants against a wider range of viruses, as has been demonstrated for poty viruses.

Notably, virus-resistant transgenic crops, which offer numerous benefits to growers and consumers, need to be deployed safely after due assessment of safety considerations. However, risk assessment studies need to be realistic to provide valuable assistance to regulatory authorities for the safe and timely release of such crops (Fuchs and Gonsalves 2007).

4 Insect Diseases

Crop losses due to insects and nematodes, estimated at 10–20% for major crops, are a significant factor in limiting crop yields. To overcome this problem modern agriculture uses a wide range of insecticides and nematocides to control pest damage. However, chemical control of pests, in addition to being expensive, frequently results in negative environmental effects. The development of insect- and nematode-resistant plants is therefore an important objective of plant breeding strategies with relevant implications for both farmers and the seed and agrochemical industries. In this section attention will mainly be given to plant response to insects, indicating some specific examples related to nematodes.

4.1 Nature of Plant Resistant Mechanisms

According to Maxwell and Jennings (1980) insect resistance is defined as "those heritable characteristics possessed by the plant which influence the ultimate degree of damage done by insects". Resistance is relative and is measured by using susceptible cultivars of some species as controls. Additionally, host-plant resistance may be the result of a series of interactions between insects and plants which influence the selection of plants as hosts and the effects of plants on insect survival and multiplication. Within this context three mechanisms of plant resistance have been described: (i) non-preference (or antixenosis), (ii) antibiosis and (iii) tolerance (Painter 1958). Tolerance differs from non-preference and antibiosis in its mechanism: non-preference and antibiosis require an active insect response or lack of response. However, tolerance is more subject to variation as a result of environmental conditions than non-preference and antibiosis. The age or size and general vigor of the plant and size of the insect-resistant population also strongly influence the degree of tolerance.

In their long association with pests and pathogens, plants have evolved an impressive arsenal of defensive tools. In this respect, natural pest resistance mechanisms occurring in higher plants have been classified into preformed and inducible resistance mechanisms and throughout the last century agricultural pest control has attempted to harness these mechanisms wherever possible (reviewed in Howe and Jander 2008). Furthermore, plant traits conferring resistance to insect pests may also be classified according to the manner in which they are regulated. Some traits are expressed constitutively under the control of hard-wired develop-

mental programs, irrespective of the insect threat level. For example, reproductive tissues typically accumulate large amounts of defensive proteins and metabolites. In contrast to these preformed barriers, herbivore-challenged plants mount defense responses at the site of tissue damage and, in many cases, systemically in undamaged tissues. Moreover, to induce defensive traits, plants can minimize the fitness consequences of tissue loss by activating physiological processes, such as sequestration of sugars in below-ground tissues, which allow plants to better tolerate insect damage.

4.2 Genetic Bases in Imparting Insect Resistance

At least 30 major or single genes for insect resistance have been tagged or mapped in various crops (e.g. maize, rice, wheat, tomato, mung bean, apple), conferring resistance to species from 5 orders: *Homoptera*, *Hemiptera*, *Diptera*, *Lepidoptera* and *Coleoptera* (reviewed in Yencho et al. 2000). Each gene is known to confer resistance to only one insect species or to closely related species within the same genus. The *Mi* gene from tomato provides an interesting example because it was originally identified as a dominant gene for resistance to a root-knot nematode, *Meloidogyne incognita*. Further studies have shown that it is located at the same locus as that previously known as *Meu-1*, which provides resistance to some isolates of the potato aphid (*Macrosiphum euphorbiae)* and to the silverleaf whitefly *(Bemisia tabaci*) (Nombela et al. 2003). Interestingly, *Mi* is one of the few examples of genes for insect resistance cloned from a plant; it is a member of the nucleotide-binding, leucine-rich (MBS-LRR) repeat family of resistance genes, many members of which have been found to confer isolate-specific resistance to viruses, bacteria, fungi, and nematodes (Hammond-Kosack and Parker 2003). Another NBS-LRR protein, encoded by the melon *Vat* gene, confers increased resistance to both *Aphis gossypii* (cotton aphid) and the transmission of plant viruses by this aphid species (Dogimont et al. 2007). By analogy to plant defense against pathogens, these findings suggest a gene-for-gene interaction between the plant and the insect. However, the presumed avirulence proteins in aphid saliva have not yet been identified. Similarly, research on nematodes has identified at least 15 genes conferring resistance to nematodes in various crop species (reviewed in Jung et al. 1998). For example 2 genes for nematodes resistance have been cloned on the basis of their chromosomal position and identified by genetic complementation. The first was $Hs1^{pro\text{-}1}$ from sugar beet that confers resistance to the beet cyst nematode (*Heterodera schachtii*). The second was *Mi-1* which is responsible for the hypersensitive reaction of tomato roots after infection with *Meloidogyne* spp.

4.3 Gene Mapping and Molecular Markers

Molecular markers have been used to map the above-cited genes for insect resistance in most of the major crop species and to map QTLs for resistance to 11 species of insects from 3 orders (*Homoptera*, *Lepidoptera* and *Coleoptera*) in 6 plant species (reviewed in Yencho et al. 2000). Traits evaluated include direct measures of insect fitness or behavior (e.g. larval weight, population growth, ovipositional preference); plant damage (e.g. scores on scales of 1–9, tunnel length, leaf area defoliated); plant morphology (e.g. trichomes, leaf toughness); and plant chemical content or enzyme activity (e.g. acyl sugars, maysin, polyphenol oxidase). For any single trait scored, the number of QTLs identified varies from 1 to 10, and the percentage of variation explained by any single QTL varies from 1.3% to 58%. More recently, Pfals et al. (2007), by mapping in *Arabidopsis thaliana* QTLs for resistance agents of 2 cruciferan specialist lepidopteron herbivores (*Pieris Brassicae* and *Plutella xilostella*), identified 6 QTLs for resistance against *Pieris* herbivory and found only a weak QTL for *Plutella* resistance. Similarly Omo-Ikerodah et al. (2008), in genetic mapping QTLs affecting resistance to flower bud thrips (*Megalurotrhips sjostedti*) in cowpea, found association between 23 DNA markers and resistance to flower bud thrips. QTLs with effects on resistance were identified in five linkage groups which accounted for 77.5% of the phenotypic variation for resistance. Moreover, molecular markers can greatly speed up the identification of new resistant genes. This aspect is well documented for the Hessian fly (Hf), *Mayetiola destructor* (Say) (*Diptera:Cecidomyiidae*), one of the most destructive pests of wheat worldwide. To date, 31 major *Hf*-resistance genes (named *H1* through *H31*) have been identified from wheat and its relatives (Williams et al. 2003), but distinguishing new genes is difficult by traditional phenotypic differentiation with biotypes. Liu et al. (2005), using molecular markers, have identified a new gene or a new allele of an *H* gene, (tentatively named *Hdic*) on the short arm of wheat chromosome 1A, which confers a high level of resistance to Hf. of a known *H* gene on chromosome 1A. The broad spectrum of resistance conferred by the *Hdic* gene makes it valuable for developing Hf resistant wheat cultivars. Selection for nematode resistance has a long tradition in potato breeding; both polygenic and monogenic types of resistance have been mapped with molecular markers (cf. Jung et al. 1998). These include the *Gro1*, genic resistance to all pathotypes of the root cyst nematode . *Globodera rostochiensis*. Similarly, in soybean cyst nematode different types of resistance to *Heterodera glycine* have been mapped with molecular markers such as the *Rhg1* and the *Rhg4* loci. In barley, the nematode resistance loci *Ha1 and Ha2* have been mapped to chromosome 2, while a new gene, *Ha4* has been mapped to chromosome 5. In *Triticeae*, two loci, *Cre1* and *Cre3*, have been mapped.

In addition to their utility as selectable markers to facilitate breeding efforts, molecular markers can be employed to increase our understanding of the mechanisms of plant resistance to insects. By mapping QTLs encoding for specific plant physical and/or biochemical attributes associated with insect resistance, and comparing the locations of these QTLs with those identified for the phenotypic expression of resistance to a pest

species, valuable insights can be obtained into the nature of resistance. Often, these insights have both basic and applied implications that can be used to develop insect-resistant crops more efficiently. The advantages of these techniques is well illustrated by researches carried out by Byrne et al. (1998). These authors have used molecular markers and QTL mapping techniques to unravel the genetic mechanisms of resistance in maize to the corn earworm (CEW), *Helicoverpa zea*, larvae which cause considerable direct yield loss as well as development of kernel-rotting fungi. Moreover, significant negative correlations were reported between maysin concentrations of fresh silks and growth of CEW larvae in dried-silk bioassays (Wiseman et al. 1996). Because C-glycosyl flavones are synthesized via a branch of the well characterized flavonoid biosynthetic pathway, Byrne et al. (1998) hypothesized that loci of that pathway would explain a large portion of the quantitative variation in maysin concentration, and by extension, resistance to CEW. These loci were proposed as "candidate genes" in a series of QTL analyses. (A candidate gene is one that is hypothesized to affect expression of the trait of interest, either *a priori* based on knowledge of trait biology, or *a posteriori*, guided by similar locations of QTLs and genes of known function).

4.4 Direct Defense Responses

Upon attacks by insects, individual plants rely on a matrix-like variety of defense mechanisms, involving physical barriers (leaf toughness and trichomes), toxic or anti-nutritive secondary metabolites, synthesis of defensive proteins, volatile attractants and extrafloral nectars, and/or recruitment of predators and parasitoids, as well as the reallocation of resources upon attack. Additionally, a plant's defense arsenal depends on various genetic, ontogenetic, and environmental factors, which together modulate the complex defensive phenotype and outcome of the interaction.

Although it is known that plants change their primary and secondary metabolism in leaves to resist and tolerate aboveground attack, there is little awareness of the role of roots in these processes (reviewed in Erb et al. 2009). This is surprising given that plant roots are responsible for the synthesis of plant toxins, play an active role in environmental sensing and defense signaling, and serve as dynamic storage organs to allow re-growth. Studying roots is therefore essential for a better understanding of resistance and tolerance to leaf-feeding insects and pathogens. Indeed roots are increasingly recognized to synthesize secondary metabolites implicated in leaf defenses. However, the active role of roots in plant resistance against leaf herbivory implies shoot-root communication. A model of a defensive shoot-root-shoot loop in plant defense reaction has recently been provided by Erb et al. (2009) to which readers are referred for details.

4.4.1 Defensive Metabolites

A remarkably diverse array of over 200,000 low-mass natural products, known as secondary metabolites, are produced by plants. These include alkaloids, furanocoumarins, tannins, saponins, glucosinolates, cyanogenic glycosides, phenolics and benzoaxinoids. This rich diversity results in part from an evolutionary process driven by selection for improving chemical defense against microbial and herbivorous predation. For instance, several terpenoids, the most metabolically diverse class of plant secondary metabolites (>40,000 known structures), play a role in plant defense (Aharoni et al. 2005). The alkaloids, widely distributed secondary metabolites that are best known for their metabolic effects in mammals likely evolved as a defense against insect herbivory.

Benzoxazinoids are also secondary metabolites that are effective in defense and allelopathy. They are abundant in grasses, including the major agricultural crops, i.e. maize and wheat, and other Gramineae, and are synthesized in seedlings and stored as glucosides (glcs) in the vacuole (see Frey et al. 2009, for a recent review). A specific glycosidase, located in the chloroplast, catalyzes the formation of the toxic aglucon when a cell is damaged and disintegrates. DIBOA [2,4-dihydroxy-2H-1,4-benzoxazin-3-one and its C-7-methoxy] derivative DIMBOA are the prevalent representatives of benzoxazinoids in plants. Figure 4.6 gives a schematic representation of the benzoxazinoid biosynthetic pathway in maize as provided by Frey et al. (2009). It has also been shown that DIMBOA is an enzyme inhibitor of α-chymotrypsin, aphid cholinesterase and plasma membrane H^+-ATPase. The correlation between DIMBOA content and protection against insect feeding damage was especially investigated, indicating that DIMBOA can act as a feeding deterrent and reduce the viability of insect larvae, with practical application in developing maize plants with improved insect resistance, as previously suggested by Klun et al. (1970).

Plants also contain significant quantities of various polyphenolic acids, as well as their glycosides and esters. These compounds are implicated in two defense mechanisms: the phenolic fortification of cell walls and the deterrent effect of fiber content (Bergvinson et al. 1995). Free phenols, mainly 4-coumaric and ferulic acid, were implicated as factors contributing to resistance of maize against ECB and the maize weevil (*Sitophilus zeamais*), and recently, to pink stalk borer (*Sesamia nonagrioides*; Santiago et al. 2006). Notably, the transgenic expression of wheat oxalate oxidase in maize significantly increased the phenolic concentrations, mainly ferulic acid. Field testing showed that the transgenic maize exhibited more resistance to ECB than its non-transgenic counterpart. It was suggested that transgenic oxalate oxidase elicits defense responses by generation of H_2O_2 and activating jasmonic acid signaling (Mao et al. 2007).

In addition to possible synergistic effects, metabolic diversity in toxin production by individual plants can also provide defense against multiple herbivores with different feeding styles or resistance mechanisms. Recent work on glucosinolates demonstrates how natural selection for a diverse profile of secondary metabolites can provide defensive specificity. Glucosinolates are found almost exclusively in Brassicales (Hansen et al. 2008); nearly 40 different glucosinolates have been found in *A. thaliana*, and more than 100 breakdown products are likely formed after activation by the enzyme myrosinase. Experiments with four insect herbivores showed

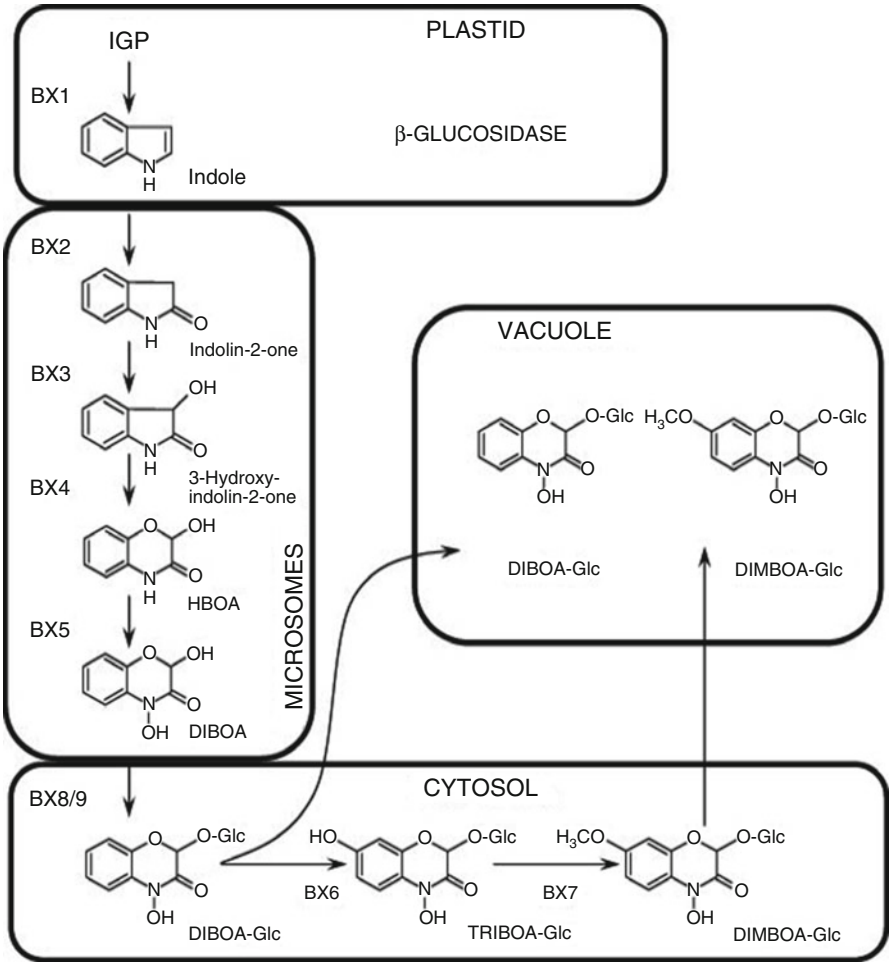

Fig. 4.6 Enzymes and intermediates of benzoxazinoid biosynthesis in maize. In this scheme the BX1 is encoded by the Bx1 gene, a homolog of the Trp synthase a-subunit, catalyses the formation of indole in the first specific pathway. The introduction of four oxygen atoms into the indole moiety that yield DIBOA is catalysed by four cytocrome P450 monooxygenases, termed BX2 to BX5. DIBOA-glc is the substrate of the dioxygenase Benzoxazinless6 (Bx6) and the produced 2,4,7-trihydroxy-2H-1,4-benzoxazin-3-(4H)-one-glc is metabolized by the methyltransferase Bx7 to yield DIMBOA-glc. The enzymatic function of BX1-BX5 is indicated. DIMBOA and DIBOA are accepted as substrates by BX8, while DIMBOA is the preferred substrate of BX9. DIMBOA-glucoside is the predominant benzoxazinoid glucoside in young maize plants. IGP; indole-3-glycerolphosphate, TRIBOA-Glc; TRIBOA-glucoside (Adapted from Frey et al. 2009)

that tryptophan-derived indole and methionine-derived aliphatic glucosinolates have differing effects on Hemiptera and Lepidoptera (Mewis et al. 2005). Indole glucosinolates, which break down in the absence of the activating enzyme myrosinase (Barth and Jander 2006), provide a better defense against *Myzus persicae* than do the more stable aliphatic glucosinolates (Kim and Jander 2007). Almost all genes

required for the production of glucosinolates, a diverse class of metabolites found in the model plant *A. thaliana* and other *Cruciferae*, have been identified (Halkier and Gershenzon 2006). As an example of how such knowledge of biochemical pathways can be applied to change plant immunity to herbivory, *A. thaliana* was engineered with three enzymes from grain sorghum to produce the cyanogenic glycoside dhurrin, thereby enhancing resistance to yellow-striped flea beetle *(Phyllotreta nemorum;* Tattersall et al. 2001).

Many defensive compounds are potentially toxic to the plants that produce them. Therefore, the storage of relatively benign precursors that are activated by herbivory is a recurring theme in plant biology. For instance, all three defensive systems mentioned in the previous paragraph include compounds that are sequestered in plants, but not activated until the onset of herbivory. DIBOA is stored as inactive DIBOA-glucoside, glucosinolates are enzymatically activated to produce toxic breakdown products, and the respiratory inhibitor hydrogen cyanide is released from cyanogenic glycosides during herbivory attack.

4.4.2 Defensive Proteins

Insect feeding triggers the expression of plant defensive proteins that exert direct effects on the attackers. The best known plant proteins supposedly involved in defense mechanisms are lectins, ribosome-inactivating proteins (RIPs), inhibitors of proteolytic enzymes, chitinases, and glycohydrolases (reviewed in Carlini and Grossi-de-Sà 2002).

Protease inhibitors (PIs), which impair various mechanistic classes of digestive proteases in the insect midgut, have been thoroughly studied for their role in the active defense response (Ryan 1990). Inhibition of gut proteases by PIs results in amino acid deficiencies that negatively affect the growth and development of the herbivore (Lison et al. 2006; Zavala et al. 2004). The effectiveness of PIs as a defense is often thwarted by the insect's adaptive ability to express digestive proteases that are insensitive to the host plant complement of PIs or that inactivate PIs (e.g. Bayes et al. 2005; Rivard et al. 2004). PIs are synthesized and stored in seeds and tubers of plants and the expression of some *PI* genes is induced in response to mechanical wounding or insect damage. For instance, local and systemic induction of expression of *MPI*, a maize protease inhibitor gene, efficiently inhibits elastase and chymotrypsin-like activities from the larval midgut of *Spodoptera littoralis* (Cordero et al. 1994); this suggests that *MPI* is a factor of maize insect resistance. Similarly, strains of tropical maize germplasm were found to exhibit resistance to Lepidoptera. In these strains, larval feeding led to the induction of a unique cysteine proteinase, *Mir1-CP*; proteinase accumulation was detected at the feeding site, localized predominantly in the phloem of minor and intermediate veins and was correlated with a significant reduction in larval growth (Lopez et al. 2007).

The plant's defensive protein arsenal also includes enzymes that disrupt insect digestive physiology and other aspects of food consumption. Members of the cysteine protease family of enzymes, for example, disrupt the chitin-rich peritrophic membrane that protects the gut epithelium (Konno et al. 2004; Mohan et al. 2006).

Plant lectins and chitinases may also target carbohydrate containing components of the insect gut (Lawrence and Novak 2006; Peumans and Vandamme 1995). Oxidative enzymes such as polyphenol oxidase (PPO) and lipoxygenase (LOX) covalently modify dietary protein through the production of reactive *o*-quinones and lipid peroxides, respectively (Wang and Constabel 2004). Because catalysis by O_2-dependent enzymes is limited by low oxygen levels in the foregut and midgut of some insect species (Thipyapong et al. 1997), an alternative possibility is that PPO and LOX act rapidly (i.e., within seconds) during tissue mastication by insect mouthparts.

The discovery of novel defensive proteins can be facilitated by proteomic analysis of gut content and feces (frass) of insect herbivores. This approach is based on the premise that defensive proteins are relatively resistant to gut proteases and, as a consequence, are highly enriched during passage of the food bolus through the insect. Application of this procedure to the tomato-reared *Manduca sexta* larvae led to the identification of isoforms of arginase and threonine deaminase , which degrade the essential amino acids arginine and threonine, respectively, in the lepidopteran midgut (Chen et al. 2005).

4.4.3 Volatile Defenses

Plants synthesize and emit blends of volatile organic compounds (e.g. terpenoids, green leafy volatiles, and ethylene) in response to damage from herbivorous insects (reviewed in Unsicker et al. 2009). The induced volatiles are proposed to serve a variety of physiological and ecological functions, including the attraction of natural enemies of herbivores, which is termed “indirect defense”. Advances in plant biotechnology have allowed investigators to manipulate plant volatile emissions and demonstrate their defensive function in laboratory studies with model plants (Schnee et al. 2006; Kappers et al. 2005). The specificity of this interaction has recently been proved by Degenhart et al. (2009), by restoring the emission of a specific belowground signal emitted by insect-damaged maize roots. According to these authors, the sesquiterpene (E)-β-caryophyllene is highly attractive to the entomopathogenic nematode *Heterorhabditis megidis.* It was shown that (E)-β-caryophyllene is emitted by ancestral maize and European lines, but most American varieties have lost this ability and do not attract the nematode, which is therefore much less effective as a control agent of the larvae of the western corn rootworm, *Diabrotica virgifera virgifera*, a serious root pest in maize cultivation. To restore nematode attractions, a non-producing maize line was transformed with a *caryophyllene-synthase* gene from oregano, resulting in constitutive emissions of (E)-β-caryophyllene. In rootworm infested field plots, in which they released nematodes, transformed plants received significantly less root damage and had 60% fewer adult beetles emerge than isogenic lines. This demonstration that plant volatile emissions can be manipulated to enhance the effectiveness of biological control agents opens the way for a novel ecologically sound pest control strategy.

4.4.4 Signal Transduction Pathways

There is relatively little information about the signal transduction pathways that connect insect-specific elicitors to the plant defense responses they generate. Evidence indicates that the calcium ion (Ca^{2+}) is involved as a second messenger in many plant signaling pathways, including responses to herbivory (Maffei et al. 2007). Transient increases in cytosolic Ca^{2+} levels activate calmodulin and other calcium-sensing proteins that subsequently promote downstream signaling events, including protein phosphorylation and transcriptional responses. Although no complete mitogen-activated protein kinase (MAPK) signaling cascades (Pitzschke et al. 2009) leading to insect resistance has been identified, there is evidence that such pathways play a role in some plant-insect interactions. In tomato, *Mi-1* mediated resistance was attenuated when expression of certain MAPKs and MAPK kinases was reduced by virus-induced gene silencing (VIGS) (Li et al. 2006). VIGS studies in tomato also showed that at least three MAPKs are required for systemin-mediated defense responses to *Manduca sexta* (tobacco hornworm) (Kandoth et al. 2007).

Many inducible defenses are expressed rapidly (i.e., within hours) in undamaged leaves of herbivore-challenged plants. This systemic response, which has been reported in a wide range of plant species, provides effective resistance to future insect attacks (Karban and Baldwin 1997). Since the discovery of this phenomenon more than 35 years ago (Green and Ryan 1972), research effort has been devoted to the identification of systemic wound signals and the underlying mechanisms by which they are produced, transported, and perceived. In this respect, it was found that systemin, which is a strong peptide elicitor of PI expression in *Solanum lycopersicum*, appears to enhance systemic defenses by amplifying jasmonate synthesis in damaged leaves (Schilmiller and Howe 2005).

The plant hormone jasmonic acid (JA) and related signaling compounds (collectively referred to as jasmonates) appear to be ubiquitous signals for tissue injury and for the subsequent activation of defense responses to many, if not most, insect herbivores (Howe and Jander 2008).

Recent studies with *Nicotiana attenuata* indicate that fatty-acid amino acid conjugates (FACs) in oral secretions of *M. sexta* elicit rapid activation of MAPK activity and defense-related genes in undamaged areas of the attacked leaf (Wu et al. 2007). FAC binding to a hypothetical receptor was proposed to generate a rapidly acting, short-distance mobile signal that triggers MAPK cascades in the damaged leaf. This intraleaf systemic response is followed by the production of a second mobile signal (e.g., jasmonate) that initiates PI expression in distal undamaged leaves. These findings are consistent with the idea that multiple intercellular signals, acting over a range of distances, mediate the complex spatiotemporal responses of plants to herbivory. The fact that both *S. lycopersicum* systemin and FACs positively regulate jasmonate synthesis via a MAPK cascade (Kandoth et al. 2007) suggests that parallel signaling pathways initiated at the plant-insect interface may converge on the jasmonate pathway. In this context, evidence has been provided in the past few years to indicate that the jasmonate family of signaling compounds is involved in endogenous regulation of plant resistance to insects.

4.4.5 Breeding Strategies for Improving Plant Pest Resistance

Although there have been many notable successes in conventional breeding for improved plant resistance to insects, the breeding process is often slow and laborious, and sufficient levels of resistance have not been achieved for some pests. However, recent progress in plant transformation technologies has made it possible to produce new genetically modified cultivars with improved resistance to insect pests by genetic engineering. In addition, with advances in biotechnology, breeding of horizontal resistance, whereby resistance is based on many genes, along with genetically enhanced sustainable pest resistance with fusion genes, offer new strategies in improving plant insect resistance (Wan 2006). Genomic tools are enabling significant progress in the understanding of nematode diseases (reviewed in Bellafiore and Briggs 2010). Genome-wide expression profiling of infected plants has revealed genes that respond to infection and functional tests show they can mediate the interaction with nematodes. Several candidate effectors from nematodes have been identified and functional tests using RNAi have supported their putative roles in pathogenesis. These will increase the possibility to design novel approaches to developing crops resistant to nematode injuries.

Marker Assisted Selection

Once a major gene or QTL has been identified and mapped, marker assisted selection (MAS) and/or mapbased gene cloning can be initiated. Particularly, MAS offers the opportunity of combining different genes for a given pathosystem in a single genotype (gene pyramiding). A prerequisite for gene pyramiding is that loci are not allelic. Moreover, it would be wise to determine if the resistance genes targeted for introgression are indeed potentially durable. In choosing the resistant parent(s) for a mapping population, or choosing among existing mapping populations for a study of insect resistance, knowledge of the mechanisms of resistance involved or prior observation of the durability of a resistant cultivar in the field or in selection experiments can identify cultivars that may be sources of promising major genes or QTLs (Alam and Cohen 1998). The results of a QTL analysis itself will indicate whether the insect resistance in the resistant parent of the mapping population indeed has a polygenic basis. Insight into whether the QTLs influence multiple resistance factors acting on multiple targets within the pest can be gained by analyzing the mapping population for a series of carefully chosen traits.

Selective breeding for QTLs conferring a particular modality of insect resistance (antibiosis, antixenosis), or tolerance (Painter 1958), is another approach to achieving more durable varietal resistance. In this respect, Alam and Cohen (1998), in a QTL analysis of six traits associated with rice resistance to the brown planthopper, found a total of seven QTLs, one of which was predominantly associated with antixenosis and a second with tolerance. Most of the other QTL analyses of insect resistance conducted to date have scored chemical or morphological antibi-

otic resistance factors, or plant damage ratings under free-choice conditions, the results of which can be influenced by all three resistance modalities (see Yencho et al. 2000, for a review).

Transgenic Plants for Pest Control

Insect-resistant transgenic crops for enhancing insect pest control is currently one of the most important successes of plant biotechnology: more than 30 million hectares are planted worldwide with crops expressing *Bacillus thuringiensis* (Bt) δ-endotoxins (James 2009).

Bt is a soil bacterium that makes crystalline inclusions (Cry proteins) during sporulation (De Maagd et al. 2001; Bravo et al. 2007). These crystals dissolve in the alkaline environment of insect midguts and release protoxin molecules (65–140 kDa) that are processed by midgut proteases to yield active insecticidal proteins (60–70 kDa). These proteins interfere with the ion channel pumps and ultimately lead to the death of insect larva that ingests the crystal. They are quite specific in their host range (determined by ligand-receptor interaction) and this property has been exploited in the development of transgenics tolerant of specific groups of insect pests. The Bt δ-endotoxins are now known to constitute a family of related proteins for which 140 genes have been characterized for Lepidopterans, Coleopterans and Dipterans, and are not toxic to other organisms (Crickmore et al. 1998). Hence, they are safe insecticides and offer an interesting alternative to chemical control agents.

Cry-1 encoding genes have been introduced into several crop species such as maize, rice, cotton, tomato, potato and tobacco, with resistance target insect pests (Hilder and Boulter 1999), the modified varieties are generally referred to as Bt varieties. Transgenic Bt varieties are in several ways better than Bt spray formulation. In Bt transgenic plants, the protein is expressed in all tissues at all times, while the effectiveness of the sprays would be affected by a lack of uniform coverage and instability of the Bt protein, especially on exposure to sunlight.

As an example, several events of transgenic Bt maize have been developed over the past decade and there are currently varieties registered able to control lepidopteran and coleopteran species including the corn borer complex (European Corn Borer- ECB), southwestern corn borer – *Diatraea grandiosella* – and sugarcane borer – *Diatraea saccharalis*-, corn earworm (ECB), fall earmyworm (*Spodoptera frugiperda*), black cutworm (*Agrotis ipsilon*), and Worm Corn Root (WCR) complex (Figs. 4.7 and 4.8; Western, Northern and Mexican rootworms; *Diabrotica virgifera virgifera* LeConte). These insects can cause significant economic damage to maize production and all these transgenic varieties have provided a more effective control than insecticides, with lower cost than traditional insecticide applications and fewer logistical, health, and environmental concerns (Head and Ward 2009). Furthermore, this technology reduces the risk associated with lepidopteran pests like the European corn borer by improving yield stability. The use of multiple Bt proteins in a single product offers the potential for an extended spectrum of pest control and reduced risk of resistance evolving in the target pests.

Fig. 4.7 Damage inflicted by WCR larval feeding on transgenic (MON863) and conventional maize hybrids: the *left* root is a conventional hybrid and has been severely damaged while the root on the *right* side of the frame is protected by event MON863 (Modified from Vaughn et al. 2005)

The benefits of using Bt crops depend on many factors, most obviously the nature or the major insect pests in the area (not all are controlled by Bt) and the insect pressure in a given season (Christou et al. 2006). However, there are concerns regarding the use of Bt transgenic crops, the two major ones being: the effect on non-target organisms, and the possibility of the target insects developing resistance to the Bt protein. In this respect, several studies showed that the effect of maize pollen from Bt crops was negligible on non-target insects, including butterflies, under field conditions (Hodgson 1999). Moreover, though Bt crops have been widely cultivated since 1995, there has been no instance of a pest developing resistance (Ferry et al. 2006). However, given the experience of the diamondback moth having developed resistance to Bt sprays, the development of resistance in the insects cannot be discounted. As a proactive measure, several strategies for insect resistance management have been developed as a package for the cultivation of Bt crops. These strategies include refugia (growing a non-Bt crop on a small proportion of the area along with the Bt transgenic crop), gene pyramiding, and a high dosage of the protein in the plant to prevent any insects escaping from the Bt field (Christou et al. 2006; Ferry et al. 2006).

As alternatives to the Bt *Cry* genes, several candidate genes have been used to develop insect-resistant transgenic plants, such as protease inhibitors (Xu et al. 1996), α-amylase inhibitors (Ishimoto et al. 1996), vegetative insecticidal proteins from Bt (Estruch et al. 1996), cholesterol oxidases (Corbin et al. 1994), and toxins from predators such as mites and scorpions (Barton and Miller 1991). Transgenic tobacco plants expressing chitinase, one of the most important enzymes implicated in insect integument, have shown increased resistance to lepidopteron insects (Ding et al. 1998). Studies on rice (reviewed in Deka and Barthakur 2010) show that some of these candidates appear promising and provide an effective alternative to the Bt

Fig.4.8 Comparison of Bt (**a**) and conventional (**b**) maize hybrids in field trials in Italy (Courtesy CRA-MAC, Bergamo, Italy)

approach. In a transgenic assay in tobacco, enhanced resistance to *Helicoverpa zea* was generated by constitutive expression of the maize ribosome inactivating protein, named RIP- b-32, suggesting that this RIP plays a role in resistance to maize-feeding insects (Dowd et al. 2003). Characteristics of b-32, the developmentally regulated expression and synthesis of a non-toxic precursor, are reminiscent of the concept of phytoanticipine in the chemical defense strategy.

A further interesting solution to the development of insect-resistant plants was provided by Baum et al. (2007) for the control of coleopteran insect pests. Through RNA interference (RNAi) technology, they demonstrated that ingestion of double stranded (ds)RNA supplied in an artificial diet triggers RNAi in several coleopteran species, most notably WCR, which may result in larval stunting and mortality. Interestingly, transgenic maize plants engineered to express WCR dsRNAs show a

significant reduction in WCR feeding damage in a growth chamber assay, suggesting that the RNAi pathway can be exploited to control insect pests via *in planta* expression of a dsRNA.

A molecular strategy for establishing nematode resistance in plant species has also been proposed with the development of artificial resistance. This can be achieved by introducing effector genes into the host plant that have a nematocidal impact. Such transgenes can encode enzymatic inhibitors that block physiological processes within the nematodes (e.g. PIs toxins) or degrading enzymes (e.g. collagenases, chitinases), toxic compounds that are ingested (cytotoxins), compounds that bind molecules (e.g. lectins, monoclonal antibodies), enzymes that interact with the nematodes, and substances that cause the breakdown of specific feeding structures (cytotoxins). More recently, RNAi was used to evaluate the role of the 16D10 secretory peptide of *Meloidogyne incognita*, which apparently interacts with the root Scarecrow protein. Expression of dsRNA in *Arabidopsis* to silence the *16D10* gene of infecting nematodes confers resistance to four *Meloidogyne* spp. (*M. incognita, M. javanica, M. arenaria*, and *M. hapla*; Huang et al. 2006). Moreover, RNAi in soybean has been used to target essential genes of *H. glycine*, causing a reduction in the number of females developing on transgenic roots (Klink et al. 2009). Despite this positive finding, results from RNAi experiments should be taken with caution: exposure to dsRNA per se is capable of causing aberrant phenotypes in both cyst and root knot nematodes (Dalzell et al. 2009). To address this problem, a novel design strategy to generate 21 bp siRNAs has been successfully applied to the potato cyst nematode, *G. pallida,* and to the root knot nematode, *M. incognita* (Dalzell et al. 2009).

5 Herbicide Tolerance

Farmers must control weeds that compete with their crops for water, nutrients and sunlight. Depending on the crop and location, weeds can decrease crop yields by 35%–100%. A number of options are available to farmers for minimizing the impact of weeds on crop productivity; one of these is the application of herbicides to the weeds. Indeed, effective weed control is a prerequisite in any crop production system if high yields and good quality are to be achieved, and herbicides have revolutionized weed control in many cropping systems and play an important role in modern agriculture. They provide economical weed control and increase the efficiency of crop production. A number of new herbicides combine high weed killing potency with low- or no-environmental persistence. However, the very effective broad spectrum herbicides available also lack selectivity, thus limiting their use in some cropping operations. On the other hand, the continuous use of the few available selective herbicides is speeding up the development of herbicide resistance in weeds; hence making effective control difficult to achieve in some crops.

5.1 Mode-of-Action and Metabolism

A large amount of knowledge exists on the mechanisms of herbicide mode-of-action and metabolism; these have frequently been described by several authors (e.g. Mazur and Falco 1989; Powles and Shaner 2001) so will not be repeated herein. Briefly, herbicides generally function by disrupting unique and essential processes in plants e.g. photosynthesis, mitosis, pigment biosynthesis or essential amino acid biosynthesis. This in turn has permitted a number of herbicide-tolerant target enzymes naturally existing in different plant species and microorganisms to be identified, as well as a number of herbicide-modifying enzymes leading to herbicide-tolerant organisms. Among the input traits offered to farmers, herbicide resistance has been the most widely adopted.

5.2 Breeding Strategies

Both crops and weeds share essential biochemical processes. Consequently, selectivity is mostly based on differential herbicide uptake between weeds and crops, controlled timing and site of application or rapid detoxification of the herbicide by the crop plants. Reliance on these natural selection processes limits the effective use of potent herbicides; hence mechanisms to impart better herbicide selectivity in crops need to be investigated.

Two approaches can be exploited. The first is the design of specific chemicals with broad selectivity for crops. This approach, however, is expensive and the products thereof may be uneconomical for use by growers, not to mention that it may also increase the already growing chemical load to the environment. Moreover, it has become increasingly difficult to discover new herbicides and even harder to come up with one that has a novel mode of action (Gressel 2002; Tan et al. 2005). The second and more popular approach to crop herbicide selectivity is the development of crop cultivars with tolerance to the already existing effective broad-spectrum herbicides so as to expand the crop options in which they can be used. Two methods can be used to develop crops with resistance to herbicides.

5.3 Conventional Methods

Conventional plant breeding utilizing strains that are known to be tolerant to specific herbicides is one approach that could confer resistance on susceptible crops from closely related species. However, this approach has limitations in that naturally herbicide resistant plants are found more among weed species than in crops. In addition, conventional plant breeding takes a long time to produce a single useful genotype.

5.4 *Biotechnology Techniques*

A faster approach is the use of biotechnology techniques such as *in vitro* cell culture, mutagenesis and selection in physiologically inhibitory concentrations of herbicides (also referred to as brute force selection) or genetic transformation of already existing crop cultivars with genes that confer resistance to herbicides.

5.4.1 Cell Culture and Selection

A number of mutant enzymes have been identified from plant cells in cultures. A trait of agronomic interest that may be expressed by cultured cells is herbicide sensitivity. Herbicides that interfere with basic metabolic activities are expected to inhibit growth of cultured cells as well as of the whole plant. In such instances, herbicide-tolerant mutants can be selected by culturing cells in the presence of a herbicide concentration that is toxic to normal cells, favoring subsequent identification of the herbicide-tolerant target enzyme.

Using cell culture techniques, BASF Inc. produced a maize hybrid that is resistant to the sulfonylurea herbicide, sethoxidim. In their analysis, a mutant cell line (named S2) was identified following continuous culture of maize embryo tissues under high sethoxidim selection pressure. Plants regenerated from this somaclonal mutant line were found to contain a form of the enzyme, acetolactate synthase (ALS, target of sulfonylureas/imidazolinones), which was insensitive to the herbicide. This resistance was subsequently transferred to the commercial hybrid (DK404SR) by backcrossing the S2 line with both of its parental lines. Further investigations showed that the sethoxidim tolerance was inherited as a single partially dominant allele. Similarly, Zambrano et al. (2003) selected a glyphosate-tolerant sugar cane cell line in liquid medium containing 0.8 mM glyphosate and regenerated plants that could tolerate up to five-fold the concentration of glyphosate that killed plants from unselected cells. Cell culture under lethal concentrations of certain herbicides also results in gene amplification in surviving cells that leads to resistance through the overproduction of enzymes targeted by herbicides. For example, a petunia cell line with resistance to glyphosate was selected in this manner and plants regenerated from it survived lethal levels of glyphosate (Steinrucken and Amrhein 1986). This resistance was found to be due to amplification of the gene encoding 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase that caused its overproduction in the cells. Similarly, Caretto et al. (1994) selected carrot cells and subsequently regenerated plants that were resistant to the sulfonylurea herbicide, chlorsulfuron. Resistance in these plants was due to amplification of the *ALS* gene. *In vitro* development of phosphinothricin (PPT) resistant rice has also been reported by inducing plantlet regeneration in explants collected from 7-day old seedlings on medium supplemented with sublethal doses of PPT (Toldi et al. 2000). Other *in vitro* cell selection studies have developed resistance to paraquat in tomato cells (Thomas and Pratt 1982), resistance to glyphosate in carrot and groundnut cells

(e.g. Jain et al. 1999) and resistance to a protoporphyrinogen oxidase (PPO) inhibitor in soybean cells (Warabi et al. 2001); however, no viable plant regeneration was reported in these studies.

5.4.2 Mutagenesis

Chemical or physical mutagenesis of seed, microspores or pollen followed by selection under herbicide selection pressure has also been used to develop crop resistance to herbicides. The most common mutagen used is ethyl methanesulfonate (EMS), which is efficient at producing chloroplast mutants (McCabe et al. 1990). In this strategy, seeds or pollen are treated with EMS then grown either *in vitro* or *in vivo* in the presence of a herbicide. Surviving plants are selected and grown to maturity to provide seed that is used for further screening with herbicides. Using this method, Sandhu et al. (2002) developed 21 rice lines that were resistant to glyphosate. Ashfaq-Farooqui et al. (1997) produced atrazine resistant *Solanum melongena* plants by mutagenizing seeds followed by germination and *in vitro* regeneration of plants from the resultant seedling cotyledons. Similarly, Mourad et al. (1993) isolated, by screening seedlings of M2 populations from EMS-treated seeds, a triazolopyrimidine (herbicide) resistant mutant. The resistance was found to be due to a single, dominant, nuclear gene mutation that encodes the ALS enzyme. ALS activity in enzyme extracts from the mutant was about 1,000-fold less sensitive to inhibition by triazolopyrimidine than in extracts from wild-type plants.

Ultra-violet (UV) or EMS treated microspores or pollen can be grown *in vitro* into haploid plantlets whose chromosome number can be doubled to create instant inbred lines bearing a specific herbicide tolerance trait. This method was applied by Ahmad et al. (1991) using microspore UV mutagenesis and haploid culture to develop canola plants that were resistant to chlorsulfuron. Syngenta Seeds Inc. produced the EXP19101T line of imazethapyr-resistant maize using pollen mutagenesis. In that work, EMS mutagenized pollen was used to fertilize the parent line, UE95, progeny plants were screened for tolerance to lethal doses of imazethapyr and resistant ones selected. Tolerance in these plants was found to be the result of a single nucleotide substitution within the ALS encoding gene, which gave a single amino acid change (Ser_{621} to Asn_{621}) in the sequence of the enzyme. This change prevents the binding of the herbicide to the enzyme active site, thus maintaining normal enzyme function. More recently, Venkataiah et al. (2005) reported the production of atrazine-resistant pepper (*Capsicum annuum*) plants regenerated from 3-week-old seedling cotyledons obtained from EMS treated seeds. They also noted maternal inheritance of the atrazine resistance trait. Finally, BASF (Ludwigshafen, Germany) markets non-transgenic CLEARFIELD® imidazolinone-resistant canola, wheat, sunflowers, maize, lentils, and rice, while DuPont (Wilmington, DE) markets non-transgenic STS® soybeans with tolerance to sulfonylurea herbicides. These crops all contain mutagenized versions of the ALS, which are not inhibited by imidazolinone and/or sulfonylurea herbicides (Devine and Preston 2000). Herbicides that inhibit ALS are considered low or very low use-rate herbicides with a good spectrum of weed control and are likely to remain an important part of weed resistance management programs.

5.4.3 Genetic Transformation

Herbicide tolerance is the most common trait in commercial transgenic crops, being part of 82% of all transgenic crops in 2009 (James 2009). Transgenesis for herbicide selectivity involves the identification of a herbicide resistance gene from a plant or microorganism, its isolation and manipulation for efficient plant expression (if it is of microbial origin) and (James 2009) its subsequent delivery, stable integration and expression in the cells of the target crop plant. For the most part, genes encoding for useful herbicide resistance in crops are isolated from herbicide degrading soil microorganisms.

Herbicide tolerance via genetic transformation can be conferred by one or a combination of these four mechanisms:

1. Introduction of a gene(s) encoding for a herbicide detoxifying enzyme(s);
2. Introduction of gene(s) encoding for a herbicide insensitive form of a normal functioning enzyme or over expression of the genes encoding for a herbicide target enzyme such that the normal metabolic functioning is still achieved in the plant even though some of the enzyme is inhibited;
3. Modification of the herbicide target enzyme in such a way that the herbicide molecule does not bind to it and;
4. More recently described engineering of active herbicide efflux from plant cells.

Glyphosate (Monsanto Technology LLC) is one of the most widely used herbicides in the world; it is relatively inexpensive and can be applied after the emergence of resistant crop seedlings. Nearly all broadleaf and grass weeds are eliminated, resulting in reduced competition, higher yields, and cleaner fields at harvest. Adoption of reduced and no-till practices, where dead vegetation is left in the field rather than plowed under, has been a significant unintended feature of herbicide-resistant crops, saving farmers money in fuel costs and reducing soil erosion.

Since 1996, glyphosate-tolerant or Roundup Ready crops have been developed and marketed for soybean and maize. Glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaf weeds and has superior environmental and toxicological characteristics, such as rapid soil binding (resistance to leaching) and biodegradation (which decreases persistence), as well as extremely low toxicity to mammals, birds and fish. Glyphosate resistance is achieved in Roundup Ready® brands by expression of a modified *Agrobacterium* gene encoding for the herbicide insensitive enzyme CP4 enolpyruvyl-shikimate-3-phosphate (Padgette et al. 1996). The GA21 trait for glyphosate-resistant maize relies on a modified maize *epsps* gene, but is largely being replaced by varieties with the NK603 trait which has two copies of CP4 *epsps* with different promoters for better expression in the meristems.

Traits for resistance to three other classes of herbicides have been developed, but have not reached the same level of diffusion as glyphosate resistance. Resistance to oxynil herbicides conferred by the BXN nitrilase from *Klebsiella pneumoniae* (subspecies *ozaenae*) (Stalker et al. 1988) was the first trait engineered in cotton (developed by Calgene, Davis, now Monsanto). Because glyphosate is less expensive and controls more weed species, interest in using the oxynil herbicides has waned and 2004 was the

final year of BXN® cotton sales. BXN canola was marketed by Rhone-Poulenc Canada (now Bayer CropScience, Monheim, Germany) and then discontinued.

Phosphinothricin acetyltransferase (PAT or BAR) detoxifies phosphinothricin- or bialaphos-based herbicides (glufosinate) by acetylation of the free NH_2 group of molecules. The *pat* gene is native to *Streptomyces viridichromogenes* and *bar* is from *S. hyhroscopicus* where they act in both the biosynthesis and detoxification of the tripeptide bialaphos (De Block et al. 1987). Like glyphosate, phosphinothricin herbicides control a broad spectrum of weed species and break down rapidly in the soil so that problems with residual activity and environmental impact are greatly reduced. Bayer CropScience markets this trait as Liberty Link® in several species. The *pat* and *bar* genes are also popular plant transformation markers in the research community.

As a technology, herbicide-resistant crops are a valuable tool for efficient weed control. However, doubts remain about the long-term viability of this strategy, particularly the emergence of herbicide-resistant weeds following widespread cultivation of herbicide-resistant crops (Sandermann 2006). Regardless, growers perceive that the benefits of the herbicide resistance characteristic outweigh the risks. It is clear that the widespread adoption of herbicide-resistant cultivars, particularly glyphosate-resistant crops, has dramatically impacted weed communities (Powles and Yu 2010). Weed population shifts to naturally resistant species, species with inherent biological characteristics that make the populations difficult to control, and the evolution of herbicide-resistant biotypes are real, as are the immediate economic issues attributable to the adoption of herbicide-resistant crops and the concomitant use of the herbicide. However, studies have shown the possibility of engineering multiple resistance in plants. In this respect strategies have been suggested to delay the development of herbicide-resistant weeds. These include combined or sequential use of herbicides with different modes of action, crop rotation, integrated weed management.

These studies have opened the avenue for the targeted development of crops that would reduce the environmental chemical load due to the use of different herbicides in crop rotation programs. A greater number of, and more various, modes of resistance have evolved in weeds than in other organisms because herbicides are used far more extensively than other pesticides, and because weed seed output is so prolific. Weeds have evolved unknown mechanisms, even antibiotic, as well as other drug and pesticide resistance. It is also possible that cases of epigenetic resistance may have appeared (Gressel 2009).

6 Conclusions and Future Prospects

Plant pests and diseases have major effects on agricultural production and the food supply. Although application of fungicides and pesticides has helped control plant diseases, chemical control is economically costly as well as environmentally undesirable. The development of new strategies based on a plant's own defense mechanisms for disease control is therefore critical for sustaining agricultural production and improving

our environment and health. Basic research on the genetic bases of pest and disease resistance in plants and of host-pathogen interactions has greatly improved the efficiency of manipulating disease resistance genes in practical breeding programs and resulted in the deployment of high-yielding genetically resistant crop cultivars that in some cases have been grown over vast areas, but much remains to be learnt at the interface of the genetics of resistance and crop physiology. The cloning of resistance genes and corresponding avirulence genes has indicated considerable complexity not only in structure but also in the way in which gene products interact and trigger resistance. Hence, our overall understanding of the process is still fragmentary. Furthermore, many gaps remain in our models of the defense signal transduction network and these must be bridged before we can design truly rational strategies to activate the network. Similarly, genetic mapping of plant mutations that alter herbivore resistance, or perhaps responses to purified insect elicitors, will almost certainly lead to the identification of previously unknown defense pathways.

A further point worth noting is that although major genes and QTLs for resistance to numerous pathogens and insect pests have been mapped, the usefulness of this information for MAS breeding programs has not yet been demonstrated. In this respect, the development of new technologies, such as high-throughput DNA sequencing and microarray analysis to facilitate the mapping and cloning of major genes and QTLs for routine use will provide an assemblage of new tools to facilitate the development of crops resistant to pests and pathogens, while analysis of signaling and metabolic pathways will be harnessed to increase the power of MAS and genetic engineering for crop improvement. Furthermore, the complexity of plant-insect interactions makes it difficult to determine which anatomical features, metabolites, and signaling pathways effectively limit pathogen and pest infestations. Genomic information from both host plants and pests and pathogens should accelerate the rate of discovery in this field. The field of genomics will provide powerful tools to investigate these critical factors. Transcript profiling techniques allow the simultaneous examination of thousands of genes, and can be utilized to study changes in gene expression that are transcriptionally regulated. Beyond transcript profiling, genomics also facilitates the functional analysis of genes implicated in resistance and susceptibility. As signaling cascades and metabolic pathways are elucidated in model systems and crop plants, key regulatory genes can be targeted for silencing or overexpression to study the role of these pathways in plant-insect and -pathogen interactions. To achieve a detailed understanding of plant interactions with pathogens and pests, it will ultimately be necessary to combine transcriptomic approaches with proteomic, metabolomic, and mutational analyses. While plant responses have been the focus of most transcriptomic studies, additional levels of complexity can also be analyzed with genomic tools. Investigating changes that occur concurrently within the pathogens and insects is essential to understand the basis of an effective plant defense. Therefore, knowledge accumulated in these studies will help us to establish economical and sustainable strategies to fight insects and diseases of many important crops. Beyond genome sequencing, additional effort should be targeted at identifying pathogens and insect genetic markers, studying natural variation in host plant utilization, and

developing methods such as RNA interference for manipulating insect gene expression. The development of such research tools will facilitate studies on both sides of the plant-insect and -pathogen interactions and thereby achieve a more complete understanding of plant response to pathogens and insects.

Since the first transgenic plants appeared almost two decades ago, this technology has contributed to develop new methods of crop protection aiming to increase world food production. It is certain that the methodology developed for creating *Bt* plants will ultimately make the objective of having highly productive, pest- and pathogen-resistant, and environmentally friendly crops, become a reality. The promising alternative of genetic engineering of insect- and pathogen-resistant plants, relying exclusively on the repertoire of plant defense genes, should be thoroughly investigated as it may provide solutions to the problem of increasing plant productivity for future needs. Success in developing transgenic organisms will also benefit from knowledge of the signal transduction pathways that regulate pathogenesis, particularly host range and the availability of a wide range of suitable genes that can be used to increase virulence. Genetic engineering strategies require information on the roles and consequences of these genes, leading to enhanced exploitation of the genetic resources present in plants.

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Chapter 5
The American Halophyte *Prosopis strombulifera*, a New Potential Source to Confer Salt Tolerance to Crops

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Contents

Abstract Salinity imposes a major environmental threat to agriculture and its adverse impact has become the most serious problem in vast regions of the earth surface. The combination of population growth and land degradation are of such

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significance that plant salt tolerance improvement has become an urgent need for the future of agriculture. Generation of salt tolerant crops requires a clear understanding of the complex mechanism of abiotic stress resistance in key species. Evolutionary processes under saline conditions gave rise to halophytes which have evolved adaptive traits to cope with the salinity of the environment. They include some specific biochemical, physiological and molecular mechanisms, on one hand, and a capability of natural association with different microorganisms called Plant Stress-Homeo-regulating Rhizobacteria (PSHR), on the other. However, a complete understanding of these processes is still lacking despite the intensive research conducted during the last decade. The genus *Prosopis* includes many important arboreal and shrub-like species that are present in saline zones of the Americas and some of them are considered to be unique terrestrial species due to their combined ability to fix nitrogen and grow under high-salinity conditions. The shrub *Prosopis strombulifera* (Lam) Benth. is distributed from the Arizona desert (U.S.A.) to Patagonia (Argentina) and is especially abundant in the salinized areas of central Argentina. This species showed a halophytic response to NaCl surviving up to 1 M NaCl in *in-vitro* experiments, but in contrast, a strong growth inhibition at lower Na_2SO_4 concentrations. These differential responses to the most abundant salts present in most salinized soils make this species an excellent model to study salt-tolerance mechanisms in halophytic plants. This chapter provides an overview of different salt tolerance mechanisms in the native halophyte *Prosopis strombulifera*, which may be considered a new useful source to improve crop salt tolerance through two biotechnological strategies: considering *P. strombulifera* as a natural gene donor to improve the genetic salt tolerance of low tolerant crops, and considering this species and its rhizosphere as natural sources of PSHR microorganisms capable of physiologically improving salt tolerance in crops.

Keywords Halophyte • NaCl • Na_2SO_4 • Differential tolerance • PSHR microorganisms • Biotechnology

1 Introduction

Nowadays salinity, which has been representing a threat to agriculture in some parts of the world for more than 3,000 years, is affecting more and more lands. Some of the most serious examples of salinity occur in arid and semiarid regions. For example, in Iran, Pakistan, Egypt, and Argentina, out of the total land area of 162.2, 77.1, 99.5, and 237.7 million hectares, about 23.8, 10, 8.7, and 33.1 million hectares are salt-affected, respectively (FAO 2008). As the world population has expanded, so there is a need to grow more food, which requires an increase in the area of land under cultivation as well as in land productivity as yields per hectare (Flowers and Flowers 2005). For this reason, genetic improvement of salt tolerance has become an urgent need for the future of agriculture (Owens 2001; Munns 2007).

The deleterious effects of salinity on plant growth are associated with (1) low water potential of the root medium which causes a water deficit within the plant; (2) toxic effects of ions mainly Na^+ and Cl^-; and (3) nutritional imbalance caused by reduced nutrient uptake and/or transport to the shoot (Munns and Termaat 1986; Marschner 1995; Serrano et al. 1999; Hasegawa et al. 2000; Ashraf and Ahmad 2000; Parida and Das 2005; Munns and Tester 2008; Hariadi et al. 2011). In addition, salt stress can induce oxidative stress, such as generation and/or accumulation of reactive oxygen species, including hydrogen peroxide (H_2O_2), superoxide anion, and hydroxyl radicals (Kovtun et al. 2000; Miller et al. 2010). For that, salt tolerance is a complex trait involving responses to cellular osmotic and ionic stresses, subsequent secondary stresses (*e.g.* oxidative stress), and whole plant coordination of these responses. Various approaches have been advocated to generate salt-tolerant crops, none of which has offered a universal solution (Flowers and Flowers 2005; Munns 2007).

During the last years, it has been proposed that improving our knowledge about the specialized physiology and biochemistry of halophytic plants, which impart their exceptional degree of salt tolerance, would give us new insight into plant salt tolerance, but it still represents a challenge for the scientists. For this purpose, the tolerant relative of *Arabidopsis*, *Thellungiella halophila*, has been considered the alternative halophytic genetic model system (Inan et al. 2004).

Nevertheless, our plant model system *Prosopis strombulifera* exhibits growth promotion up to 500 mM NaCl and its growth is slightly inhibited at higher salt concentrations such as 700 mM NaCl. This exceptional degree of tolerance makes this species a perfect candidate to deepen our knowledge on halophytic salt tolerance mechanisms.

2 *Prosopis strombulifera*, a Halophytic Legume

Within the family Leguminosae, virtually all of the important annual legumes that belong to the subfamily Papilionaceae such as soybeans, beans, peas, and cowpeas are highly salt sensitive and suffer yield reductions from salinities with conductivities as low as 2–3 dS m^{-1} (Richards 1954; Ayers and Westcott 1985). Alfalfa is the most salt tolerant of the commercial legume species showing yield reductions of 50% at salinities of 9.6 dS m^{-1} (Ayers and Westcott 1985). In contrast, the genus *Prosopis*, subfamily Mimosoideae, includes many important arboreal and shrub-like species that are present in saline zones of the America (Burkart 1937, 1952, 1976); these species present a rapid growth at seawater salinity or 45 dS m^{-1} which is nearly 20 times greater than salinities that can be tolerated by annual temperate legumes. The *Prosopis* genus is considered to have unique terrestrial species with the ability to fix nitrogen and grow under high-salinity conditions (Rhodes and Felker 1987).

The spiny shrub *Prosopis strombulifera* (Lam.) Benth. (Burkart 1976) is distributed from the Arizona desert (U.S.A.) to Patagonia (Argentina) and is especially abundant

Fig. 5.1 *Prosopis strombulifera* plants in their natural habit (Saltland in San Luis, Argentina) and plants growing in hydroponic culture

in the salinized areas of central Argentina (Fig. 5.1) (Cantero et al. 1996). The germination of this species is strongly influenced by the nature of the ions in the salt solutions and their interactions (Sosa et al. 2005).

In the salinized soils of southern Cordoba and southwestern San Luis, Argentina where we can find *P. strombulifera* populations, the proportions of NaCl and Na_2SO_4 are similar, although, in some samples, Na_2SO_4 was up to three times more abundant (Sosa et al. 2005). This and other recent studies have suggested an increasing need to compare the effects of Na_2SO_4 and NaCl on plant growth in order to better understand plant responses to the major salts found in the salinized soils of several countries such as Pakistan, China, India and Tunisia (Iqbal 2003; Bie et al. 2004; Shi and Sheng 2005; Naeem and Quereshi 2005; Manivannan et al. 2008; Tarchoune et al. 2010).

Comparative studies of the effects of Cl^- and SO_4^{2-} on *P. strombulifera* germination have demonstrated that SO_4^{2-}-based solutions are considerably more inhibitory than Cl^--based solutions at iso-osmotic concentrations (Sosa et al. 2005; Llanes et al. 2005). Species like *Hordeum vulgare* (Huang and Redmann 1995), *Medicago sativa* (Redmann 1974), *Triticum aestivum* (Hampson and Simpson 1990), *Pinus banksiana* (Croser et al. 2001), and *Ocinum basilicum* (Tarchoune et al. 2010) were found to be more inhibited by Na_2SO_4 than by NaCl. For other species like *Brassica napus* (Huang and Redmann 1995) the reverse was found.

The response of *P. strombulifera* to salinity was different depending on the type of salt used and the osmotic potential in the culture medium. Shoot growth was stimulated up to $\Psi_o = -1.88$ MPa (500 mM) NaCl; this is an interesting halophytic response that differs from other *Prosopis* species (Felker 2007). The genus *Prosopis* occurs worldwide in arid and semiarid regions and includes about 44 species grouped in 5 sections and 8 series (Burkart 1976). Many species of this genus, particularly those belonging to the Algarobia Section, have economic and ecological potential, often being major components of native North and South America

ecosystems wherein they offer shade, firewood, food, and forage for wildlife and livestock. Some *Prosopis* species, especially *P. pallida*, *P. juliflora*, *P. tamarugo*, and *P. alba* have individual plants with rapid growth at seawater salinity or 45 dS m^{-1} (Felker 2007). Notwithstanding, according to the literature *P. strombulifera* NaCl-tolerance exceeds the limits described for most *Prosopis* species being closer to tolerance levels observed in some Chenopodiaceae.

On the other hand, this species is much less tolerant to Na_2SO_4, showing strong and sustained shoot growth inhibition from the beginning of the treatment, accompanied by senescence symptoms such as chlorosis, necrosis, and leaf abscission (Reinoso et al. 2005; Reginato 2009). Root growth stimulation was also observed in NaCl-grown *P. strombulifera* plants showing a generalized adaptive mechanism to favor water and nutrient absorption. The SO_4^{2-} anion had a detrimental effect on root growth in comparison with the Cl^- anion, which was also partially alleviated when both anions were present in bisaline solutions.

Similar results were obtained in comparative salinization studies with Na_2SO_4 and NaCl in the halophyte *Chenopodium rubrum* by Warne et al. (1990) who reported full growth inhibition at $\Psi_o = -1.6$ MPa with SO_4^{2-}-based solutions but Cl^-mediated growth inhibition at Ψ_o lower than −2 MPa. These results differ from those obtained by Egan and Ungar (1998), who registered shoot growth stimulation with Na_2SO_4 in *Atriplex prostata* seedlings and growth inhibition in the presence of iso-osmotic NaCl solutions.

Nevertheless, none of these authors analyzed growth responses to iso-osmotic mixtures of both salts. Partial alleviation of SO_4^{2-} toxicity by bisaline solutions is an interesting response in our experiments, confirming previous reports on germination of this species (Sosa et al. 2005; Llanes et al. 2005).

3 Mechanisms of Salt Tolerance in *Prosopis strombulifera*

3.1 Ion Exclusion, Accumulation and Compartmentation

Sodium exclusion (Garcia et al. 1995), K^+/Na^+ discrimination (Asch et al. 2000; Houshmand et al. 2005), and Cl^- exclusion (Rogers and Noble 1992) traits have proven valuable in screening germplasm for salinity tolerance. Some halophytes make an osmotic adjustment by accumulating equivalent amounts of Na^+ and Cl^- in vacuoles (Kefu et al. 2003). *Prosopis strombulifera* does not allow the entrance of great amounts of Na^+ (compared with Cl^- levels) which correlates with the particular characteristics observed in the radical system, such as precocious lignification and suberization of endodermis for salt exclusion (Reinoso et al. 2004, 2005); this observation would imply that *P. strombulifera* falls within the category of salt-excluding plants (Yensen and Biel 2005), which are able to exclude salts from the roots. Similarly, the economically important relatives *Prosopis* species are Na^+ excluders (Villagra et al. 2010).

Nevertheless, Na^+ content in the leaves of *P. strombulifera* was 2–3 fold higher than K^+ content (0.48 and 0.185 mmol g^{-1} DW, respectively) when optimal growth was observed in NaCl treated plants ($\Psi_o = -1.9$ MPa, 500 mM). These results suggest that *P. strombulifera* is a sodium exporter like *Atriplex*. Thus, a good explanation for the unusual salt tolerance of this species may be a combination of exclusion mechanism in the roots and ion accumulation/compartmentation in the leaves. It is remarkable to note that *P. strombulifera* preferentially accumulated Cl^- over Na^+ reaching significant levels of Cl^- anion (2.24 mmol g^{-1} DW) in roots as well as in leaf tissues without toxicity symptoms when optimal growth was observed in NaCl treated plants (Reginato 2009). The total ion content pattern determined at the end of the experiment (48 days) is shown in Fig. 5.2.

In Na_2SO_4-treated *P. strombulifera* plants, there was SO_4^{2-} accumulation in roots and leaves from the beginning of salinization, reaching amounts equivalent to those of Cl^- at the highest salt concentration tested in our experiments (530 mM Na_2SO_4, osmotically equivalent to 700 mM NaCl). Chrispeels et al. (1999) affirmed that SO_4^{2-} causes acidification of the medium, generating membrane depolarization and favoring SO_4^{2-} uptake. The SO_4^{2-} levels in leaves were lower than those of Cl^- in NaCl-treated plants, but they were sufficient to cause metabolic disorders and visible signs of toxicity, as reported above. In bisaline-treated plants a preferential accumulation of Cl^- over SO_4^{2-} was observed, corroborating the fact that SO_4^{2-} is absorbed slowly by the roots of higher plants (Bie et al. 2004). The deleterious effect of SO_4^{2-} on leaf development and root and shoot elongation may be a consequence of several metabolic reactions, such as sulfide formation in the process of sulphate assimilation in the chloroplast, where sulphite reductase catalyzes the reduction of sulphite to sulphide using reduced ferredoxin as an electron donor (De Kok et al. 2005). Free sulphide can be incorporated to L-cysteine by cysteine synthase. If this assimilatory step does not consume all free sulphide, the sulphide could be emitted to the environment or could effectively bind to cytochromes, thus inhibiting mitochondrial respiration (Schmidt 2005).

Some species accumulate NO_3^- anion for charge balance and osmotic adjustment (Martinez-Ballesta et al. 2004). In halophyte *Suaeda salsa* NO_3^- plays an important osmotic role in high salinity (Song et al. 2009). In *P. strombulifera* NO_3^- anion does not seem to have such a role. This species synthesizes compatible solutes like proline and pinitol in high concentrations for charge balance and osmotic adjustment (Llanes 2010).

On the other hand, Na^+ accumulated in *P. strombulifera* leaves at the expense of K^+ in a quantitatively different manner depending on the salinizing agent used. In roots, SO_4^{2-}-based treatment caused greater Na^+ accumulation at the beginning of salinization limiting sodium's transport to the shoot. Kefu et al. (2003) reported that when external Na^+ concentration is very high, it can block K^+ transporters (including high-affinity transporters) causing a decrease in K^+ entrance. Although most plants have an absolute requirement for K^+, and Na^+ is toxic for many biological reactions in the cytoplasm. This does not apply to vacuolar processes, because Na^+ accumulates preferentially in vacuoles being more suitable than K^+ for osmotic adjustment in several species; the replacement of K^+ by Na^+ in the vacuole does not produce toxicity

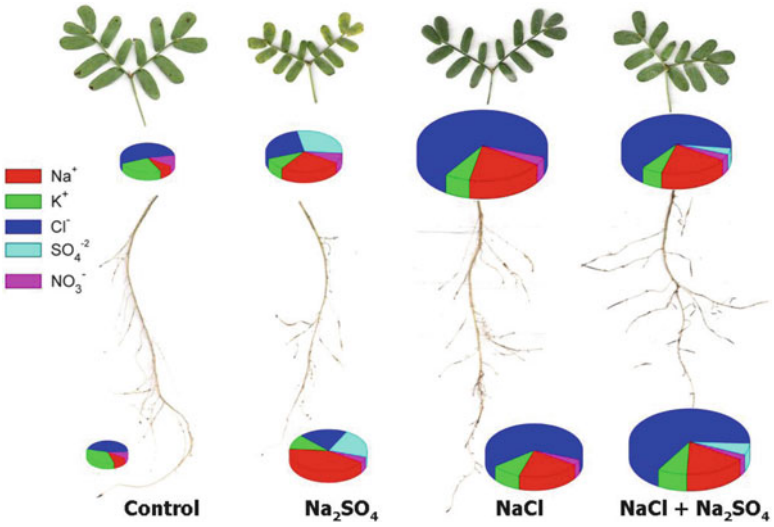

Fig. 5.2 Ion composition in *P. strombulifera* plants. Control plants (48 days); Na_2SO_4, NaCl and NaCl + Na_2SO_4 treated plants ($\Psi_o = -2.6$ MPa, 48 days). The area of the circles designates the total ion content in different organs of the plant. The size of the *circle* in leaves of control plants corresponds to 3.158 mmol/g DW (100%). The sizes of *circles* (and their respective portions) in the other plant organs and treatments are proportionally depicted

(Subbarao et al. 2003). Therefore, the use of inorganic ions is efficient and significantly more economical than the synthesis of compatible organic solutes for plants under salt stress (Song et al. 2009).

The long-distance Na^+ transport and, especially, the mechanism of Na^+ loading into the xylem play a substantial role in the formation of water potential gradient in *Suaeda altissima* (Balnokin et al. 2005); efficiency in long-distance Na^+ transport to stems and leaves is a required mechanism for salt tolerance in halophytes (Patel and Pandey 2007). Then, limitation of sodium's transport to the shoot by SO_4^{2-} anion seems to be one of the reasons for the lack of tolerance of this species to SO_4^{2-} treatment, discarding the possibility that unfavorable plant growth in Na_2SO_4 resulted from Na^+ toxicity due to the higher Na^+ concentration in this solution.

Salt treatments caused a decrease in Ca^{2+} levels at all osmotic potentials tested in our experiments, showing a great difference between controls and treated plants in both leaves and roots (data not shown). The lower Ca^{2+} than Na^+ and K^+ contents in roots may be explained by membrane nonselective cation channels that allow monovalent or divalent cation movement indistinctly. These would constitute the primary way of Ca^{2+} influx to cells (Demidchik et al. 2002).

These results demonstrate that NaCl and Na_2SO_4 differentially affect ion accumulation by plants, generating specific distribution patterns of ions in different organs (Fig. 5.2). This fact suggests an important specific anionic effect on membrane permeability. Increasing concentrations of bisaline solutions allowed higher salt tolerance than increasing concentrations of individual Na_2SO_4 solutions through

various phenomena such as ionic antagonism and/or mutual competence, which bring about specific physiological responses, confirming previous results (Sosa et al. 2005; Llanes et al. 2005).

3.2 Water Relations and Water Use Efficiency

Salt stress affects plant water relations by reducing water potential; decreased osmotic potential and increased turgor pressure are common responses in plants exposed to salt. The water use efficiency is addressed by several authors as a relevant mechanism of salt tolerance in plants (Verlues et al. 2006; Grewal 2010). Decreased water consumption per accumulated biomass unit implies a reduced amount of salt absorbed by plants and a reduced energy cost for compartmentation or extrusion of salt itself.

Prosopis strombulifera has the ability to maintain a higher relative water content (RWC) in leaves of seedlings growing in high NaCl concentrations without succulence. Thus, it appears that the extreme tolerance of this species to NaCl would be due to its capability of osmotic adjustment (Fig. 5.3). Also, the high water content in its leaves help ease the impact of Na^+ accumulation in tissues, which is also favored by leaf anatomical adaptations such as the particular distribution of chlorenchyma around the leaf veins (Reinoso et al. 2004) and the modifications on stomatal area and distribution (Reginato 2009) as well as stomatal conductivity that enable *P. strombulifera* seedlings to make a more efficient use of water under these experimental conditions.

Plants treated with high Na_2SO_4 concentrations ($\Psi_o = -2.6$ MPa) failed to make an efficient osmotic adjustment (Fig. 5.3) showing extremely negative osmotic and water potentials in leaves causing water imbalance, which, in comparison with NaCl-treated plants, correlates directly with the marked decrease in individual and total leaf area at $\Psi_o = -1.88$ MPa and lower treatment, reaching final values of 30% and 60% inhibition of these parameters, respectively. An increase in stomatal density and epidermal cell density with smaller stomata, maybe additional efforts of Na_2SO_4-treated plants to survive (Reginato 2009). However, leaf area was not modified in NaCl-treated plants.

3.3 Metabolism of Protection-Compatible Solutes Production

One of the most common stress responses in plants is overproduction of different types of compatible organic solutes (Serraj and Sinclair 2002; Parida and Das 2005; Munns and Tester 2008). Compatible solutes are low molecular weight, highly soluble compounds that are usually nontoxic at high cellular concentrations. Generally, they protect plants from stress through different courses. Multiple functions for these compounds have been suggested. In addition to the conventional role of

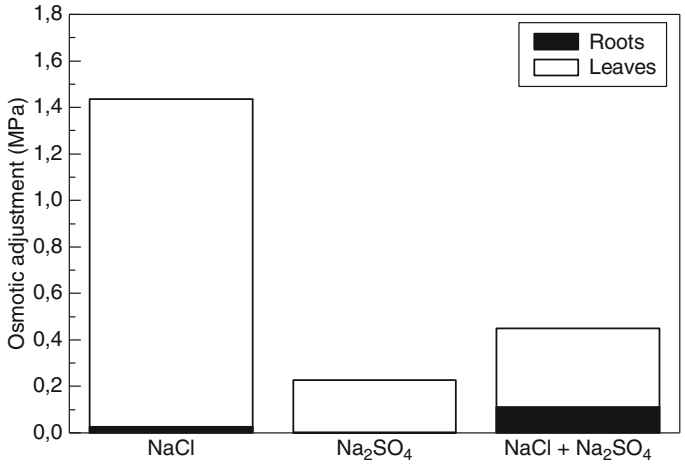

Fig. 5.3 Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on osmotic adjustment in leaves and roots of *Prosopis strombulifera* plants. Osmotic adjustment was calculated from two linear regressions for all treatments as the difference between osmotic potential (OP) and the estimated OP of tissue ascribed to mere loss of water at each given RWC (OP_0) at RWC of 70%

these compatible solutes in cell osmotic adjustment, they are also reported to act as low-molecular-weight chaperones, stabilizing the photosystem II complex, protecting the structure of enzymes and proteins, maintaining membrane integrity and scavenging ROS (Bray 2002; Manivannan et al. 2008; Türkan and Demiral 2009). Recently, it was also shown that some of these compatible solutes are very efficient in reducing the extent of K^+ loss in response to both salinity and oxidative stress (Cuin and Shabala 2007) in barley and *Arabidopsis* roots.

Compatible solutes fall into three major groups: amino acids (e.g. proline), quaternary amines (e.g. glycine betaine, dimethylsulfoniopropionate) and polyols/sugars (e.g. mannitol, trehalose). Many plant species accumulate significant amounts of glycine betaine, proline, and polyols in response to high salinity (Di Martino et al. 2003; Silveira et al. 2009). Different varieties of a particular plant species exhibit a high degree of variation in salt tolerance (Chen et al. 2007) and a possible causal link between these responses and the differential accumulation of glycine betaine and proline among cereal genotypes has been proposed (Yang et al. 2003). Indeed, the introduction of genes involved in the synthesis of proline, betaines, and polyols in plants contributes to an increased abiotic stress tolerance (Chen and Murata 2002); numerous genetic engineering attempts to manipulate compatible solutes biosynthetic pathways have been made in order to enhance salt tolerance (Wang et al. 2003; Chen et al. 2007; Verbruggen and Hermans 2008).

In *P. strombulifera* seedlings a remarkable proline accumulation occurred under increasing salinity. The proline content increased by 200% since the start of all salt treatments that is in line with the earlier suggestions that proline content in plants

increases in a linear relationship with conductivity in the medium (Llanes et al. 2010). In *Medicago truncatula,* proline accumulation in response to NaCl stress was higher in aerial parts (13 fold) than that in roots (8-fold) compared to controls (Armengaud et al. 2004). A similar effect was observed in *P. strombulifera* where proline accumulation in leaves was much more important than in roots. This fact could be related to the high ion accumulation in salt-treated leaves, raising the need for organic molecules production to counteract osmotic balance and/or ion sequestration, scavenging free radicals and buffering cellular redox potential under stress conditions. The results with NaCl and Na_2SO_4 treated plants indicate that proline would be a stress intensity signal but not a salt tolerance indicator, as it increased concomitantly with decrease in osmotic potential, independently of the ion composition of the solution (Llanes et al. 2010).

In view of the literature, there is also a linear relationship between glycine betaine content and salt stress intensity in several species. However, *P. strombulifera* seedlings did not accumulate glycine betaine under any salt treatment in our experiments in line with the statement by Tipirdamaz et al. (2006) that 'species that behaved as proline accumulators contained little betaines and vice versa'.

A number of sugar-alcohols are present in some but not all halophytes from several families, as is the case of pinitol (Ishitani et al. 1996; Murakeozy et al. 2003; Arndt et al. 2004). The phreatophytic but desert-dwelling *Populus euphratica* accumulated the cyclitols chiro-inositol and pinitol to significant concentrations when growing over saline groundwater in the Taklamakan desert in China (Arndt et al. 2004). Sorbitol and mannitol are not widely reported in halophytes, but are present in several families of flowering plants, including the mangrove *Lagunculana racemosa* (Noiraud et al. 2001; Koyro 2006).

Mannitol accumulation in *P. strombulifera* seedlings only occurred in leaves of NaCl-treated plants with moderate to high salinity, while in those treated with sulphate and salt mixture, manitol level was lower than that in controls. By contrast, sorbitol accumulated in greater amounts in sulphate and salt mixture-treated plants, while under NaCl treatment sorbitol level was similar to controls. Thus, it appears that this species uses mannitol + pinitol for osmotic adjustment in the presence of NaCl, while in the presence of the toxic sulfate it synthesizes sorbitol + pinitol. Thus, sorbitol biosynthesis might be a possible manifestation of a carbon metabolism disorder (Llanes et al. 2010).

Mannitol and sorbitol have been targeted for engineering compatible-solute overproduction. Tarczynski et al. (1993) introduced a bacterial gene that encodes mannitol 1-phosphate dehydrogenase into tobacco plants, resulting in mannitol accumulation and enhanced salinity tolerance. In addition, transgenic tobacco plants carrying a cDNA encoding myo-inositol *O*-methyltransferase (*IMT1*) accumulated D-ononitol and, as a result, acquired enhanced photosynthesis protection and increased recovery under drought and salt stress (Shelevela et al. 1997). On the other hand, abnormal phenotypes associated with sorbitol accumulation were also found in transgenic tobacco transformed with *stpd1*, a cDNA encoding sorbitol-6-phosphate dehydrogenase from apple. Plants with low levels of sorbitol (less than 2–3 mmol g^{-1} fresh weight) developed normally, but necrotic lesions and

reduced shoot and root growth, as well as low fertility, were associated with an excessive sorbitol accumulation. The adverse effects observed in these sorbitol overproducers may have resulted from a disturbance in carbohydrate transport and allocation (Shelevela et al. 1998) which confirms our observations in *P. strombulifera.*

More generally, the roles of compatible solutes are still being vigorously debated, especially in relation to crop yield in water-limited environments (Ashraf and Akram 2009; Hamdia and Shaddad 2010). After analyzing a large body of work, many authors suggested that 'the purely osmotic contribution of these metabolites to stress tolerance may not describe their function completely, and that the pathway leading to a particular osmolyte may be more important than accumulation *per se*. Several examples of other significant stress-relieving functions served by either the solute or the associated enzyme pathway have been reported, *e.g.*, free radicals scavenging. Irrespective of the exact function of osmolytes or their biosynthetic pathways, the identification and estimation of their significance to cell turgor maintenance would be an important step forward (Adams et al. 2005; Flower and Colmer 2008).

3.4 Anatomical Modifications

In addition to producing metabolic changes, soil salinity can also affect the plant general growth, particularly its morphology and anatomy (Serrato Valenti et al. 1991; Reinoso et al. 2004, 2005; Tattini et al. 2006; Wang et al. 2008). It is generally known that plants that grow well in highly salinized environments have specific structural adaptations to allow altered physiological and biochemical mechanisms while maintaining their reproductive capacity (Shannon 1994).

Prosopis strombulifera does not possess salt glands in its leaves. When grown under high NaCl salinity some tissues of this plant develop great vacuolization and the radical system acquires particular characteristics such as precocious lignification and (or) suberization of endodermal cells, with casparian strips found much closer to the root tip than that in glycophytes. Consequently, these plants have greater potential to efficiently filter the soil solution to prevent an excessive ion loading onto the xylem (Reinoso et al. 2004). On the other hand, Na_2SO_4 treatment induced structural alterations in the cells and tissues of *P. strombulifera* seedlings, which modified the growth pattern at different levels of organization. These alterations included anatomical and histological differences in roots, stems, and leaflets from Na_2SO_4 -treated plants compared to NaCl -treated plants and those grown without any salt (Reinoso et al. 2005). Although the low water potential and the ion toxic effect had strong incidence on cellular growth in these plants, the photosynthetic apparatus might have not loosened efficiency thus providing the carbon skeletons necessary for neoformed parenchymatic layers in the stem, neo-synthesis of lignin, suberin and tannins, whose concentrations increased proportionally with salinity increase (Reinoso et al. 2005). All these complex compounds of high molecular weight greatly influenced total dry weight of treated seedlings, mainly those treated with Na_2SO_4, because this parameter was not modified in spite of the marked growth inhibition observed in the latter.

An important feature in salt-treated plants is the significant increase in tannin content in all organs, which increased with salt concentration independently of light intensity and temperature conditions (Reinoso et al. 2004, 2005; Reginato 2009). Results of several studies suggest that tannin accumulation occurs in response to different stressing conditions (Fahn and Cutler 1992; Grossoni et al. 1998; Hwang et al. 1995) and may be involved in cell protective roles such as scavenging of reactive oxygen species.

3.5 Antioxidant Defense

Salt stress leads to increased reactive oxygen species (ROS) production in plant cells, which are known to produce a secondary oxidative stress under these conditions (Sreenivasulu et al. 2000). To counteract oxidative stress induced by salinity, plants have developed different strategies among which the stimulation of synthesis of secondary metabolites, particularly polyphenols, may be involved in cell protective roles such as ROS scavenging (Simic and Jovanovich 1994).

As reported earlier, there is an important increase in tannin content in salt-treated *Prosopis strombulifera* plants. Leaf and root content of total phenols, total flavonoids, total flavan-3-ols, condensed tannins (or proanthocyanidins), tartaric acid esters and flavonol analysis showed that salt treatments increased the accumulation of several polyphenols in both leaves and roots (Fig. 5.4).

Sodium sulphate (Na_2SO_4) treatment sharply induced an increase in total polyphenols showing the highest levels of these compounds in our experiments, especially flavonoids and total flavan-3-ols. Bisaline treated plants also accumulated high levels of polyphenols. HPLC analysis showed high levels of rutin, catechin, epicatechin and proanthocyanidine in these plants.

These increases in total flavonoids and flavan-3-ols, when SO_4^{2-} anion is present in the growth solution, may indicate a role for these compounds in counteracting the oxidative damage induced by severe salt stress (Reginato et al. 2010). Agati and Tattini (2010) reported that antioxidant mesophyll flavonoids, at micromolar range, may effectively avoid generation of reactive oxygen forms (e.g. by chelating transition metal ions).

3.6 Changes in Photosynthetic Pigments

Salinity results in both qualitative and quantitative changes in photosynthetic pigment composition depending upon the species tested and quality and quantity of salts used. Chlorophyll concentration in leaves is an important biochemical indicator that represents the potentiality of the photosynthetic apparatus in plants, and an increase in chlorophyll content has been observed in salt tolerant species (Joshi 1976; Reddy et al. 1992).

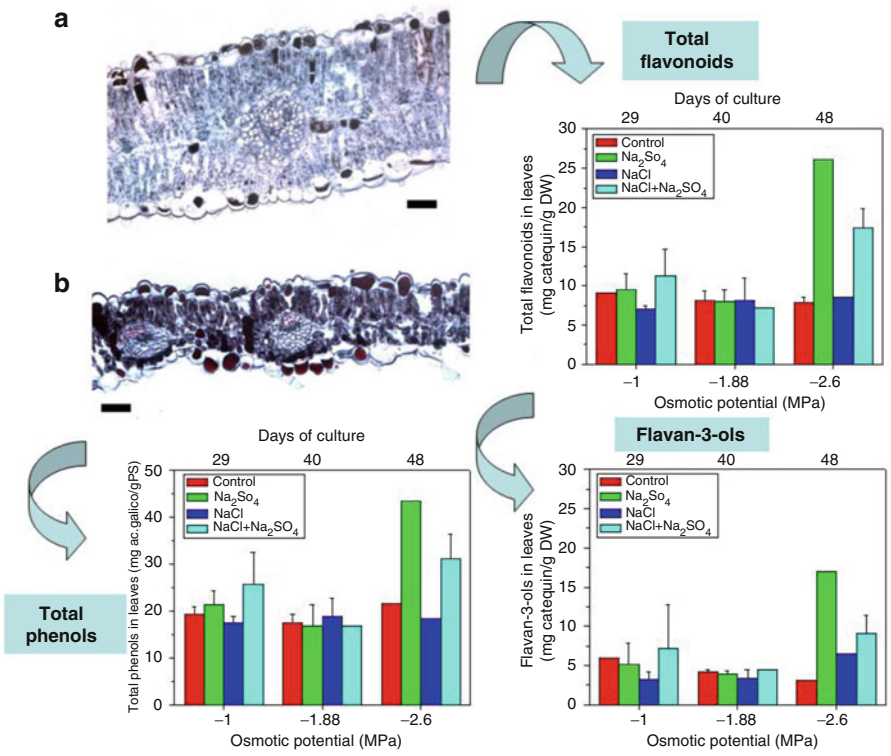

Fig. 5.4 Transverse section of 48 day-old plant leaflets showing the accumulation of condensed tannins under salt stress (Control plants (**a**) and Na_2SO_4 treated plants ($\Psi_o = -2.6$ MPa) (**b**); Scale = 50 μm). The associated graphics show the respective quantification of polyphenols in control and treated plants

The photosynthetic pigment content in *P. strombulifera* was also differentially affected by NaCl, Na_2SO_4, and their mixture. Chlorophyll *a* content remained constant in all treatments. On the contrary, chlorophyll *b* content significantly increased under all treatments of low salinity, with a sharp increase after Na_2SO_4 treatment. Reddy et al. (1997) proposed this increment as a cellular adaptation to satisfy the increasing demand of energy due to salt toxicity. One could speculate that the antennae pigments need to be more effective to deliver sufficient energy to deal with energy-consuming adaptations to salinity. Similar to our results, the halophyte *Aster tripolium* L. showed high tolerance to soil salinity and increased chlorophyll contents to get higher light harvesting capacity and, hence, higher rates of photosynthesis (Ramani et al. 2006).

Carotenoids play three major in the photosystems of thylakoid membranes are light harvest, photoprotection and maintenance of structural stability (Nayak et al. 2002). The effect of salinity on biosynthesis of carotenoids in plants is little known.

In our experiments, Na_2SO_4 treatment caused a 100% increase in carotenoid production from the beginning of salinization. Similarly, *Plantago coronopus* leaves showed salt-induced increase of carotenoid content when plants were cultured in 500 mmol L^{-1} NaCl (Koyro 2006). The proposed function for this carotenoid increase is excess energy dissipation as heat or non-damaging chemical reactions in photosystem I and II (Lu et al. 2003), and chloroplast membranes stabilization (Havaux 1998). Additionally, the increase in the carotenoid pool in *P. strombulifera* is correlated with a marked increase in the phytohormone abscisic acid (ABA).

3.7 Polyamine Accumulation and Metabolism

Polyamines (PAs) play an important role in plant responses. Free PAs are small organic cations essential for eukaryotic cell growth, and have been proposed as a new category of plant growth regulators involved in a wide variety of physiological processes (Liu et al. 2007). The three main PAs in plants are putrescine (Put), spermidine (Spd), and spermine (Spm). Less common PAs are diaminopropane (Dap) and cadaverine (Cad).

Accumulation of PAs under salt stress was reported for mono- and di-cotyledonous species (Bouchereau et al. 1999; Simon-Sarkadi et al. 2002). *Lupinus luteus* seedlings accumulated Put and Spd in leaves in response to increased NaCl (Legocka and Kluk 2005). Salt-tolerant cultivars of rice and tomato accumulated Spd and Spm, whereas salt-sensitive cultivars accumulated Put (Krishnamurthy and Bhagwat 1989; Santa Cruz et al. 1999). PAs are presumed to have protective functions, *e.g.*, scavenging free radicals (Besford et al. 1993), cellular pH maintenance, cellular ionic balance and membrane stabilization (Kakkar and Rai 1997; Aziz et al. 1999; Groppa and Benavides 2008), cationic channels regulation in cells (Liu et al. 2000; Janicka-Russak et al. 2010), prevention of lipid peroxidation (Verma and Mishra 2005; Zhao and Yang 2008) and bioenergetics of photosynthesis (Ioannidis and Kotzabasis 2007). These various processes may occur separately, or be combined as a unified strategy to minimize membrane damage, promote cell growth, or enhance cell survival in response to stress (Liu et al. 2007).

Stressful conditions may alter the content of endogenous PAs, particularly Put, with variation according to type of stress, species, and time of exposure (Kakkar and Rai 1997; Ali 2000). In *P. strombulifera,* leaves of Na_2SO_4-treated plants showed the lowest Put content, which was associated with a strong inhibition of shoot growth and possibility associated with a general metabolic alteration caused by SO_4^{2-} anion (Reginato 2009). The physiological significance of stress-induced Put accumulation has been linked to maintenance of cellular pH, cation/anion balance, and membrane stabilization (Kakkar and Rai 1997). In contrast to leaves, Na_2SO_4-treated roots showed the highest Put contents, together with significant increases in Spd and Spm. PA accumulation has been correlated with adventitious and lateral roots formation

(Altamura et al. 1991) in coincidence with our previous observations in *P. strombulifera* (Reinoso et al. 2005). Perhaps Put is involved in the plasticity of root development, *i.e.,* part of an attempt by Na_2SO_4-treated plants, to optimize ions and water uptake under stressful conditions imposed by this salt.

The low Spd content in *P. strombulifera* leaves, in all treatments, suggest a rapid degradation of this amine to Put, as a part of an interconversion pathway well known in animals and also characterized in higher plants (Duhazé et al. 2002). That part of the high Put content in NaCl-treated plant leaves in our studies could be due to Spd catabolism. On the other hand, plants that accumulated the highest Spd content in leaves were those with higher toxicity and deterioration symptoms caused by Na_2SO_4. Spd accumulation in these Na_2SO_4-treated plants may represent a metabolic disorder, rather than an adaptation mechanism. In addition, Spm content in leaves was not altered by changes in salinity; hence, this PA, at least in its free form, may not play a significant role in salt stress response in this species.

Salinity plant response is often associated with stimulation of PAs oxidation and catabolism (Cona et al. 2006), which play an important role in the regulation of cellular PAs level. Accumulation of Dap and Put could result from the presence of polyamine oxidases (PAOs) acting on Spd, as reported by Duhazé et al. (2002) in various halophytic species of the genus *Limonium*. The specific effect of Dap is poorly understood, but may be involved in biosynthesis of uncommon PAs and/or β-alanine, via oxidative deamination pathway, in some species (Cohen 1998). Biosynthesis of uncommon PAs such as 1.3 Dap, Cad, norspermidine, homospermidine, and norspermine, has been associated with the capacity of some biological systems to grow or function under extreme environmental conditions (Flores 1991). In salinized *P. strombulifera*, several unknown chromatographic peaks were observed, mainly in Na_2SO_4-treated plants at high salinity. The analysis of these peaks, which may correspond to uncommon PAs as above, deserves further research.

The presence of diamine Cad is sporadic and uncommon. Cad has been found in several other genera of Leguminosae, and also in Gramineae and Solanaceae (Flores 1991). Although Cad in roots has been associated with cell elongation and adventitious root formation, similarly to Put (Shevyakova et al. 2001; Carrizo et al. 2001), its accumulation in Na_2SO_4-treated roots of *P. strombulifera* may reflect a stress symptom rather than an adaptative response.

PAs content in leaves and roots of control and salt-treated plants at −2.6 MPa (day 48) is shown in Fig. 5.5 for a more comprehensive understanding of the distribution pattern of these compounds.

In summary, the limited variation in free PAs content in salinized *P. strombulifera* suggests active PA catabolism and conjugation. This could be related to observations that in higher plants subjected to salt stress, PA profile is disturbed in glycophytes but remains stable in halophytes. Internal level of PAs in *P. strombulifera* may remain constant because of equilibrated rates of synthesis and degradation, which are differentially affected by different salts (NaCl, Na_2SO_4, and their iso-osmotic mixture) in roots vs. leaves.

3.8 Hormonal Changes

Hormonal changes in response to environmental abiotic stress have been well-studied in glycophytic plants, but such information is much more limited for halophytic plants. Plants respond to biotic and abiotic stresses by generation of various lipid-based signals. Jasmonates (JAs) are key signaling molecules in plant stress responses and development (for review see Wasternack 2007). This group of compounds includes jasmonic acid (JA), its methyl ester (JAME), its amino acid conjugates, and metabolites such as 12-OH-JA and 11-OH-JA. The octadecanoid cis (+) 12 oxophytodienoic acid (OPDA) is the precursor of JA.

Changes of jasmonate profiles have been reported in response to abiotic stresses such as NaCl, mannitol/sorbitol, and water stress (Kramell et al 2000; Pedranzani et al. 2007). In glycophytes, tomato, barley, and others, involvement of JAs in salt responses has been well established (Pedranzani et al. 2003; Wasternack and Hause 2002).

The same jasmonate family members (OPDA, JA, 12-OH-JA, 11-OH-JA) found in glycophytes are present in the extremely salt tolerant halophyte *Prosopis strombulifera*. In this species, we observed large differences in total amount of JAs between organs, as well as in the ratios of these compounds. Similarly, Miersch et al. (2008) reported differences in the content of JAs among different tissues of various plant species. The major components in *P. strombulifera* roots were OPDA and JA, whereas those in leaves were OPDA and 12-OH-JA, confirming the existence of specific metabolic JAs profiles depending on the organ analyzed, as also observed in glycophytes. Hence, the concept of an "oxylipin signature" (Weber et al. 1997) or a "jasmonate signature" (Miersch et al. 2008), can be applied to the halophyte *P. strombulifera*.

OPDA is synthesized in leaves (in chloroplasts), and there is an evidence of a similar pathway in roots (Hause et al. 2000; Abdala et al. 2003). The high OPDA content in 29-day-old control roots of *P. strombulifera* suggests active biosynthesis in this organ. Contrarily to what it was expected, given the fact that *P. strombulifera* is a halophyte, its roots responded to high salinity by decreasing total content of JAs. OPDA content was higher than that of the other JAs, and was more strongly affected by salt stress. JAs are known to inhibit primary root elongation (Woodward and Bartel 2005; Wasternack 2007). However, a few reports demonstrated that exogenously applied JAs enhance rooting *per se* (Moons et al. 1997; Toro et al. 2003). In *Arabidopsis*, exogenous MeJA positively regulates lateral root formation (Sun et al. 2009). In *P. strombulifera,* NaCl-treated plants showed the lowest OPDA content at −1 MPa, concomitantly with a significant root growth promotion.

The differential response to salt treatments of roots vs. leaves could be attributed to the root's function as a "stress-receptor organ", which responds to stress with more dramatic hormonal changes compared to leaves. Another difference is the higher content of 12-OH-JA in leaves than in roots; hence, the higher content of this compound in leaves may result from active hydroxylative pathway in these organs or transport of root-generated 12-OH-JA. For many years, 12-OH-JA was detected

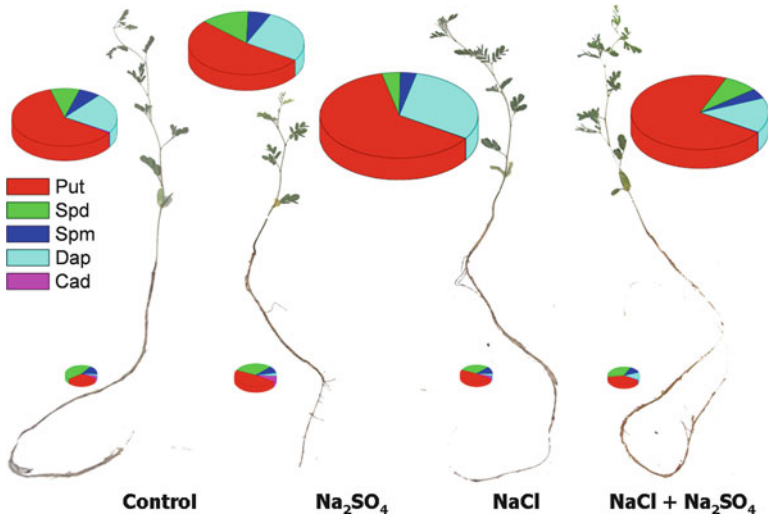

Fig. 5.5 Total PAs levels in *P. strombulifera* plants. Control plants (48 days); Na_2SO_4, NaCl and NaCl + Na_2SO_4 treated plants ($\Psi_o = -2.6$ MPa, 48 days). The area of the *circles* designates the total PAs levels in different organs of the plant. The size of the circle in leaves of NaCl-treated plants corresponds to 846.66 nanomol/g FW (100%). The sizes of *circles* (and their respective portions) in the other plant organs and treatments are proportionally depicted

only in solanaceous species, although more recently analysis of monocotyledons and dicotyledons revealed the presence of 12-OH-JA in a wide variety of species and organs, including tomato flowers (Hause et al. 2000), *Arabidopsis thaliana* leaves (Gidda et al. 2003), potato tubers and stolons (Cenzano et al. 2005), tomato seeds (Andrade et al. 2005), sunflower seeds (Vigliocco et al. 2007), and soybean seeds (Miersch et al. 2008). Clearly, 12-OH-JA must be involved in physiological processes other than tuberization. On the other hand, 11-OH-JA is usually present in much lower concentration than that of 12-OH-JA, except in the seeds of *Cucurbita pepo* (Miersch et al. 2008). In *P. strombulifera* salt treatments had no effect on both hydroxylates content. As for PAs, Fig. 5.6 shows JAs distribution pattern under salinity.

On the other hand, upon salinity or drought stress, plants accumulate the phytohormone abscisic acid (ABA), which in turn controls many adaptive responses such as salinity-induced stomatal closure and induction of gene expression and tolerance to drought. Results obtained previously in our group (Sosa 2005) demonstrated that optimal growth was observed in *P. strombulifera* seedlings when the salt concentration in the medium was 400–500 mM NaCl (Ψ_o: −1.8 MPa). Thus, higher or lower salt concentrations could be registered as stressful by the plant, which could explain the high ABA levels found in plants growing in the salt-free Hoagland solution (controls). Moreover, a marked decrease in ABA level at Ψ_o: -1.88 MPa in NaCl treated plants compared to controls was found, which could mean that such NaCl

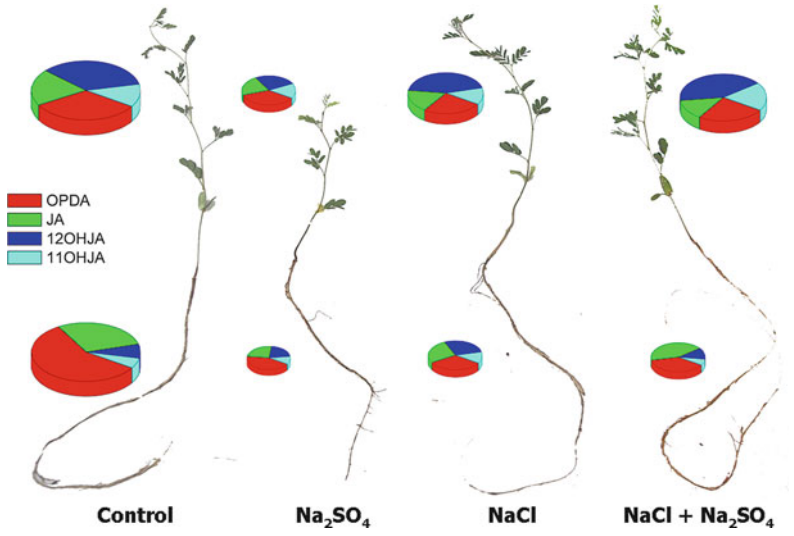

Fig 5.6 Total JAs levels in *P. strombulifera* plants. Control plants (48 days); Na_2SO_4, NaCl and NaCl + Na_2SO_4 treated plants ($\Psi_o = -2.6$ MPa, 48 days). The area of the *circles* designates the total content of JAs in different organs of the plant. The size of the circle in leaves of control plants corresponds to 12,005 pmol/g DW (100%). The sizes of *circles* (and their respective portions) in the other plant organs and treatments are proportionally depicted

concentration is not a stressful condition for this species, showing a true halophytic response. Higher NaCl concentrations lead to higher ABA accumulation (Sosa 2005; Llanes 2010).

In Na_2SO_4 -treated plant leaves the highest free ABA peaks were quantified, mainly at the highest salt concentration, in coincidence with growth inhibition, toxicity symptoms and significant levels of Spd. As mentioned earlier, a correlation between higher ABA levels and higher PAs accumulation was observed in our experiments. Similarly, Liu et al. (2005) reported ABA-induced PAs synthesis in salt stressed maize plants. Furthermore, Hanzawa et al. (2002) and Urano et al. (2003) informed that exogenous ABA application stimulated transcription and accumulation of genes involved in polyamine biosynthesis.

With respect to ABA metabolism, our results showed that both roots and leaves of *Prosopis strombulifera* seedlings accumulated mainly ABA-GE (glucose conjugated) in relation to phaseic acid (PA) and dihydrophaseic acid (DPA) whose levels were very low. This pattern was especially notable in salinized plants at highest Na_2SO_4 concentration. The greater ABA-GE accumulation recorded in roots under high salt concentrations (−2.6 MPa) could be related to ABA transport to the leaves where highest levels of free ABA were detected. It is noteworthy that in the presence of Na_2SO_4, both roots and leaves showed the highest concentration of ABA-GE as well as free ABA, which correlates with the severe stressful conditions imposed by this salt (Llanes et al. 2008).

4 Biotechnological Approach

4.1 P. strombulifera as a Natural Gene Donor to Improve Salt Tolerance in Crops

Development of stress tolerant crop plants requires, among others, knowledge of the physiological mechanisms and genetic control of the contributing traits at different plant developmental stages. In the past two decades, biotechnological research has provided considerable insights into abiotic stress tolerance mechanisms in plants at the molecular level (Munns 2007). Nevertheless, for halophytic species this knowledge is still very incomplete.

The exploration for highly salt-tolerant *Prosopis* species is important for both theoretical and applied considerations. Within the family Fabaceae to which *Prosopis* belongs, virtually all of the important annual legumes of the Papilonoideae subfamily, *i.e.* soybeans, common beans, peas *etc*., are highly salt sensitive to salt and only some alfalfa varieties have a moderate degree of salt tolerance. Thus, identification and full understanding of salt tolerance mechanisms in *Prosopis* species may have relevance to current commercially important legumes (Felker 2007). The search for cDNAs and differentially expressed RNA sequences in this highly salt-tolerant *Prosopis* species, *P. strombulifera,* is being carried and in our lab with the hope that this information would be useful for improving stress tolerance in common annual legumes.

In *P. strombulifera* seedlings four sequences were isolated and identified in our lab, showing homology with proteins encoding genes involved in salinity responses. They were named *PSALl, PSAL3*, *PSAL11* and *PSAL5*, and were selected for further studies of gene expression and functional analysis with the aim of contributing to understand the mechanisms responsible for the extreme salt tolerance of this species. The isolation of *PSAL1* sequence, which has homology with an ABA-dependent atypical LEA protein, is an interesting result related to cellular protection against osmotic stress (Bray 2002; Parida et al. 2007). Preliminary studies of this gene expression showed that *P. strombulifera* roots constitutively accumulate high levels of the corresponding transcript, whereas gene induction was observed only in Na_2SO_4-treated plant roots at $\Psi_o = -1.88$ MPa, which coincides with ABA and ABA-GE accumulation. *PSAL5* sequence encodes a myo-inositol-1-P synthase enzyme, which participates in the formation of the pinitol precursor; its further study will give us valuable information considering that pinitol is the main polyalcohol accumulated in our species under salinity. *PSAL11* sequence shows homology with a potassium and sodium transporter HKT1 from *Mesembryanthemum crystallinum*. Functional analysis of this transporter would provide important information on osmotic potential regulation in *P. strombulifera* seedlings. Similarly, *PSAL3* sequence shows homology with a sodium/proline transporter, which may play a key role in stress tolerance considering that both, sodium and proline, accumulate largely in this species (Llanes 2010).

4.2 *P. strombulifera and Its Rhizosphere as Natural Sources of PSHR Microorganisms Which Are Capable of Improving Salt Tolerance in Crops*

4.2.1 Plant Growth Promoting and Stress Homeo-Regulating Rhizobacteria

Bacteria living in the rhizosphere are commonly named rhizobacteria, and have the ability to associate and colonize a large number of plant species (Garate and Bonilla 2000). Depending on the type of relationship established with plants, they could be classified into *symbiotic* or *free-living* rhizobacteria; whichever, all of them are associated to the endorhizosphere (*i.e.* endophytic inside plant tissues as bacteroids), rhizoplane or ectorhizosphere in a mutualistic relationship (Kloepper et al. 1989). Among the most successful partnerships in nature, there are some members of Order *Rhizobiae* with legumes, and some *free-living* rhizobacteria of the genera *Pseudomonas, Bacillus* and *Azospirillum* with grasses and other non-legumes (Döbereiner and Pedroza 1987). If the rhizobacteria are beneficial for plant growth, they are named plant growth promoting rhizobacteria (PGPR). Cassan et al. (2009a) suggested that microorganisms could be re-classified into three groups, according to their effect on the plant: (a) direct plant growth promotion, (b) indirect plant growth promotion or phytopathogens biocontrol, and (c) regulation of plant growth through homeostasis regulation under abiotic stress conditions.

The first group, PGPR, was proposed by Kloepper and Schroth (1978) and represents rhizobacteria that stimulate plant growth through direct mechanisms such as biological nitrogen fixation, supply or solubilization of essential mineral elements as iron (Fe) or phosphorus (P), and phytohormone production as auxins (Patten and Glick 1996), cytokinins (Tien et al. 1979) and gibberellins (Cassán et al. 2003), as well as certain polyamines particularly cadaverine (Cassán et al. 2009a).

The second group, known as biocontroling-PGPB (Bio-controlling-plant growth promoting bacteria) was defined by Bashan and Holguin (1997) for bacteria (not necessarily rhizospheric ones) that can stimulate plant growth through several biocontrol mechanisms such as biosynthesis of antibiotics or antifungal compounds, which activate under biotic (bacterial or fungal) stressing agents.

The third group, known as PSHR (Plant stress homeo-regulating rhizobacteria), was proposed by Cassan et al. (2009a) for those microorganisms which support or promote plant growth under abiotic stress conditions. The main plant promoting mechanism proposed for the third group is the production of active molecules that regulate abiotic stress tolerance in plants, such as abscisic acid (ABA) (Cohen et al. 2008), jasmonic acid (JA) (Forchetti et al. 2007) certain polyamines as cadaverine (Cassán et al. 2009b), and activities of enzymes such as ACC deaminase (Glick 2005). Another important mechanism, less studied, but of great interest from the ecophysiological point of view, is related to the bacterial ability to immobilize or neutralize toxic compounds, such as heavy metals, salts or ions (rhizoremediation) (Sgroy et al. 2010).

The halophyte *P. strombulifera* has various physiological and biochemical mechanisms that allow optimal growth under high saline conditions as reported earlier. Nevertheless, its adaptive success may depend, at least in part, on its ability to establish and maintain effective associations with plant growth-promoting endophytic or rhizospheric bacteria. Studies carried out in our lab (Sgroy et al. 2010) reported for the first time the isolation and molecular characterization of *P. strombulifera* endophytic bacteria as well as the principal PSHB mechanisms under controlled growth conditions. Twenty-nine endophytic strains were isolated from established *P. strombulifera* plants in their natural habits, with a 69% frequency of positive spore-forming bacilli, including *Bacillus* sp. representing the 80% of gram-positive isolated strains. The genotypic 16 S rDNA sequencing showed a prevalence of 18.75% for *L. fusiformis* (Ps7); 68.75% for *B. subtilis* (Ps8)*;* 6.25% for *B. licheniformis* (Ps14); and 6.25% for *B. pumilus* (Ps19). On the other hand, 31% of isolations were gram-negative bacilli, and genotypic identification showed a prevalence of 55% for *Achromobacter xylosoxidans* (Ps27) and 45% for *Pseudomonas putida* (Ps30).

The physiological evaluation of the potential PHSR mechanisms present in these rhizobacteria showed that all the strains were able to express *ACC deaminase* activity to produce high amounts of several phytohormones functionally associated with the abiotic stress tolerance, such as ABA and JA, and to immobilize the most toxic anion (SO_4^{2-}) for *P. strombulifera* plants grown under controlled conditions (Sgroy et al. 2010).

The current literature has identified a strong relationship between the bacterial ACC-deaminase activity and plant salt-stress tolerance (Ma et al. 2008). Similarly, bacterial production of ABA (Cohen et al. 2008) and JA (Forchetti et al. 2007) was correlated with the microorganism capacity to increase stress tolerance in inoculated plants. Little information is published on the rhizobacterial capacity to promote or regulate plant growth through sulphate immobilization under saline stress conditions. In this regard, our results could be considered the basis for a reconsideration of the rhizoremediation concept under salinity conditions.

The existence of the plant stress homeo-regulating mechanisms described earlier in rhizobacteria, could be the result of a co-evolutionary relationship allowing plants to select their most convenient partners for association, so as to improve their natural capacity to face adverse environmental conditions. Our findings suggest the existence of an "endophytic consensus" among all the strains colonizing the same plant, each of them expressing one or more different interacting mechanisms that jointly determine a global answer of plant growth regulation under salt stress.

5 Conclusions and Perspectives

The great opportunity for salinity tolerance research now is the ability to consolidate new molecular techniques with the body of literature on whole plant physiology. This new opportunity in salinity tolerance research provides exciting prospects for ameliorating the impact of salinity stress on plants through genetic engineering, and

improving native halophytic species performance for agricultural and environmental sustainability and human health care.

Halophytes have recently been pointed out as a useful approach for generating salt tolerant crops in the future by using them as alternative crops, like *Aster tripolium* and *Salicornia rubra* (Erdei and Bogemans 2009) or as gene donors for genetic manipulation of economically important species, representing a very good model for future research.

The present review is a contribution to a better understanding of the physiological responses of a native halophyte to salinity, taking into account the chemical nature of the major salts present in soils of several countries, not only Argentina. Beside this, a new approach emerging from this work is the consideration of the rhizosphere of halophytes as natural sources of PSHR microorganisms capable to help plants to improve salt tolerance, giving rise to a new biotechnological tool for the inoculants industry.

Finally, we consider that more interdisciplinary work is needed, and the interaction and cooperation between plant physiologists, bacteriologists and molecular biologists should be emphasized.

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Chapter 6
Breeding for Biotic Stress Tolerance in Plants

L.F. De Filippis

Contents

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M. Ashraf et al. (eds.), *Crop Production for Agricultural Improvement*,
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Abstract Modern agriculture is concerned with the production of crops used primarily for human and animal food, but in so doing there is often the need (in some cases by law) to protect the environment. In crop production there is also the need to lower production costs, and especially reduce the use of expensive pesticides

and fertilizers. It is often an important aim, which is not always fulfilled to apply fertilizers and pesticides only when needed, but in order for this strategy to succeed, a better understanding of biotic stress and associated influences from plant breeding achievements is required. Therefore the impact of biotic stress and injury to plants and plant yield is not only of economic importance to agriculture but is directly related to other biological and environmental questions. For example, biological and economic decision made over the control of biotic stress forms an important part of Integrated Pest Management (IPM). In this chapter, we deal with the latest results and conclusions of yield losses in plant pathology, entomology and weed science, and successful application of breeding approaches to limiting such yield reductions. We intend to cover all biological classes of biotic stressors, and plant breeding methods commonly used for the diversity of organisms involved. It will focus on current knowledge of yield and fitness loss in agricultural ecosystems, and improved approaches in order that crops can better tolerate these biotic factors. Therefore in the first part of the chapter we intend to cover agricultural crops, production and limitations, conventional and molecular breeding, and where DNA-based molecular markers have been used with advantages over traditional phenotype trait selection. Molecular markers can be used to tag biotic resistance genes, and they can serve for improvement of the efficiency of selection in plant breeding, by so called marker assisted selection (MAS). The potential benefits of MAS are discussed, especially with the use of MAS to overcome some of the problems faced by classical phenotypic screening approaches in conventional plant breeding programs. In the second part of the chapter we intend to discuss biotic stress within the context of each biological class of organisms involved in crop losses, and attempt to evaluate the knowledge available in breeding and control of biotic stress damage. Abiotic stress (dealt with elsewhere in the book) will be mentioned from time to time and we certainly make a strong argument for an integrated approach to these two types of stresses in agriculture whenever possible.

Keywords Agricultural crops • Biotic stress • Crop improvement • Crop production • Crop security • Environmental protection • Plant breeding

1 Introduction

Population increase and food security is now an issue of concern. The United Nations have projected that the world population will increase by 25% to 7.5 billion by 2020. On average, an additional 73 million people are added annually to the population of the world (an average of more than two persons every second); of which 95% will live in developing countries. At the moment, nearly 1.2 billion people live in a state of 'absolute poverty' (Oerke and Dehne 2004), of which 800 million people live under uncertain food security, and 160 million pre-school children suffer from malnutrition (Alexandratos 1999). A large number of people also suffer

from deficiencies of micronutrients such as iron, zinc and vitamin A. Food insecurity and malnutrition results in serious public health problems, and lost human potential. The amount of land available for crop production is decreasing steadily due to urban growth and land degradation, and the trend is expected to be much more dramatic in developing countries than in developed countries. In 1990, only Egypt, Kenya, Bangladesh, Vietnam, and China had per capita cropland availability below 0.25 ha. However by 2025, countries such as Peru, Tanzania, Pakistan, Indonesia, and the Philippines are likely to join this group (Sharma et al. 2002). The decrease in the amount of land available for crop production and an increase in human population will have major implications for food production and security over the next two to three decades. There had been a remarkable increase in total grain production between 1950 and 1980, but only a marginal increase was realized during 1980–2000 (Myers 1999). Much of the early increase in grain production resulted from an increase in area under cultivation, irrigation, better agronomic practices, and improved cultivars. Yields of several food crops have already reached a plateau in developed countries, and therefore, most of the productivity gains in future will have to be achieved in developing countries.

Rice, maize and wheat provide 60% of the energy consumed as food in the world making them the most important food crops. The global dimension of loss in crop production due to biotic stress is substantial. Oerke et al. (1994) estimated that between 1988 and 1990 from the total attainable production of the eight major crops (wheat, corn, rice, barley, soybean, cotton, potatoes and coffee) worth US$580 billion, about 42% or US$ 240 billion was lost due to insects (total 15%), followed by pathogens (total 13%) and weeds (total 13%). To protect plants from pests and diseases, farmers had spent worldwide in 1998 a total of $34 billion on chemicals, and will spend more in coming years with an annual growth rate in pesticide consumption of 4.4% (Yudelman et al. 1998). However, growing consumer concern about the use of agrochemicals to protect cultivated plants from pests and diseases has led to seriously consider alternatives to keeping plants ‘healthy’. Simply prohibiting the use of agro-chemicals would have a devastating effect on agricultural production. Schmidt (2003) estimated that without the use of agricultural chemicals in Germany alone the yield of the major crops would decrease by 35–48%, variation in yield could be high from year to year (32–47%) and farmers’ profitability would decline by 32–57%. The most efficient, cost-effective and environment friendly approach to prevent the losses caused by biotic disease epidemics is the development of genetic resistance to these biotic stresses. Various biotic factors inflict a wide range of problems in cultivation. Of these, fortunately only a few are known to cause serious concerns and almost complete loss of agricultural crops, however all have been shown to decrease production. Among them, some are of historical importance, like coffee rust caused by *Hemileia vastatrix*, which caused the catastrophic closure of the coffee plantation industry in Ceylon (Sri Lanka) during the second half of nineteenth century, paving the way for the development of the tea industry. As well, the South American leaf blight (SALB) by *Microcyclus ulei* in rubber, which caused the shifting of rubber cultivation to South and South-East Asia in the late nineteenth century from native

Brazil (Simmonds 1989; Wilson 1999). Biotic stresses therefore can take a heavy toll on crop productivity in agricultural ecosystems.

Most of the biotic stresses in agriculture are seasonal in nature. Field crops, which can also be seasonal may come under the threat of a stress when the periods of the crop and the stress coincide with each other. Whereas, perennial crops are non-seasonal in nature and are subjected to all kinds of biotic stresses irrespective of the seasons. Therefore, perennial crops undergo a sort of natural selection *in situ* against any adverse factors, year after year, and only the fittest must be advanced forward and encouraged to be planted in the years to follow. However, there would be a great deal of geographic variation in the stresses, as well as in the reaction of crops to different kinds of stresses. In practice, many of the biotic factors are controlled in most crops in a seasonal manner, but this method only becomes sustainable in the long term when newly released varieties are tested in different environments and are bred quickly after a 'new' disease arrives. Selection over many environments and for a number of years therefore can be one of the most important breeding strategies utilized in breeding new crop varieties, but this is ever seldom done in a systematic manner.

Crop improvement is important from both a scientific and socio-economic perspective, and the ways in which these factors interact and impact on agricultural development is important; including debates on genetically modified (GM) foods. Horst (2008) described resistance in a host-pathogen relationship as the ability of plants to limit the penetration, development and/or reproduction of invading pathogens. Tolerance of host plants is measured in terms of the ability to maintain growth and yield production, in spite of infection or invasion of pathogens. Although both factors are genetically controlled, the environment of the host and plant can modify expression and production to a certain extent, especially in moderately susceptible or resistant genotypes/varieties. The use of cultivars with single-gene resistance permits the selection of mutations at a single locus to render the resistance effective in a relatively short period of time. However, due to loss of variation and selection pressure, and evolution, new virulent races of a pathogen appear which increases the need to develop durable resistance. Hence, the use of combinations of resistance genes has been suggested as the best method for genetic control of some of the biotic stress organisms (Miklas et al. 2006). This can be achieved by pyramiding effective resistance genes, but expression of individual and group resistance genes is difficult to monitor in the field. Agricultural crops of perennial nature have had few focused attempts in breeding against biotic stress factors. However, the approach is usually to integrate breeding efforts combining all desirable attributes, including yield, quality and resistance (Todorovska et al. 2009). There is a great advantage with these perennial crops, in the way that at any time during the breeding process, emergence of any superior individual can be fixed by vegetative multiplication or cloning in tissue culture.

Crop productivity gains are essential for long-term economic growth, but in the short-term these gains are even more important for maintaining adequate food supplies for a growing world population. It is in this context that biotechnology and plant breeding will play an important role in food production in the

near future. In this review, we attempt to take a critical but practical look at the prospects and constraints of various types of plant breeding methods and biotechnologies, and their application in increasing crop production and improving nutritional quality. Within this chapter, we also briefly address the critical issues of biosafety and the impact of genetically engineered crops on the environment. Genetic engineering offers plant breeders access to an infinitely wide array of novel genes and traits, which can be inserted through a short-term single event into high-yielding and locally adapted cultivars. This approach offers rapid introgression of novel genes and traits into elite agronomic backgrounds (Beaver and Osorno 2009). New crop cultivars with resistance to insect pests and diseases, combined with bio-control agents should lead to a reduced reliance on pesticides, and thereby reduce farmers' crop protection costs, while benefiting both the environment and public health. Similarly, genetic modification for herbicide resistance is set to achieve efficient and cost effective weed control to increase farm incomes, while reducing labor demand for weeding and herbicide application. Labor released from agriculture can then be used for other profitable endeavours. In addition, there is an urgent need for less labor-intensive agricultural practices in countries significantly affected by human immune deficiency virus (HIV) and other chronic diseases (e.g. malaria). By increasing crop productivity, agricultural biotechnology can substitute for the need to cultivate new land and thereby conserve biodiversity in areas that are marginal for crop production (Pinstrup-Andersen and Cohen 2000). The potential of these technologies has been extensively tested in the model crop species of temperate and subtropical agriculture. However, there is an urgent need for an increased focus on crops relevant to small farm holders and poorer consumers in the developing countries of the humid and semi-arid tropics. The promise of biotechnology can only be realized by utilising the information and products generated through research on genomics and transgenics to increase the productivity of crops, through enhanced resistance to biotic and abiotic stress factors and improved nutritional quality.

Diseases and pests can cause significant losses to most plant production systems (Beaver and Osorno 2009). Control of these biotic constraints using agrochemicals can increase production costs and create the potential for contamination of both the food and the environment. Resistance to pesticides also represents a valuable disease and pest management tool. Therefore the development of crop cultivars with greater levels of disease and pest resistances is a primary objective of most breeding programs. Examples of plant breeding achievements in individual plants are many and beyond the scope of this chapter, therefore I intend to concentrate on the methods best suited for crop breeding. Finally, conventional breeding methods do have limitations, these will be outlined and this will give way to more discussion and rationale for the use of molecular breeding. Molecular markers can be used in improving the efficiency of selection in plant breeding by marker assisted selection (MAS). The potential benefits of MAS have been widely discussed, especially for solutions to overcome some of the problems faced by classical phenotypic screening approaches in plant breeding programs (Backes and Ostergard 2008).

2 Part A: Crop Breeding Techniques and Production

2.1 Agricultural Crops, Production and Constraints

2.1.1 Introduction

Human population is projected to grow at about 80 million per year to 7.7 billion by 2020, then levelling off at about 10 billion (Tilman 1999; Pinstrup-Andersen and Cohen 2000). This increase population density together with changes in dietary habits and increase use of grains for livestock feed is projected to cause the demand for grain production alone to more than double. However land suitable for agricultural production is limited and most of the soils with high potential production are already under cultivation. Water availability in many regions is restricted and suitable cultivated areas are actually shrinking (Nelson-Smith 1995). Sustainable production for high yield are urgently required, and the availability of fertile soils and the development of high yielding varieties are major challenges to agriculture. Safeguarding crop production from damage from pathogens, pests and weeds are also a major requirement to meet these challenges.

Rice, maize and wheat already use 40% of cropland, and as yields of these three crops, plus some other crops like soybean, cotton and sugar beet respond to high production levels and mechanization more stress will be placed on arable soils. In the last decade world-wide crop production increases have focused on an ever limiting number of plant species and cultivars. Diverse ecosystems have been replaced in many regions by simple agrosystems which are more vulnerable to disease and pests. In order to safeguard productivity of these crops and reach levels of production to meet demand, these crops have to be protected more and more from diseases, pests and weeds. Plant production loss data, including the importance of pathogens and pests, the pest organisms involved and the control and use of pesticides are a prerequisite for economic management and for evaluating efficacy of present practices. Estimates of actual losses in crops worldwide have been difficult to obtain, and since crop production technology and especially crop protection are changing constantly, loss data for all major food and cash crops can be important. However, in the last two decades only revised figures and estimates have been available, and it is with this in mind that most of the loss potential data used below are exactly that (i.e. are only estimates and projections) (Oerke et al. 1994; Oerke and Dehne 2004).

2.1.2 Crop Loss and Trade

(a) **Wheat** – grown in most countries around the world and is the most important cereal crop in the Northern Hemisphere, Australia and New Zealand. The major wheat producing countries are China, India, USA, France and Russia. International wheat trading nations include USA, Canada, France, Argentina

and Australia. In wheat, weeds are the most important pests worldwide, and pathogenic rust fungi, smuts and bunts, and some soil-borne pathogens are next. Total losses can vary considerably around regions of the world from 15% to over 35%. Nematodes, rodents, birds, snails and arthropods can cause significant loss in some regions, whereas world-wide losses due to viruses are of minor importance.

(b) **Rice** – production is largely concentrated in Asia, where it is considered a major source of food. Rice is mostly grown for subsistence or local markets, and only about 4% of global production reaches international markets. Bacterial pathogens are very important in rice but there are a diverse set of regional conditions which influences this, and weeds and insects can be more and more important. Weed control, whether by mechanical or chemical means can be very effective in all regions, whereas control of pests and diseases is heavily reliant on synthetic pesticides; and these can have variable effects, from 16% loss to 35% loss.

(c) **Corn** – staple food for human consumption in East Asia, Latin America and parts of Africa, but its production is highest in the Americas and international trade in corn can reach about 12% of production. Weed competition is the most important problem which can depress production the most, followed by animal pests, fungal and bacterial pathogens, with viruses being least significant. In Central Africa and South-East Asia crop protection is largely restricted to weed control, and despite this over 30% of attainable production can be lost.

(d) **Barley** – mostly grown in the Northern Hemisphere, especially in dry areas and areas short of cropping periods. Barley is hardly grown for human consumption anymore but rather for animal feed and malting. The international trade amounts to about 12% of production. Weeds are the cause of most production loss, followed by fungal pathogens, animal pests and viruses. Prevention of barley loss due to pest control measures can be as high as 20% of total production. This however can differ widely amongst regions (from 5% to 15% loss), and under intense cultivation this is mainly due to good weed control.

(e) **Potato** – the production of potatoes has gone through a period of expansion in recent times and is ranked number five in the food crops. Potato can produce more starch than any other crop and is only second in protein production to soybean. Vegetative propagation dominates potato planting therefore all pests and diseases are of importance to control. Major pathogens are bacteria and viruses, and nematodes. Between soil practices, mechanical and chemical control methods, prevention of production losses of near 30% can be achieved, yet despite this, actual losses can approach 40% world-wide.

(f) **Soybean** – an annual member of the legumes and satisfies about half the global needs for vegetable oils and protein. Most important producers are USA, Brazil, China and Argentina. International trade value can be over 25% of world production. Weeds are the most important production dangers followed by fungi, bacteria, viruses and pests. Control measures can protect about 30% of production and attained production can be high, but this is heavily dependent on regions.

Table 6.1 World regional differences in the overall efficacy of actual crop protection practices in eight major crops (wheat, corn, rice, barley, potato, soybean, sugarbeet and cotton)

World area	Percent reduction in total crop loss potential
North Africa	49
Central Africa	32
South Africa	36
North America	59
Central America	46
South America	47
Near East	47
South Asia	41
Southeast Asia	46
East Asia	56
Northwest Europe	70
South Europe	60
Northeast Europe	48
Southeast Europe	46
Oceania	45

Values are estimates for the period 1996–1998 as detailed by Oerke and Dehne (2004)

(g) **Sugar beet** – the most important sugar producing crop after sugar cane, and is mostly a Northern Hemisphere planted crop. It only accounts for about 35% of sugar production world-wide where the most important producers are France, USA and Germany. Seedling production can be slow and the plant can suffer losses from weed competition, but this can vary from 5% to 50% production loss. Pathogens are minor causes of losses in sugar beet crops and actual loss is usually restricted to below 10%.

(h) **Cotton** – not a food crop but one of the most important fibre crops globally. It is grown in almost all tropical and sub-tropical countries. Most important producers are China, USA, India and Pakistan, where in many developing countries it is an important cash crop. Cotton is especially vulnerable to insect attack and then weeds where losses can approach 35–40%. In most countries protection of cotton crops has increased dramatically and can approach on average 50% of production, but this can vary due mainly to the intensity of the growing system and money spent on protection chemicals.

2.1.3 Efficacy of Crop Protection

World-wide, the efficacy of success in crop protection was lowest in wheat, potato and barley (about 45% prevention), moderate in rice, maize and soybean (about 55% prevention) and highest in cotton and sugar beet (about 65% prevention) (Table 6.1). Regional variation in these figures was lowest in sugar beet and cotton,

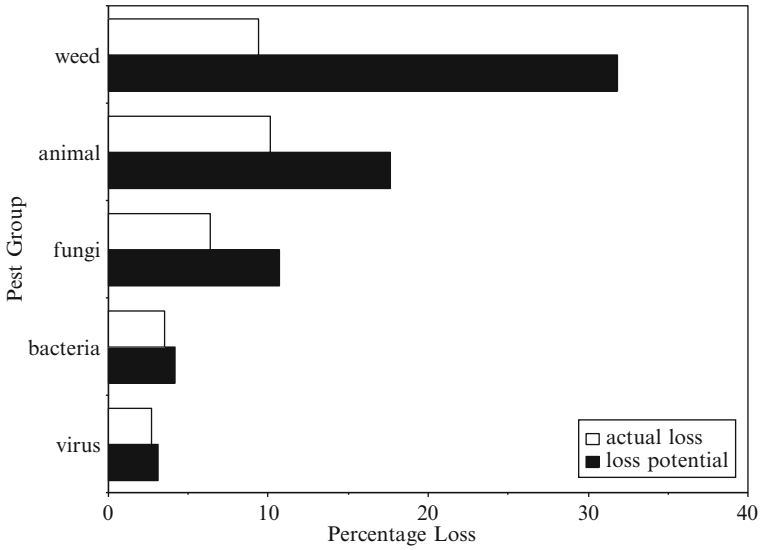

Fig. 6.1 World data on the percentage loss potential estimated, and the actual loss percentage of major agricultural crops according to the five major groups of pests and diseases. Values are estimates for the period 1996–1998 as detailed by Oerke and Dehne (2004)

and highest in potato and wheat. The percentage of loss prevention according to areas around the world ranged from 32% in Central Africa and Eastern Europe to 70% in North West Europe. In East Asia, North America and South Europe it was between 41% and 60% (Table 6.1). In terms of pest control measures by pest group and practice, mechanical or chemical weed control achieved an overall efficacy of almost 70%. Control of animal pests, bacteria and fungi were considerably lower and averaging about 20–45%. Virus control reached an efficacy on average of only about 15% (Fig. 6.1). In order to achieve even these efficacy figures considerable expenditure on herbicides, fungicides and insecticides was necessary, and there was considerable regional differences in such outlays and expenditure on agrochemicals; this is detailed in Fig. 6.2.

2.1.4 Safeguarding Crop Production

Much of the observed yield increase in recent decades can be attributed to better control over biotic stress rather than an increase in yield potential. But progress in improving food security has been uneven, and many developing countries have failed to see such progress. The persistence of food insecurity reflects regional differences rather than to a lack of overall capacity. It is worth noting that at present the world already produces sufficient food, but many people are undernourished because they are poor in terms of agricultural resources, education, technology etc;

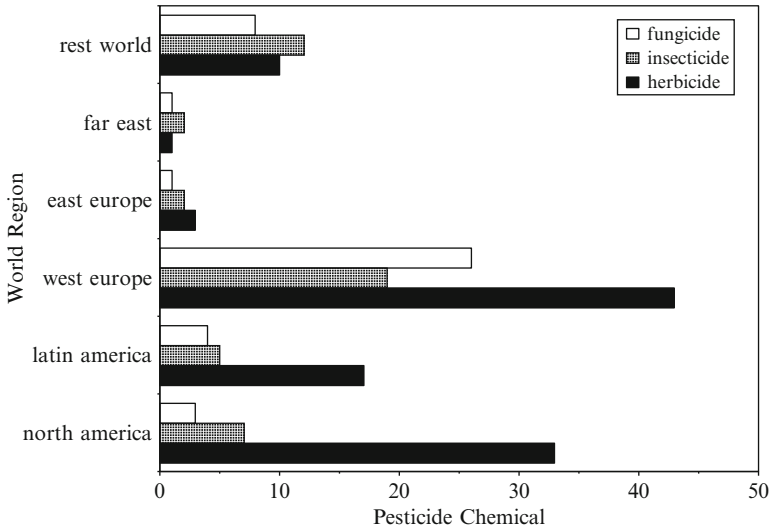

Fig. 6.2 World regional data on the percentage of pesticide used on major agricultural crops according to the three major groups; fungicides, insecticides and herbicides. Values are estimates for the period 1996–1998 as detailed by Oerke and Dehne (2004)

and thus are not able to produce their own food (Alexandratos 1999). The increasing trend of cereal yield increases alone should be sufficient to cope with most of the demographically projected increase in population until about 2025. World trade is expected to increase and expand considerably (Dyson 1999) but after 2025 the growth rate in food crop production of about 1.1% per annum achieved in the past 35 years is below the 2.0% per annum required.

Increase in cultivated land will contribute less than ever before, and in present high crop production systems any rise may be impossible to achieve; e.g. in rice where 70% attainable yield is being achieved in Japan, Korea and parts of China. Further yield increases must now depend on the availability and increase use of genetic high yield potential crop varieties and hybrids (Cassman 1999). However, losses due to weeds, animal pests, pathogens and viruses continue to reduce yields worldwide, and although advances have been made the picture is variable. Development of new compounds that are highly effective against former less controllable pests and the use of GM crops must be considered. Training of farmers by government and non-government organizations have contributed to a better outcome in the past 10 years, especially in Asia and Latin America; the situation is still not favourable in parts of the former Soviet Union and especially in sub-Sahara Africa for example. The problem of food allocation can in part be alleviated to some extent by intensification of crop production where the demand is highest, but this will not always be possible.

2.1.5 Integrated Pest Management (IPM)

Integrated pest management includes various techniques suitable for maintaining pest infestations below economically acceptable levels, rather than attempting to eradicate all pests. Therefore IPM is a management approach that encourages natural control of pest populations by anticipating problems and preventing them from reaching critically high levels. All appropriate techniques can be used in such an approach, such as:

(a) Enhancing numbers of natural enemies
(b) Planting pest resistant crops or cultivars
(c) Adopting appropriate cultural management and practices
(d) Using chemical pesticides judiciously
(e) Using organic 'natural' chemicals as deterrent

IPM programs have been established in various crops around the world and have proven especially suitable in developing countries (Cuyno et al. 2001). Where food supply often suffers from poor crop production technology and crop losses are high due to inadequate pest control, the intensification of food production can only be realized in part by implementation of IPM into current cropping systems. IPM has been used successfully in perennial and annual crops in both temperate and tropical conditions.

2.2 Conventional Phenotype Breeding

2.2.1 Introduction

The crop plants we see today are the result of spontaneous mutations followed by millions of years of evolution through natural selection; and more recently human intervention by selection which has artificially accelerated the pace of evolution. Plant breeders have developed desirable traits in plants resulting in the creation of numerous modern crop varieties. Modification of crop plants by man to improve cultivation has persisted for at least 10,000 years. Early farmers produced better crops by simply saving the seeds of their most desirable plants, unknowingly modifying the crops genetic make-up. Since the re-discovery of Mendel's laws in the early 1900s plant breeders have become more rigorous in this approach. Hybrid varieties and selection for increased yield and increased resistance to biotic and abiotic stresses have increased agricultural production consistently (Akhond and Machray 2009).

2.2.2 Backcross Breeding

The method is useful in preserving important cultural and seed traits of plants, yet incorporating simple inherited traits into new lines. However the method is less suitable for quantitatively inherited traits (e.g. tolerance to most abiotic stresses).

Backcrossing is inefficient in removing portion of the genome closely linked to the genes to be targeted in backcrossing. Linkage drag can impede effects to using backcrossing to introgress desirable traits into the recurrent parent. The Inbred Backcross Line (IBL) method developed by Bliss (1993) can produce near heterozygous lines that can be used for replicate testing of traits. One or two backcrosses are made after the initial set of crosses. The backcrosses are followed by a few generations of single seed descent (SSD – see Sect. 2.2.6) to produce an inbred backcross line. This method is well suited to marker assisted selection (MAS) and has been used to identify quantitative trait loci (QTL) conditioning resistance to *Fusarium* root rot (*Fusarium solani*)(Roman-Haviles and Kelly 2005). However, it has many limitations when used in most types of plant breeding, and other methods below appear better.

2.2.3 Gamete Selection

The method has been proposed to simultaneously select plants for multiple traits (Singh 1994). In breeding for multiple traits, gamete selection permits the early generation evaluation of potential resistance in populations. Populations that do not segregate for desired traits in early generation testing can be discarded, this avoids the loss of valuable time and resources. The method can be labour intensive and therefore permits evaluation of few populations, and care is required in selecting parents that possess the degree of resistance required (or desired trait). Gamete selection has been successfully used to develop high-yielding bean lines with resistance to leafhoppers and some diseases (Singh et al. 1998). Asenio-Manzanera et al. (2006) also used gamete selection to select for common bacterial blight and halo-blight resistance. Molecular markers can facilitate gamete selection and the method may be most efficient in pyramiding simple inherited traits or QTL traits to have large effects. The evaluation of large populations can be expensive and time consuming, unless an automated cheap method of screening is available.

2.2.4 Recurrent Selection Breeding

The method permits the accumulation of favourable alleles as a result of recombination in each cycle of selection. Because F_2 plants could be evaluated for plant resistance and seed trait, each cycle could be completed in a shorter period of time than most other methods. Nevertheless at least three cycles at a minimum are required to break up undesirable linkage (Singh 1999). Garcia et al. (2003) used recurrent mass selection to select bean populations with greater resistance to soil-born diseases caused by *Pythium, Rhizoctonia* and *Fusarium*. Breeding lines were developed to produce greater seed yield and higher survival rates than the parents. However, lines developed from inter-gene pool crosses usually have poor performance due to the breakup of some favourable gene complexes within each pool. So, recurrent selection provides additional opportunities for the plant breeder for recombination and formation of new gene complexes that could lead to disease resistance.

2.2.5 Pedigree Breeding

This is a very common method used to develop improved cultivars to diseases. But an important limitation to pedigree selection is the amount of time needed (Fehr 1987). In warm tropical areas, breeders can accelerate cultivar development by planting most of the year round, even in the dry season by using irrigation. Also breeding can be speeded-up by growing additional generations in nurseries or greenhouses. Whilst growing one generation, breeders can screen other breeding lines for disease resistance. Resistance to diseases such as anthracnose and bean common mosaic necrosis virus (BCMNV) have been successful, and in recent years pedigree breeding has also used marker-assisted selection to identify specific genes for disease resistance (Miklas et al. 2001). Exclusion of susceptible lines is necessary and Beaver and Macchiavelli (1998) have noted that screening breeding lines in F_4 or later generations can improve the probability of identifying lines with desirable genotypes; and would reduce the number of lines that need to be evaluated in early generations.

2.2.6 Single Seed Descent (SSD)

The method is recommended when working with crosses between elite lines within a set of varieties, and can provide a way of maintaining genetic variability. SSD can be conducted in the field and target environments, or in nurseries and greenhouses. Macchiavelli and Beaver (2001) noted that breeders often bulk seeds rather than use one single seed. Although bulking reduces genetic variability, on average every third F_6 line would be derived from a different F_2 plant. Concentrating on improvement of a single disease trait favours the use of SSD. SSD is a rapid method to develop recombinant lines for diseases that cannot be phenotyped in early generations. SSD can be costly, therefore it is primarily used in molecular and mapping techniques that have the aim of identifying parents that have increased disease resistance and are useful for later crossing. SSD is quite difficult to manage on a large scale and is most useful at breeding for specific purposes (Fehr 1987).

2.2.7 Bulk Breeding

An old and well established method where multiple generations can be grown each year. It is most appropriate for crosses between elite lines where little segregation for seed type or adaptation would be expected. If the bulked population can be grown in a target environment, some natural selection may occur for traits such as disease resistance, however it is more suited to increasing yields of plants and seeds. Plant breeders would need to advance large samples of bulked populations to avoid genetic drift. Molecular plant breeding techniques might be used to monitor genetic variability and determine an adequate sample size for breeding. Plant breeders must also avoid planting bulked populations in situations where undesirable traits may be favoured, and bulk breeding has been used extensively to obtain disease resistance in lentil to a number of important diseases (Renato-Corte et al. 2002).

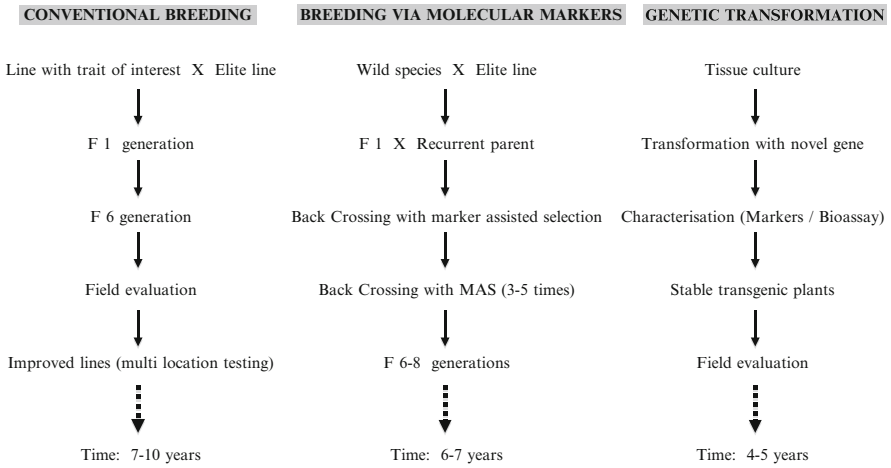

Fig. 6.3 A schematic diagram outlining approaches in crop improvement, comparing the steps required for the most commonly used methods; conventional breeding, breeding via molecular markers and using transgenic technology. Times taken for each method is only an approximation

2.3 Molecular Breeding

2.3.1 Introduction

In a number of situations conventional breeding may be assisted by molecular methodology in order to quicken selections, but also molecular approaches attempt to be more precise and bear lower costs. Figure 6.3 summarises essential differences between conventional breeding and molecular marker breeding with approximate times needed for an improved cultivar to be realised. A number of molecular methods have been employed to improve breeding objectives; these include:

(a) Key enzyme and/or metabolite measurements as a result of expression or even over-expression of genes
(b) Molecular signal changes between the host plant and the pathogen, but these have seldom been used
(c) Quantitative trait loci (QTL) and genomic maps (see Sect. 2.3.4 below)
(d) Molecular markers in MAS (see Sect. 2.3.5)
(e) Association mapping (Muehlbauer et al. 2006)

Using such molecular methods, breeders can screen for genes for disease resistance without running the risk of introducing the disease agent. The risk is that normally disease resistance genes are in clusters, so the breeder must be aware of the risk of introducing susceptibility to a different disease if these genes are linked for example (Michelmore 1995). Other problems include the specificity of some markers to only one plant genome, however as more and more genes are cloned,

documented and sequenced it may be possible to design more specific markers to specific alleles. Ideally a good marker useful for routine molecular screening should be specific, reliable, rapid and inexpensive. However as Broughton et al. (2003) noted that at the moment molecular plant breeding techniques (including molecular markers) are only an additional set of tools available for breeders. A suitable combination of conventional and molecular tools is still the best application for agricultural plant breeding, but needs should always be evaluated carefully as should resources (Bernardo 2008).

2.3.2 Key Enzymes and Metabolites

Knowledge of changes in a specific plant metabolic function induced by different treatments or observed during breeding has led to the development of methods to identify genes involved in regulating metabolic pathways and associated physiology and biochemistry. With the availability of tight mutant lines, the use of elegant biochemical screening systems based on this knowledge can be considered a relatively easy approach compared to gene isolation and selection. Many secondary plant metabolites such as flavonols have been implicated in plant pathology, including resistance to biotic stress factors (Sharma et al. 2002). The biochemical pathway to the synthesis of flavonols is well understood, however the use of such biochemical markers has been relatively unsuccessful. Fatty acid analysis and isoenzymes have also had limited success in plant breeding towards biotic stress resistance (Chawla 2009; Davey and Anthony 2010).

2.3.3 Molecular Signals

Responsive Induced Expressed Genes (micro RNA-miRNA and small interfering RNA-siRNA) code for at best small polypeptides that are induced due to biotic stresses. miRNA and siRNA are a class of small size non-gene-coding RNA that can act as regulators of gene expression in both animals and plants. The induction of miRNA may play a role in adaptation and resistance to biotic stress, although abiotic stress related miRNA and their targets are better known. At least a number of miRNA are known to be induced under the action of fungi and bacteria (miRNA 393 and miRNA 159), and more are induced due to viral infection (miRNA 156, miRNA 160, miRNA 164, miRNA 166, miRNA 169 and miRNA 171) (Lin et al. 2009). The mechanism of pest-transcription regulation is not well known or understood. In this regard mi RNA 399 induced during low phosphorus nutrition and miRNA 395 induced during sulphur homeostasis are better understood (Bartel 2004). miRNA and siRNA have been estimated to constitute about 1% of plant genomes, which means that more unknown miRNA and siRNA functions await discovery, and these small RNA structures appear to be another useful tool to enhancing crop resistance or tolerance to pests and diseases.

2.3.4 QTL and Genomic Mapping

When a trait is governed by multiple and/or co-dominant inherited genes, quantitative trait loci (QTL) maps are very informative, which is a more holistic approach to genome interaction. Subsequently molecular markers identified this way are closely linked to the trait under investigation. In this way molecular marker assisted selection (MAS) has possible merit by identifying the important regions of the genome associated with the trait (Torres et al. 2006). Simple inherited traits can be located on genetic maps. It is widely recognized that the development of saturated genetic linkage maps provide a most valuable tool in plant genetics and breeding. Using genetic maps, it is possible to estimate the number of loci controlling genetic variation in a segregating population and to estimate the effects of the loci. Identification of favourable alleles at each locus is then possible for many traits, including disease resistance.

2.3.5 Marker Assisted Selection (MAS)

Some molecular markers available allow the indirect selection of interesting genotypes, and they constitute an essential tool for the development of MAS. Molecular markers fall into a number of categories, each having positive and negative features (also summarized in Table 6.2):

(a) **Restriction Fragment Length Polymorphism (RFLP)** – requires hydrolysis of probe DNA from samples. Can provide high quality data but has severe restrictions on throughput because large amounts of DNA are required and because it is not PCR-based.
(b) **Random Amplified Polymorphic DNA (RAPD)** – is a method based on PCR but uses arbitrary short primers for identity of plant DNA regions. No knowledge of the genome is needed, but by the same token markers can be at many places in the genome. Results can be inconsistent and only dominant genes can be identified.
(c) **Simple Sequence Repeats (SSR)** – are high quality and consistent DNA markers, but they are the most expensive to develop. SSR markers require extensive band sequencing data for each marker developed, and often the markers are species and even cultivar specific.
(d) **Amplified Fragment Length Polymorphism (AFLP)** – requires enzymatic degradation of DNA and careful fragment separation, where only a sub-fraction of the population is sampled by PCR. It can provide too much information at any time. It is more technically demanding and information can be difficult to interpret. It produces very good high quality data which is suitable for high output sources and suitable for automation.
(e) **Single Nucleotide Polymorphism (SNP)** – relies on the fact that the vast majority of differences in eukaryotic organisms are surprising point mutations in their DNA. So there are a vast number of polymorphisms that are SNP.

Table 6.2 The major classes of molecular DNA markers used for indirect selection in marker assisted selection (MAS) in plant breeding

Criteria	RFLP	RAPD	SSR	AFLP	SNP
Principle	Endonuclease restriction and Southern blot	DNA amplification with random primers (in PCR)	PCR of simple sequence DNA repeats	PCR of endonuclease restriction sites	Sequencing part of genome looking for base changes
Polymorphism type	Single base changes (insertion/deletion)	Single base changes (insertion/deletion)	Changes in length of simple repeats	Single base changes (insertion/deletion)	Single base changes (insertion/deletion)
Genomic abundance	High	High/very high	Medium high	High/very high	High
Polymorphism level	Medium	Medium	High	High	Very high
Dominance	Codominant	Dominant	Codominant	Codominant	Need testing
Amount of DNA required	2–10 μg	10–25 ng	50–100 ng	20–50 ng	ng amounts
Sequence information required	No	No	Yes most times	Yes it can help	Yes
Radioactive detection required	Yes/no	No	No	No	No
Development and start-up costs	Medium	Low	High	Medium	Very high
Continual costs per sample	Medium/high	Low	Medium	Medium	High

The list is data compiled from personal experience by the author in areas of criteria, development and costs of the respective methods

The biggest advantage is automation and techniques that do not require electrophoresis to separate fragments.

(f) **Expressed Sequence Tags (EST)** – requires cDNA synthesis to develop and apply, therefore it is the only method listed above which is based on RNA. Preferences for this method may be for plant species where there is already extensive sequencing data.

The use of DNA markers (and indirectly EST markers from RNA) for direct selection offers greater potential gains in breeding for QTL and traits with low heritability, and these can be the most difficult to work with in breeding. However these traits are also amongst the most interesting and the most difficult to develop. The expression of the trait can be greatly affected by 'genotype X environment' interactions and 'epistasis' which can complicate the development of MAS to almost the same extent as compared to traditional field based phenotypic selection (Backes and Ostergard 2008).

2.3.6 Pyramid Multiple Resistance

Pyramiding of multiple resistance genes to foliar fungal pathogens in lentils has been developed (Muehlbauer et al. 2006) and should provide a broader and more durable resistance, as also demonstrated in rice against bacterial blight (Singh 2001). Durable resistance to stem rust in wheat has also been demonstrated and pyramiding resistance genes for several diseases has been reported. Pyramiding multiple resistance in breeding programs now constitutes a major tool for the advancement of MAS breeding (Todorovska et al. 2009).

2.3.7 Association Mapping (AM)

Association or linkage disequilibrium (LD) mapping has been used successfully in lentil, but has been used more successfully for studies of traits in humans for some time now (Flint-Garcia et al. 2003; Muehlbauer et al. 2006). Entire genomes can be examined for markers for quantitative and qualitative traits, and may allow breeders to 'break-out' of the restrictions of F_1-derived mapping populations, and employ other populations available. Gebhardt et al. (2004) summarized the four potential benefits of AM:

(a) Allows assessment of genetic potential of specific genotypes before phenotypic evaluation.
(b) Allows identification of superior trait alleles in germplasm.
(c) Can assist in high resolution QTL mapping.
(d) Can be used to validate candidate genes responsible for individual traits.

However some important issues must be considered before implementation of AM:

(a) Determination of the population structure.
(b) Estimation of nucleotide diversity.

(c) Estimation of haplotype frequencies and LD (ie non-random association of alleles at different loci).
(d) Precise evaluation of phenotypes.

Often nucleotide diversity and LD are not possible on the species investigated, but may be inferred from very closely related species, especially if the two related species have been sequenced. AM also constitutes a major tool for the advancement of plant breeding, but careful attention is required in many crop plants where population structure and precision on phenotypic expression can be poorly documented.

2.4 Tissue and Cell Culture

2.4.1 Introduction

The term "tissue culture" is often used to describe all types of aseptic plant culture procedures leading to growth, and falls into various types depending on the growth of plant cells, organs and tissues as end points of the culturing process (Chawla 2009; Neuman and Kumar 2009). Tissue culture can involve any of:

(a) Plant protoplasts (cells without the cell walls).
(b) Callus (loose or compact cell arrangement).
(c) Tissues (such as apical or root meristems, or pith segments).
(d) Organs (such as buds or roots).
(e) Embryos (usually for more specialized use).
(f) Plantlets (usually having both roots and stem/leaves).

Because the growth takes place in a sterile, artificial and often controlled environment we tend to call these techniques *in vitro* techniques. It is fair to point out that the eventual outcome of tissue culture must have in mind always to try to acclimatize these plantlets to greenhouse and eventually open field conditions, otherwise the time, efforts and resources put into tissue culture may not be realized. Details of the various and specific techniques involved in the whole area of tissue culture is beyond the scope of this chapter and will not be dealt with here, but I will refer you to various recent books that address this (Razdan 2003; de Fossard 2007).

Plant tissue culture is not a science in itself. It is merely a range of enabling techniques in which the knowledge of many different scientific disciplines are put to use when a particular type of conventional method cannot be used, or a type of end-user plant product is required; such as:

(a) Stock plant material must be made disease free.
(b) Breeding and/or propagation must be speeded up.
(c) Storage of germplasm and whole genotypes must be preserved.

(d) Plant genotypes must be manipulated, such as it is difficult or impossible *in vivo*.
(e) Increase production of desired plant metabolites.

2.4.2 Use of Tissue Culture

The use of plant tissue culture has had a rapid period of expansion, but recently there are signs of stabilization. In the Netherlands alone in 1988 there were 67 companies producing 62 million plants a year. The number of companies today has decreased from this point due to consolidation and increase in size of operations, but the number of plants produced has in fact increased (de Fossard 2000). Similar consolidation of tissue culture companies is evident in many other countries of the world, and this technology is a major export from developed countries to developing countries in agricultural and horticultural production. It is the emerging developing countries in the area of agriculture and horticulture that are quickly developing such plant tissue culture facilities and technologies. The most important developed countries in tissue culture are the US, Japan, Israel, Italy, Holland, France, Spain, Belgium, UK, India and South Africa. The main developing countries for tissue culture include China, Columbia, Brazil, Kenya and Vietnam (De Filippis 1999). The expansion and use of tissue culture has become very important for some food crops because high quality is very important as one of the most costly aspects of crop production, especially in the planting material used; and especially so if the product is to be exported to international markets. A good example of this is potato where the traditional mode of propagation is by planting tubers or sliced tubers, and this is prone to diseases such as *Erwinia, Rhizoctonia*, leaf roll virus and spindle tuber viroids. The process of accumulation of any of these organisms causes stock degeneration and eventually poor availability of disease-free stocks for further planting. The application of tissue culture for pathogen elimination and superior planting stock and rapid multiplication in potato has lead to its widespread use in both developed and developing countries with excellent results. The use of tissue culture in transformation of plants is now considered essential no matter what transformation system is used, and genetically modified (GM) crops will be dealt with later.

2.4.3 Tissue Culture in Plant Breeding

In vitro techniques can be used very successfully to aid conventional and molecular breeding programs because they have a number of advantages over traditional use of greenhouses and field methods. These include disease elimination and selection of elite breeding lines, and the rapid multiplication of these. Use of 'somaclonal variation' and induced mutation for new genotypes, and screening of breeding stocks is quicker and exact, especially with MAS. There are some disadvantages to

consider in that in culture, diseases if present could quickly kill all material, it can be expensive, requires more skills, and material must be conditioned to grow in the field because the plantlets out of tissue culture tend to be metabolically 'weak'; and difficult to grow or establish in unprotected environments (Herman 2009; Davey and Anthony 2010).

2.4.4 Conservation of Germplasm

Conservation of tissue culture material is often easier to protect, but this is very much more of importance for vegetatively propagated plants; ie plants not normally propagated from seeds. The material is easily available in small tubes and can be multiplied quickly. This conservation can be in two forms:

(a) **Cryopreservation** – that is in the frozen state where the techniques developed for some material have been difficult, and water content of the tissue can be a problem, and resolution of this can be costly.
(b) **Lower Temperatures** – this means temperatures above freezing and at a temperature where growth is drastically reduced, and can also be costly on material and space for new culturing conditions.

2.4.5 Embryo Rescue

A method of working and breeding via zygotic embryos in tissue culture where they are usually aborted or become not viable in the plant itself. This is usually due to poor endosperm development or failure, where the media components in tissue culture replace the nutrients normally in the endosperm. Embryo rescue can increase the chance of incorporating new genes into breeding programs and the development of elite lines which otherwise would not be possible. This method has already been used successfully in a number of crops, including potato, adding new and valuable genetic material (Malik 2008).

2.4.6 Microspore, Anther and Ovule Culture

These types of culture material are sometimes called 'haploid cultures' because the number of chromosomes in them has been halved, and in some cases haploid plants can be obtained for breeding purposes. The number of chromosomes can be doubled later by chemical means. This technique is increasing in importance but has limitations in that the method is difficult to master and not successful in many plants, where much depends on the ploidy level of plants; for instance tetraploid plants are very difficult to handle. The advantages of using homozygous diploids after haploid

culture is that parents derived this way have lower recombination events and there is a better chance of recovering viable plants afterwards (Vinterhalter et al. 2008b).

2.4.7 Somatic Embryogenesis and Organogenesis

Somatic embryos are independent bipolar structures which are not physically attached to the tissue in culture but are produced from a somatic line of cells. The embryos can grow to complete plantlets. Somatic embryogenesis can even occur spontaneously, and as long as the genetic make-up is stable they can be multiplied very rapidly, and therefore they have great potential. However it is still a method most suitable for breeding rather than rapid multiplication. Organogenesis can be defined as the transformation of a single cell, callus or tissue into an organ-like structure capable of independent growth, but are organ-like and not embryo-like. Plants can eventually be produced from somatic embryos and organs by a sort of 'germination' of them in their own defined culture medium (Murphy 2007; Basha and Sujath 2009).

2.4.8 Somaclonal Variation and Induced Mutations

When plants are regenerated in culture, especially from callus it often happens that new genotypes arise. This phenomenon is called 'somaclonal variation' and there appears to be many mechanisms leading to this, but it occurs in many plants in tissue culture. These genotypic changes can be very useful but may be unstable in culture and must be stabilized otherwise they become useless. In culture it is also easy to induce mutations via conventional means of radiation or mutagenic chemicals. The use of variations in culture and induced mutations have become popular and has produced new stable and useful genotypes for many purposes, including crop breeding (Navratilova et al. 2008; Hoang et al. 2009).

2.4.9 Protoplast and Cell Culture

Protoplasts are true somatic cells which although quite difficult to work with can be grown into a whole plant. Isolation of protoplasts is quite simple now by enzymatic digestion of the cell wall, however culturing protoplasts into a callus, tissue and whole plants is technically demanding, very difficult and perhaps almost impossible for some plants; especially the monocotyledon group. Hybridisation of protoplasts and even cells is relatively simple by fusion, which can be affected by either chemicals (eg PEG) (De Filippis et al. 1996, 2000) or electric fields (electrofusion) (Hampp et al. 1997; De Filippis et al. 2000). Hybridisation of cells and protoplasts has led to new genotypes in which desirable traits have been identified. Hybrids and cybrids (where the cytoplasms only are fused) can be produced and these can be used in many situations, but perhaps the best use of this material is to develop and test genetic markers in MAS.

2.5 *Marker Assisted Selection (MAS)*

2.5.1 Introduction

A molecular genetic marker (sometimes shortened to molecular marker) is a DNA sequence which can be identified by relatively simple biochemical/molecular techniques and localized at a certain position (locus) on the chromosome. In some cases it can be statistically associated with a certain trait through a target gene located at or nearby the position of the marker. So selection based on molecular marker patterns (marker assisted selection – MAS) may complement or replace phenotypic selection for the respective trait (Collard et al. 2005). The main difference between classic phenotypic selection and MAS is the target for selection. In phenotypic selection the direct target is the trait itself and its phenotypic expression in a specific environment, so that alleles of specific genes behind the trait are selected indirectly. In MAS on the other hand the marker allele associated with the favourable trait related gene is the direct target, while the phenotypic traits to be found in a specific environment are the indirect targets.

2.5.2 Development of MAS

Development of markers for MAS includes several steps:

1. The detection of an association between markers used and linked loci affecting the trait. If the trait of interest is qualitative a simple linkage analysis can be used to determine the association between marker and target gene. If on the other hand the trait is quantitative (quantitative trait loci – QTL) a statistical analysis of trait and marker data in segregating populations is needed; ie QTL analysis.
2. The selection of a subsample of markers with expected high usefulness in MAS.
3. The evaluation of the marker in different genetic backgrounds.
4. Eventually a technical optimization of the marker for practical application.

In the case of a qualitative trait with no dominance, phenotypic selection and MAS roughly give the same results, even though incomplete linkage between marker and gene can lead to a certain percentage of errors in prediction of trait expression. The situation is much more complicated in the situation of a quantitative trait where a single trait is influenced by several other genes, and some of these may also influence other traits. Here the combination of MAS and phenotypic selection can have an advantage relative to selection based solely on phenotype; especially traits with modest heritability and high genotype-environment interactions (Moreau et al. 1998).

2.5.3 Application of MAS

In conventional plant breeding, MAS is a well established selection tool (Tuvesson et al. 2007). MAS has its biggest advantage when applied in early stages of the

breeding process on single plants. In this case markers can reduce effectively the number of lines to be tested and save planting area and labour in field trials. In breeding of cereals, the success of markers is due to the simultaneous introduction of double haploid technologies (DH). In DH lines the genetic constitution is fixed after haploidisation and selection efficiency is no longer reduced by genetic recombination in the following generations. Thus molecular markers allow for selection that would be difficult, very costly or just not possible when based solely on phenotype; and a good example is disease resistance in the absence of the pathogen (de Oliveira et al. 2005). In perennial plants, MAS can be carried out on seedlings and an earlier selection for traits, and therefore achieves quicker breeding progress (Dilrewanger et al. 2004). Finally, the increased use of automation in marker approaches allows large-scale screening for DNA markers within a short time and has contributed real advantages to this technology (Dayteg et al. 2007).

Up until now the application of MAS in plant breeding has mostly concerned qualitative traits. Well known examples are genes for virus resistance in barley and wheat where molecular markers have been used in pyramiding (stacking) genes against several virus strains (Werner 2005). This effective use when pyramiding several highly effective genes and MAS may be even higher when applied to quantitative traits. Only a few examples of success have been found for MAS in quantitative traits. The reason may be that many issues can complicate or even prevent the use of MAS for QTL (Charcosset and Moreau 2004) such as:

(a) If the target trait is influenced by many QTL with relatively small effects, MAS is inefficient and the sub-population possessing all optimal marker alleles will be small; so a relatively small effect is seen.
(b) Effects of QTL found in analysis can be overestimated due to the influence of undetected QTLs. A possible solution is cross-validation in an independent sample.
(c) Epistatic effects (*i.e.* QTL X QTL interactions) can cause a QTL effect to be highly dependent on genetic background of the respective genotype. This problem can be partially circumvented by the use of several crosses at the same time.
(d) Interactions between the genotypes (*i.e.* QTL) and the environment can be important. This is especially true when the trait is heavily influenced by strong environmental factors. QTL are seldom independent of the environment and success may lie in being able to include genetic environment interaction in analysis (Francia et al. 2005; Christiansen et al. 2006). But a considerable reduction in the number of usable QTL can be expected, especially when the trait is under strong environmental influence.

In vitro selection systems enable an increase rate and a more precise level of MAS. Selection can be affected on numerous individuals and can be done at any stage of plant growth, and this is a real advantage for tissue culture. Therefore MAS in tissue culture has become more important and more efficient, and more exact than any greenhouse or field selection system.

2.6 *Biotechnology and Genetic Manipulated (GM) Crops*

2.6.1 Introduction

Improvements of plants by conventional breeding involves crossing parents holding part of the desired traits while selecting progeny of individuals showing the proper combination of a superior set of traits. Selection however has its limitations and some of these can be removed by molecular biology techniques, but in other cases only the use of transgenic plants can provide success. For a full comparison of steps in conventional breeding, MAS breeding and genetic transformation see Fig. 6.3. Transgenic plants and molecular breeding has opened a number of possibilities:

(a) Isolating and amplifying genes encoding for a particular trait *in vitro.*
(b) Equipping an isolated gene with regulatory signals recognized by the plant.
(c) Introduction and expression of a modified gene into plant cells and tissues.
(d) Regeneration of transgenic plant cells into mature fertile lines of plants.

The main advantage is that it offers the possibility of adding a new trait directly to an existing valuable variety. In such systems potential species barriers to gene incorporation and expression do not exist. Applications using this approach have already been well documented in many dicotyledon plants, and some fewer monocotyledon plants (eg tobacco, potato, tomato, rapeseed, corn, wheat, barley oats, soybean and banana) (Jackson et al. 2006; Akhond and Machray 2009). Genes can be accessed from exotic sources; plants, animals, bacteria, viruses or even humans. The methods used in plants however have some limitations, and there has been limited success because:

(a) Few reliable protocols for introduction and expression of the foreign DNA exist.
(b) Number of documented genes of interest is still limited, although expanding all the time.
(c) Regulation of gene expression is vital for success, but is limited in plants.
(d) Tissue culture methods for plant regeneration are still inadequate.
(e) Long periods of time are required for full technology development and transfer.
(f) Lack of long-term funding for such programs.
(g) Absence of a co-ordinated approach by the scientific community.
(h) Unfavourable public perception towards transgenic plants.

It is interesting to note that GM technology was first developed for plants as early as 1983, but the first GM crops reached the markets only during the mid-1990s. During the next two decades the development of transgenic technology has proceeded rapidly. The global area planted to biotech crops has increased over the last 20 years by an annual rate of 10–12% each year, and the major biotech growing nations are detailed in Table 6.3. However the biotech crops planted in agriculture are still dependent on four GM crops (ie soybean, corn, cotton and canola) in most

Table 6.3 Global area of biotech crops in 2009 by country, and note the top 15 mega-countries that are growing 50,000 ha or more

Rank	Country	Area (million ha)	Biotech crops
1	USA	64.0	Soybean, corn, cotton, canola, squash, papaya, alfalfa, sugarbeet
2	Brazil	21.4	Soybean, corn, cotton
3	Argentina	21.3	Soybean, corn, cotton
4	India	8.4	Cotton
5	Canada	8.2	Canola, corn, soybean, sugarbeet
6	China	3.7	Cotton, tomato, poplar, papaya, sweet pepper
7	Paraguay	2.2	Soybean
8	South Africa	2.1	Corn, soybean, cotton
9	Uruguay	0.8	Soybean, corn
10	Bolivia	0.8	Soybean
11	Philippines	0.5	Corn
12	Australia	0.2	Cotton, canola
13	Burkina Faso	0.1	Cotton
14	Spain	0.1	Corn
15	Mexico	0.1	Cotton, soybean
16	Chile	<0.1	Corn, soybean, canola
17	Colombia	<0.1	Corn
18	Honduras	<0.1	Corn
19	Czech Republic	<0.1	Corn
20	Portugal	<0.1	Corn
21	Romania	<0.1	Corn
22	Poland	<0.1	Corn
23	Costa Rica	<0.1	Cotton, corn
24	Egypt	<0.1	Corn
25	Slovakia	<0.1	Corn

The data was compiled by James (2007)

countries, but the number of countries planting Biotech crops is expanding all the time (Fig. 6.4). The process of genetic transformation of plants involves several distinct stages; namely insertion, integration, expression and stable inheritance of the new DNA, and details of the steps involved are adequately covered in a number of books (Herman 2009; Neuman and Kumar 2009).

2.6.2 *Agrobacterium* System

The plasmid containing soil bacterium *Agrobacterium tumefaciens* can be considered a natural gene transfer system. It is one of the most efficient methods employing the naturally evolved 'crown gall' causing mechanism of DNA transfer. *Agrobacterium* transformation results from the stimulation of plant cell division by gene products encoded by a segment of DNA (T-DNA) which is transferred from the bacterium to the plant.

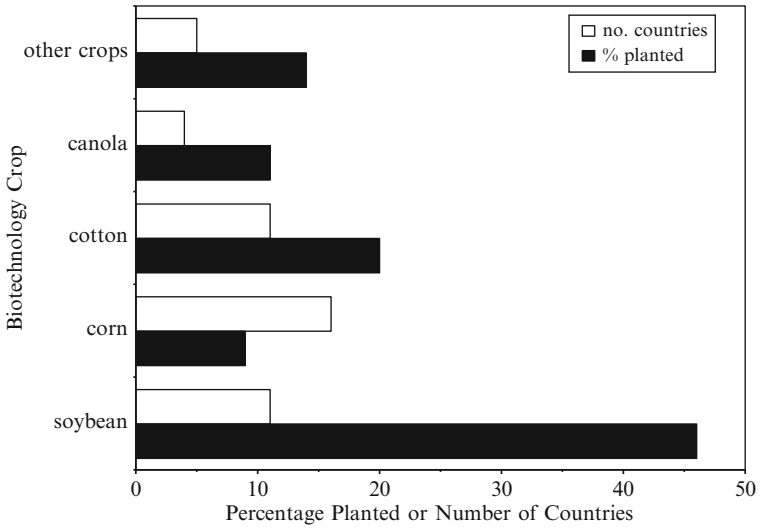

Fig. 6.4 Percentage of the major biotech crops planted, and the number of countries involved for each crop planted in 2009. The data was compiled by James (2007)

The T-DNA and '*vir*' regions required for this transfer are located on the tumor inducing plasmid (Ti). The '*vir*' system will process and transfer any DNA between two flanking regions that delimit the T-DNA, hence its utility. The strain of *Agrobacterium* used as a carrier for DNA transfer into plants has been modified (disarmed) to remove the gall-inducing (tumor producing) ability and engineered to carry the gene of interest, together with a selectable marker. Inside the plant the T-complex is imported into the nucleus of the plant, the T-strand becomes stably inherited into the plant chromosomes, and the genes (including foreign genes) are expressed (Broothaerts et al. 2005).

The *Agrobacterium* system has the ability to integrate into a variety of plants, some monocotyledons have shown some resistance to the method but now has become a routine method even in these crops (Smith and Hood 1995). More important is that the site of integration of the T-DNA is random, it can be rearranged or truncated, but the biggest problem is making sure that the carrier bacterium itself does not persist internally in transgenic plants (Basha and Sujath 2009).

2.6.3 Biolistic or Microprojectile Bombardment

The method was developed in the mid-1980s and has been widely used as a more direct gene transfer system in a variety of plants; including previously hard to transform monocotyledon plants (Southgate et al. 1995). The technique is based on the rapid acceleration of a metal microprojectile to deliver nucleic acids, including

specific genes (or DNA) into the intact nucleus or even just into plant cells and tissues. Other biological material and compounds apart from DNA could be delivered, and a commercial particle delivery system for gene transfer has been available on the market (Biolistic PDS-1000) for some time. Due to the genotype-independent physical nature of DNA delivery, the methods employed are simple, efficient and nearly the same regardless of the nature of the foreign DNA or the target cells used. It is therefore a highly valuable and adaptable technique potentially applicable over many different types of cells and tissues. During the last two decades it appears that microprojectile bombardment has become more routine and more reliable a method; so much so that it is replacing the *Agrobacterium* method quickly (Hansen and Wright 1999).

However, there are still problems to be resolved, in particular, more direct and efficient targeting of the nucleus, and tissue culture related totipotency and low regeneration of plants in crops like soybean and others. The results of the biolistic method can be a complex pattern of transgene integration which is undesirable, but can be mitigated by using 'Agrolistic' approaches. Agrolistic transformation allows integration of the gene of interest without the undesired vector sequences. In other words, deliver by biolistic means *Agrobacterium* containing the foreign DNA and only some virulence genes (eg *vir* D1 and *vir* D2) but not *vir* E2 in the T-DNA. It has been demonstrated that only these two '*vir*' genes are required for '*in planta*' stable transformation (Hess and Dressler 1989; Beranova et al. 2008).

2.6.4 Alternative Methods

(a) **Protoplast transformation** – protoplasts can be transformed either by the *Agrobacterium* system or by direct DNA uptake using electrofusion or PEG treatments. The method has been used routinely to study transient expression of transgenes. Transgenic plants have been obtained in very few cases, and only where suitable protocols were already available for culturing of protoplast to whole plants (Shillito 1999; Murphy 2007).

(b) **Chloroplast transformation** – chloroplast transformation has generated much interest as a more 'environmentally friendly' approach to plant genetic engineering. It provides a system where it can avoid out-crossing of transgenes to weeds or other non-target crops; thus reducing the potential toxicity of transgenic pollen to non-targeted species. Biolistic gene delivery is probably the most reliable and reproducible method to use, but the method is only possible in a limited number of species (Daniell et al. 2002; Murphy 2007).

(c) **Floral dip method** – a method that could circumvent the problemsome time consuming steps in previous methods, and is a simple method to master. There are a number of such methods, and they are all placed into the category of 'in plant transformation'. One such method has been well developed in *Arabidopsis* and has achieved high rates (0.5–3%) of transformation, and there are reports of success in a number of other crop plants (eg *Brassica campestris, Medicago trunculata* and radish)(Liu et al. 1998; Trieu et al. 2000; Curtis 2005).

(d) **Pollen mediated transformation** – has attracted significant interest and there have been a number of reports claiming success (Harwood et al. 1996; Heberle-Bors et al. 1996). Basically it involves incubating pollen in DNA solution and electroporation (Hess and Dressler 1989), direct particle bombardment (van der Leede-Plegt et al. 1995) or co-cultivation with *Agrobacterium* (Langridge et al. 1992). Many of the methods were later proven to be not reproducible and only in the report of Smith et al. (1994) have transgenic plants shown to be produced. A report of an enhanced *Agrobacterium* mediated transformation system in flax has been described recently by Beranova et al. (2008).

2.6.5 Future of Plant Transgenics

Introduced DNA in plants usually integrates more or less randomly into the host genome, even when the introduced DNA shows homology with plant endogenous DNA sequences. This is referred to as 'illegitimate recombination' or 'non-homologous end joining'; a very common method for joining DNA ends that are created in cells by double stranded DNA breaks. When an introduced DNA sequence can target an identical sequence in the host genome and integrates itself by homologous recombination (HR) the process is known as 'Gene Targeting' (GT). This event is a prerequisite to application of the most sophisticated tool of 'reverse genetics'; ie gene disruption and allele replacement (Berg 1991b).

In such GT technology virtually any cloned gene, even of unknown function can be specifically mutagenised *in vitro* and re-introduced to its own chromosomal location in order to study its function. But the targeted recombination of incoming DNA offers so many advantages and overcomes many of the problems with gene expression of transgenic plants. In plants only a few reports of GT have been published in *Arabidopsis*, tobacco and maize (Nguyen et al. 2007; Munkacsi et al. 2008). The reported frequency of GT can be low (10^{-3}–10^{-4}) and if it is true that in plants, as in mammals, there appears to be little or no negative consequences to the organism (Drobnik 2008; Vinterhalter et al. 2008a, b) it could be very exciting. More recently large scale GT has been reported in rice using an *Agrobacterium* system (Iida and Terada 2004), and a zinc finger nuclease based technique (Kumar et al. 2006) also shows some promise.

Crucial steps and decisions need to be made in gene targeting (GT) in plants, and these include:

(a) The design of the gene targeting construct.
(b) The choice of tissue to be transformed.
(c) Selection of transformants.
(d) Regeneration of transformants.
(e) Screening for clean, targeted transformants.

During the 1990s and 2000s the genomes of a few different plants have been completely sequenced (Jackson et al. 2006; Amano et al. 2010). Many more plants

are well on the way to being sequenced. Nowdays plant molecular research is being directed more and more towards an understanding of gene expression and function. Proteomics with a high through-put is such an approach in this post-genomic period. Reports of proteomic applications in plants and biotic stress are still rather limited compared to other biological and molecular approaches (Xing et al. 2002; Jorrin et al. 2006). But proteomics is becoming important and justifiable because:

(a) Gene function is carried out by proteins and polypeptides.
(b) A large number of genes (an important 80–90%) have no apparent assigned function.
(c) Information obtained by using transcriptone approaches is incomplete.
(d) Correlation between mRNA and protein levels is remarkably and surprisingly low.

2.6.6 RNA Mediated Silencing

Viral coat mediated resistance to some plant viruses have been reported over the last 20 years by conventional breeding, but this technology is fast being replaced by the recently recognized small interfering RNA (siRNA) and micro RNA (miRNA) mediated gene silencing (Ritzenthaler 2005). A recent report for example has demonstrated how artificially constructed miRNA can confer virus resistance in plants (Niu et al. 2006). Transcriptional factors that regulate the expression of several genes related to biotic stress resistance have been described, and belong to the stress signaling pathway. Transcriptional analysis using microarray technology has demonstrated that these are important in abiotic stress tolerance such as drought, cold and salt, but they may also be expressed as a result of biotic stress. For example *Arabidopsis* inoculated with growth promoting *Rhizobacteria* also have enhanced protection against *Erwinia* (Agarwal et al. 2006), and the *'adr*1*'* gene was involved (the so called activated disease resistance 1 gene).

2.6.7 Safety of Bitech (GM) Crops

There has been considerable public debate and concern regarding the safety of biotech (GM) crops. This has lead to an approval protocol in many countries where rigorous testing and safety assessment must be carried out before any such crops are approved for commercial cultivation. The Codex Alimentarius Commission, under FAO and WHO adopted specific guidelines in 2003 for risk assessment of plants derived from biotechnology in the global market (CAC 2003), and these guidelines are quite strict. There appears to be three concerns by the public about genetically engineered crops (Clark 2006):

(a) **Marker genes** – used in biotech crops could introduce toxicity and allergy. Experimental evidence suggests that the genetic approaches and methodology used have little potential for this (Magana-Gomez and de la Barca 2008).

(b) **Perception that there is a safety concern to diet** – gaining access to a wider range of genetic diversity in food crops, crossing species barriers and the introduction of foods with additional proteins are not safe. It is fair to say that most biotech foods are equivalent to existing and traditional foods, and there is no documented proof that any biotech crop has caused toxicity or allergies. Additionally, there is no proof that successive generations of such biotech crops have caused a biologically significant increase in endogenous allerginicity from foods (Goodman et al. 2008).
(c) **Transgenes will spread in the environment** – mathematical models and empirical experimental evidence suggest that genetic approaches used in GM crops have the potential to effectively prevent transgenes from escaping into wild populations, ie those crops that are not reproductively isolated from genetically engineered crops (Lee and Natesan 2006).

3 Part B: Biotic Factors and Crop Production

3.1 Viruses and Viroids

3.1.1 Characteristics and Symptoms

Viruses and viroids are nucleoproteins that have the ability to multiply in living plant cells and cause disease. Viruses can be specific as to infect only one species of plant or infect dozens of different plants, and each plant species may be attacked by many different types of viruses. Viruses always contain nucleic acids (RNA or DNA) plus one or more different kinds of proteins. The composition and structure of the viral proteins is know only for a few plant viruses, and most plant viruses have their nucleic acid in the form of RNA; but about 80–100 plant viruses contain DNA. When a virus infects a plant in the field it moves from one cell to another and multiplies in most of these cells (systemic infection). In all important agricultural and economic infecting viruses they reach the phloem where they are rapidly transported over long distances. Almost all viral diseases appear to cause some degree of dwarfing or stunting of the entire plant; where total crop yields are frequently reduced (Agrios 1997). The most obvious symptom of a viral infected plant usually appears on the leaves, but fruit, stem and roots may also be affected. In the case of latent viruses, infection may proceed without causing visible symptoms. The most characteristic leaf symptoms are grouped into the two categories below:

(a) **Mosaics –** characterised by appearance of light green, yellow or white areas intermingled by normal green tissue on leaves or stems.
(b) **Ringspots –** characterised by appearance of chlorotic or necrotic rings on the leaves, sometimes also on stems and fruit.

A large number of other, less common symptoms have been described, including stunt, dwarf, yellows, streak, tumors, pitting, flattening and distortion, and curling

of usually the leaves and fruits of infected plants. The present methods of detecting viruses is transmission of the virus and disease from a diseased plant to a healthy plant by budding, grafting or rubbing sap into a healthy leaf. The most definitive methods however are expensive like electron microscopy, serology and more recently molecular (DNA/RNA) probes.

Viroids are small, low molecular weight ribonucleic acids (RNA) that have the ability to infect plants, replicate themselves in plant cells and cause disease. Viroids are different to viruses in at least two main characteristics:

(a) Size of the RNA in viroids is only between 250 and 370 bases, compared to 4,000–20,000 bases in viruses.
(b) Viruses are always enclosed in a protein coat, while viroids lack this protein coat (only free RNA).

Because of their small size, viroids cannot code for even one small protein due to insufficient number of bases; even for example a replicase-type enzyme essential for viroid replication (Horst 2008).

3.1.2 Important Diseases

Viral and viroid diseases of plants have been loosely categorised into eight classes, primarily based on characteristics of the viruses rather than the plant they infect or the severity of the symptoms, or for that matter affect on production. It is my aim below to just detail the diseases in each of these categories below, and the more important viral diseases of food crop plants are summarised in Table 6.4 and Strange and Scott (2005):

(a) **Rigid Rod-shaped Viruses –** includes tobamoviruses, tobraviruses, furoviruses and hordeiviruses. These viruses are single stranded RNA type and only tobamoviruses and tobraviruses cause serious loss of production in tobacco and some Solonaceae group plants, especially potato, tomato, pepper and pea by damaging leaves, flowers and fruits. Furoviruses at times also reduce production in potato, sugarbeet and peanut however symptoms and losses can be variable. Hordeiviruses are rare and have only been detected in barley, and in general causes only minor losses in production.
(b) **Filamentous Viruses –** includes potexviruses, carlaviruses, capilloviruses, trichoviruses, potyviruses, rymoviruses, bymoviruses, and closteroviruses. These viruses are also RNA viruses but the number of strands is not always certain. Potyviruses and closteroviruses are the main classes that cause serious loss in production and severe diseases in many plants where they appear to be manifested as mosaic, mottling and chlorotic spots on leaves, stems and fruits. Some of the more serious diseases of potyvirus include lettuce mosaic, celery mosaic, bean mosaic, bean yellowing, papaya ringspot, plum pox, potato virus Y, sugarcane mosaic, turnip mosaic, watermelon mosaic and zucchini yellow mosaic. The more serious diseases of closterovirus include beet yellow, citrus

Table 6.4 The world's staple crops and their principal diseases according to the biological class of organism causing the respective diseases

Crop	Biological classes of casual organism
Barley (*Hordeum vulgare*)	**Virus**: Yellow dwarf; Stripe mosaic
	Fungi: Mildews (downy/powdery); Spot blotch and Net blotch; Scald; Scab; Barley stripe
	Nematode: Root-knot; Cyst; Root lesion
Cassava (*Maniot esculenta*)	**Virus**: Cassava mosaic, Geminivirus
	Bacteria: Bacterial blight
	Fungi: Anthracnose
Lentil (*Lens culinaris*)	**Fungi**: Wilt and Vascular wilt; Blight; Rust; Anthracnose
Maize (*Zea mays*)	**Virus**: Chlorotic dwarf; Streak Geminivirus; Yellow dwarf
	Bacteria: Stewart's wilt (*Erwinia*); Corn stunt
	Fungi: Leaf blight; Rust and Smut; Downy mildew; Stalk and ear rots
Common millet (*Panicum miliaceum*)	**Fungi**: Downy mildew
Pearl millet (*Pennisetum glaucum*)	**Fungi**: Ergot
Oats (*Avena sativa*)	**Virus**: Yellow dwarf; Golden stripe; Mosaic potyvirus
	Bacteria: Halo blight
	Fungi: Crown rust and Stem rust; Powdery mildew; Leaf blight/ Seedling blight; *Fusarium* root and crown rot; Snow mould; Leaf blotch; *Alternaria*
Potato (*Solanum tuberosum*)	**Virus**: Leafroll; Mosaic virus
	Bacteria: Bacterial wilt; Bacterial soft rot *Erwinia;* Common scab; Bacterial ring rot
	Fungi: Early blight; Black scurf; Late blight; Pink rot
Rice (*Oryza sativa*)	**Virus**: Rice tungro disease; Yellow dwarf
	Bacteria: Bacterial leaf blight
	Fungi: Blast; Brown spot; Sheath blight
Rye (*Secale cereale*)	**Virus**: Yellow dwarf
	Fungi: Snow mold; Brown rust and Stem rust; Ergot; Sharp eyespot; Powdery mildew; Glume blotch/leaf blotch
	Nematode: Eelworm
Sorghum (*Sorgum bicolor*)	**Virus**: Streak disease
	Fungi: Anthracnose; Leaf blight; Zonate leaf spot; Tar spot; Charcoal rot; Rust; Ergot; Downy mildew
Soybean (*Glycine max*)	**Virus**: Mosaic; Yellow mosaic
	Bacteria: Bacterial pustule
	Fungi: Rust; Anthracnose; Purple seed stain; Pod and stem blight; Downy mildew
Sweet potato (*Ipomoea batatas*)	**Virus**: Feathery mottle
	Bacteria: Soil rot; Little leaf
	Fungi: Scab; Fusarium wilt; Black rot; Java black rot; Scurf
	Nematode: Root-knot

(continued)

Table 6.4 (continued)

Crop	Biological classes of casual organism
Wheat (*Triticum aestivum*)	**Virus**: Yellow dwarf **Bacteria**: Bacterial leaf streak **Fungi**: Stem rust and Leaf rust; Stripe or yellow rust; Spot blotch; Head scab; F*usarium* foot/root rot; Sclerotium foot rot; Tan spot; Powdery mildew; Speckled leaf blotch; Glume blotch; Loose smut; *Rhizoctonia* root rot
Yam (*Dioscorea alata*)	**Virus**: Yam virus complex **Fungi**: Anthracnose; Tuber rots

The list is modified from data compiled from Agrios (1997), Strange and Scott (2005) and Horst (2008)

tristeza and lettuce infectious yellow. The remaining virus diseases in this group can be rare, affect ornamental plants like orchids and carnations, with the exception being the rymovirus and bymovirus which have been shown to have varying severity of effects on grain crops like barley, oats, rice and wheat.

(c) **Isometric Single-stranded RNA Viruses –** includes waikaviruses, luteoviruses, comoviruses, nepoviruses, bromoviruses, cucumoviruses and ilaroviruses. Waikaviruses, luteoviruses, cucumoviruses and nepoviruses are the main classes that cause serious loss in production and severe diseases in many plants where they appear to be manifested as mottling, chlorosis, leaf roll and stunting of plants. Some of the more serious diseases of waikavirus include rice tungro and maize chlorotic dwarf. Some of the more serious diseases of luteovirus include beet western yellow and potato leafroll. The more serious diseases of nepovirus and cucumoviruses include tomato ring spot, cherry leafroll, grapevine fanleaf, raspberry yellow dwarf, ringspot or leafcurl dwarf and cucumber mosaic. The remaining virus diseases in this group are usually rare and do not affect plant production.

(d) **Isometric Double-stranded RNA Viruses –** includes the one group of rheoviruses, which can also infect humans and animals. The more serious diseases cause wound tumor induction except rice dwarf virus. The viruses have a very limited host range amongst grasses, and although the symptoms appear severe the plants are usually young when infected. Therefore overall production loss can be moderate and localised.

(e) **Negative Single-stranded RNA Viruses –** includes diseases caused by rhabdoviruses, which is a large group of 80–100 viruses with a limited host range; but also includes the rare groups of tospoviruses and tenuiviruses. They are mostly viruses in vegetables, weeds and grasses where the disease causes mosaic, vein clearing, yellowing and dwarfing. The most notable are lettuce necrotic yellow, potato yellow dwarf, rice transitory yellowing and wheat striate mosaic. Production losses are quite low.

(f) **Double-stranded DNA Viruses –** includes two groups of caulimoviruses and badnaviruses which are important groups mostly in vegetables and ornamental

plants where the diseases cause mosaics and mottles. The most notable are cauliflower mosaic, dahlia mosaic and carnation etch ring where they cause poor growth, poor quality and reduced yields. Badnaviruses affect rice, banana and cacao and in many parts of the world it affects production severely causing large losses.

(g) **Single-stranded DNA Viruses –** includes the large group of geminiviruses which is an important group that has been extensively studied where the disease causes mottles to yellow mosaics with leaves curled and distorted. The most notable diseases are maize streak, beet curly top, African cassava mosaic, bean golden mosaic, squash leaf curl, tomato mottle, tomato yellow leaf curl and banana bunchy top. Geminiviruses affect many plants and can cause devastating losses in many of the crops, particularly in tropical and sub-tropical regions.

(h) **Viroids –** at least 20–30 plant disease have been described with the most important being coconut cadang-cadang, potato spindle tuber, citrus exocortis, avocado sunblotch, apple scar skin, chrysanthemum stunt and chloritic mottle, hop stunt and cucumber pale fruit. How viroids cause disease is largely unknown, but when they do cause a disease, symptoms can be varied and resemble symptoms of true viruses. Viroids are easily spread from plant to plant primarily by mechanical means through sap transfer on hands or tools.

3.1.3 Control Measures

Viral and viroid diseases are difficult to control and the best way is by keeping them out of the area, through quarantine, inspection and certification of crops used. Eradication of diseased plants and control of vectors is good practice, but this can be difficult if not impossible. Periodic virus indexing of crops can help in not introducing viruses but it can be expensive. Incubation of material by heat can be useful and especially hot water dipping of seeds and propagation material. No effective chemical treatments are available for plant viruses. Virus indexing and use of resistant plants are still the best options (Strange and Scott 2005).

3.2 Bacteria

3.2.1 Characteristics and Symptoms

Bacteria and Mycoplasma Like Organisms (MLO) are prokaryotes; single cell microorganisms whose genetic material (DNA) is not bound by a membrane and therefore do not possess a nucleus. In bacteria, the cells are enclosed by both a membrane and a cell wall, however MLO are surrounded by a cell membrane only. Most plant pathogenic bacteria are rod shaped with the exception being *Streptomyces* which are filamentous. Most plant pathogenic bacteria have delicate thread-like

flagella, commonly longer than the cells themselves. Based on specific cell wall staining bacteria can be divided into gram-positive or gram-negative bacteria for classification. However if bacteria are allowed to grow (and multiply) on the surface of a solid or semi-solid nutrient medium they will form colonies. Colonies are easier to study and identification of species may be possible based on colony shape, smoothness, elevation from the media, colour and how grainy they appear. Often (but not always) bacteria have single or multiple copies of smaller circular material called plasmids in the cytoplasm. Each plasmid consists of several 'non-essential' genes which can move or be transported between bacteria and even between bacteria and plants; as happens in crown gall disease (see Sect. 2.6.2). Rod-shaped bacteria reproduce by an asexual process known as binary fission or just fission. Bacteria can divide every 20–30 min. Almost all plant pathogenic bacteria develop mostly in the host plant as parasites, on the plant surfaces, in plant debris and in the soil as saprophytes.

Some bacterial pathogens after infection produce most bacteria within their plant host and have reduced numbers present in the soil. These bacteria have evolved very efficient plant-to-plant infection mechanisms often using vectors to sustain high infection rates. Other bacteria are typically soil inhabitants, however they do build-up numbers in plant cells under suitable conditions while still keeping high bacterial numbers in the soil. Dissemination and infection by these bacteria are with the aid of water, insects, animals and humans. Bacterial have flagella but in most cases move only very short distances (Agrios 1997).

The most commonly used method of detecting bacteria is still by serological methods employing metabolites as substrates or fluorescent stains. Another excellent method is by promoting growth and colony identification on selected growth supplemented media, and bacteria can be identified through genetic relatedness or genetic fingerprinting methods analogous to human fingerprinting methods on their DNA. Plant pathogenic bacteria induce as many kinds of symptoms on plants they infect, as do for example pathogenic fungi. They cause leaf spots and blights, soft rots of fruit, roots and storage organs, wilts, excessive growth, scales and cankers (Agrios 1997). What is more important than this is that any one type of symptom can be caused by bacteria belonging to several genera, and each genera may contain pathogens capable of causing quite different symptoms.

3.2.2 Important Diseases

Diseases of plant pathogenic bacteria have been characterised by two different methods below:

(a) **Taxonomic Families –** identification based on correct placement into their bacterial families.
(b) **Disease Symptoms –** families and genera grouped together based on similarity of symptoms.

The more important bacterial diseases of crop plants are summarised in Table 6.4 and Strange and Scott (2005).

(a) **Taxonomy**

1. **Family Enterobacteria –** includes *Erwinia* causing fire blight of pear and apple, Stewart's wilt of citrus and soft rots of fleshy vegetables.
2. **Family Pseudomonads –** includes *Pseudomonas, Rhizobacter, Xanthomonas, Xylophilus*, and manifested as leaf spots, blights, wilts, rots, cankers and galls in many plant species.
3. **Family Rhizobia –** includes *Rhizobium* which causes root nodules in legumes.
4. **Family Firmibacteria –** includes *Bacillus* and *Clostridium*; causing rots of tubers, seeds and stripe in wheat and a number of other cereals.
5. **Family Thallobacteria –** includes *Anthrobacter, Clavibacter, Rhodococcus, Streptomyces*, showing up as blight, wilts, scab and fasciation in a number of plants.

(b) **Disease**

1. **Bacterial Spots and Blights –** symptoms produced mainly by *Pseudomonas* and *Xanthomonas*.
2. **Bacterial Vascular Wilts –** disease produced by *Clavibacter, Erwinia, Pseudomonas* and *Xanthomonas*.
3. **Bacterial Soft Rots –** symptoms produced by Erwinia, Pseudomonas, Bacillus, Clostridium and Streptomyces.
4. **Bacterial Galls –** includes at least 20–30 plant disease primarily due to *Agrobacterium, Pseudomonas, Rhizobacter* and *Rhodococcus*.
5. **Bacterial Cankers –** symptoms produced mainly by *Pseudomonas* and *Xanthomonas.*
6. **Root Nodules of Legumes –** produced by *Rhozobium* and *Frankia*.
7. **Plant Vascular Bacteria –** diseases mainly caused by *Clavibacter* and *Xylella*.
8. **Micoplasma Like Organisms (MLO)** – diseases mostly due from *Mycoplasma.*

3.2.3 Control Measures

Bacteria and MLO diseases of plants are usually difficult to control and a combination of measures are commonly required. Sanitation is essential of equipment and tools. Steam pasteurisation of soil and growing media is one of the best methods to use. Seed sterilisation by hypochlorite soaking is effective and so can hot water treatment. Use of chemicals for control of bacterial diseases is generally less effective than in controlling fungi for example, and foliar spays for bacterial control are the most effective. The use of crop varieties resistant to bacterial diseases is one of the best methods to use (Horst 2008).

3.3 Fungi

3.3.1 Characteristics and Symptoms

Fungi are small, generally microscopic, eukaryotes, usually filamentous, branched, spore-bearing organisms that lack chlorophyll. Fungi have cell walls that contain chitin and glucans embedded in a matrix of polysaccharides and glycoproteins; but a small group (the oomycota) that also cause plant diseases contain cellulose and no chitin. Most of the 100,000 species of fungi are saprophytic, that is they live on dead organic matter which they help to decompose. Very few species cause disease in plants. All plants can be attacked by some kind of fungus, and each of the parasitic fungi can attack one or more kinds of plants. Most plant fungi have a filamentous vegetative body called a mycelium. The mycelium can branch out in all directions. The individual branches are called hyphae. The length of the mycelium may be only a few nanometres in length in some fungi but in others it may reach several metres in length. Some lower fungi however lack true mycelium and produce a system of strands called rhyzomycelium (Agrios 1997). Fungi reproduce chiefly by means of spores; the reproductive bodies. Spores may be formed asexually by budding or as a result of sexual fertilisation. Almost all plant pathogenic fungi spend part of their life cycle on the host plant, in the soil or in plant debris. During the parasitic phase of their life cycle fungi assume various positions and grow in various organs of their host:

(a) Some grow outside their hosts, but send their feeding organs (haustoria) into the plant cells.
(b) Some only grow between the cuticle and epidermal cells.
(c) Some grow only in the intercellular spaces between cells.
(d) Others grow between and within plant cells almost indiscriminantly.

Infection, survival and severity of diseases of most plant pathogenic fungi greatly depend on environmental conditions; as outlined below:

(a) Mycelium survives within a certain range of temperatures and only in contact with moist surfaces.
(b) Spores survive a much broader range of both temperatures and moisture. However spores also require favourable conditions in order to germinate.
(c) Zoospores are the only structures that can move by themselves, but only for very short distances (a few mm).
(d) Most spores depend on their spread from plant to plant, or different parts of the one plant by agents such as wind, water, birds, insects, animals and humans.

Fungi cause local or general symptoms on host plants and such symptoms may occur at any time or may follow a set time pattern. In general, fungi cause local or general necrosis (killing of plant tissue areas) and they often result in reduced growth or stunting of plant organs or the whole plant. Some fungi cause excessive growth in infected plants or plant parts, which can be manifested as growth which is very

different and distorted to normal plant growth. Another group of symptoms characteristic of fungal infection include wilting, rusts, smuts and mildew on plant parts (Horst 2008).

3.3.2 Important Diseases

A summary of the most important fungal diseases of crop plants are summarised in Table 6.4 following Strange and Scott (2005).

(a) **Pseudofungi (Fungal-like Organisms)**

1. **Myxomycetes**
   Includes slimemoulds on leaf surfaces in many plants. Clubroot of crucifers, powdery scab of potato, black smut of potato and brown spot of corn. No control is usually necessary or effective, and best to avoid planting such crops in infected areas.
2. **Oomycetes**
   Includes several of the most important plant pathogens known, and these are *Pythium, Phytopthora*, several fungi causing Downy Mildews and white rust (*Albuga*) on crucifers. Disease by Oomycetes are of two types; one which affect plant parts in contact with the soil and another which affect above ground parts, like leaves, stems and fruits.
   Pythium – a major cause of seed rot, seedling damping-off and root rot in all types of plants and seedlings. Occasionally it may cause soft rots of fleshy fruits when in contact with soils.
   Phytopthora – the cause of a late blight of potato, and root rots and blights of many other plants, especially hard to control in forest trees.
   Downy mildews – caused by a number of fungal species in dicotyledon plants which can be destructive in lettuce, tobacco, grape and cucumber. In monocotyledon plants other fungal species are involved in mildews but with similar destructive effects.
   Albugo – can be common but usually exhibit a white rust symptom in crucifers, and is not destructive.

(b) **Ascomycetes (Imperfect Fungi)**
Many Ascomycetes and imperfect fungi cause a variety of diseases in all types of plants. The most important plant pathogens are detailed below.

1. **Sooty Moulds** – appear on leaves and stems of plants as superficial black growths in warm humid weather. Most are not parasitic and live on dew and sugars of leaves. Rarely damage plants and no control is required.
2. **Leaf Curl** – several species of *Taphine* cause curls on leaves, flowers and fruits; even stored fruits. Damage can result to the fruit and even the plant but is easily controlled by a single fungicide spray.
3. **Powdery Mildew** – one of the most common, conspicuous, widespread and easy to recognise plant disease. The fungi causing powdery mildew are

all obligate parasites and they can be so common that they may cause each year losses that surpass any other single type of disease. They seldom kill the plant but by depleting reserve nutrients they may reduce growth the following year of up to 40%. Control is difficult and may involve resistant varieties and systemic fungicide application.

4. **Foliar Diseases** – there are a variety of plant pathogenic diseases of foliage which are economically important due to Ascomycetes. These include *Alternaria*, Rice Blast, Black Rot of Grape, Needle and Blight of Conifers, Banana Leaf Spot (Sigatoka Disease), Cucurbit Stem Blight, *Cernospora* Disease and *Pyrenospora* Disease of Cereals and Grasses.
5. **Stem and Twig Cankers** – poplar canker, Black But of plum and cherry, Chestnut Blight, *Nectria* Canker and *Leucostoma* Canker.
6. **Anthracnose Diseases** – Black Spot of Roses, *Glomerella* disease of annuals and Fruit Rots, Leaf Spot Disease of Gramminacea, *Colletochritum* Disease. Some anthracnose disease can have a serious effect on crops.
7. **Fruit and Seed Diseases** – Ergot of Cereals and Grains, Apple Scab, Brown Rot of Stone Fruit, *Botrytis* are the more important diseases which can cause large production losses.
8. **Vascular Wilts** – *Fusarium* Wilts, *Verticillium* Wilts, Dutch Elm Disease can all be serious.
9. **Root and Stem Rots** – *Gibberella* Stalk, Seedling Blight of Cereals, *Fusarium* Rot, Take-all of Wheat, and some other stem and root rot diseases are examples, and some that can cause production losses.
10. **Postharvest Disease** – *Erwinia* and *Pseudomonas, Rhizobacteria* and *Sclerotium* (Basidiomycetes see below), *Alternaria, Botrytis, Fusarium, Penicillium* also may be involved in serious post-harvest loss of crops.

(c) **Basideomycetes (True Fungi)**

Most Basideomycetes are fleshy fungi, such as the common mushroom. They are mostly saprophytic and/or cause wood decay, which includes root and stem rots of trees. However two very common and very destructive groups of these plant pathogens are present; the rusts and smuts.

1. **Rusts** – cereal rusts, stem rust of wheat and other cereals, Apple Rust, Coffee Rust, Rusts of Fruit Trees, White pine Blister, Fusiform Rust and Rusts of Bean and Roses are of commercial importance.
2. **Smuts** – Corn Smut, Kernel Smut of small grains, Covered Smut, But of Wheat, Sorghum Head Smut, Leaf Smut and Stripe Smut can be considered the most important in crop production loss.
3. **Root And Stem Rots** – *Rhizoctonia* and *Sclerotium* Diseases, *Armillaria* Root Rot can be important.
4. **Wood Rot and Decay** – Can have huge losses in timber and are usually divided into Brown-Rot Fungi and White-Rot Fungi depending on the cell wall components the fungi are preferentially degrading. Some of these fungi are simply wood staining fungi that are just surface moulds, but they can

cause wood discolouration, lines and splits which have some commercial implications in timber sales.

(d) **Mycorrhizal Fungi**
The feeder roots of many flowering plants (Angiosperms) growing in native soils can be infected by these symbiotic fungi. The fungi generally do not cause a root disease but instead can be beneficial to their host plants. There are two types of mycorrhizal fungi described Ectomycorrhizae and Endomycorrhizae. However details of this special plant-fungal relationship is beyond the scope of this chapter.

3.3.3 Control Measures

There are numerous and diverse methods to try to control fungal diseases, and the complexity of many of these methods can appear endless. Few fungal diseases can be controlled just with one method and usually a combination of methods are necessary. Some common methods and practices include the use of resistant plant cultivars, clean tools and machinery, however these are not always possible or effective. But for many plants and their fungal diseases the most effective method of control is the application of chemical sprays and dusts (Strange and Scott 2005). Soil fungi can be controlled by steam pasteurisation. Very often a combination of resistant varieties and good chemical spray programs are essential.

3.4 Nematodes

3.4.1 Characteristics and Symptoms

Nematodes belong to the Animal Kingdom. Nematodes are worm-like in appearance but are quite distinct taxonomically from the true worms. There are several thousand species of nematodes and they live freely in fresh or salt waters or in the soil, where they generally feed on microscopic plants or animals and microorganisms. Only a few hundred species are known to feed on living plants, and only some cause a variety of plant diseases worldwide with considerable economic losses. Plant parasitic nematodes are very small and therefore mostly invisible to the eye. They are easily observed under the microscope as eel-shaped, round in cross section and with longer mostly unsegmented bodies, without legs or appendages. Their bodies are more or less transparent and most penetrate their host plant via a hollow stylet or spear to withdraw nutrients from their host. Their life cycle can be short (2–4 weeks) under optimal conditions but usually will take longer under cool temperatures. When the infection stage is reached they must feed on a suitable host plant otherwise they will die. Lack of suitable hosts may result in complete loss or death of a species within months, but in some species the juvenile stages may dry-up and remain quiescent as the egg can survive for years (Agrios 1997).

Nematodes spread through the soil without aid but generally travel only a few metres at best in one season. They can be faster in more open soil, but they do not survive well if soils are waterlogged. Nematodes are easily spread quickly by anything that moves and can carry soil particles. Farm equipment, irrigation, flooding drainage water, animal feet, birds, dust and humans are primary spreading agents. Plant diseases caused by nematodes can be complex. Root feeding decreases the ability of the plant to take-up water and nutrients from the soil, but also they interact with the plant biochemical mechanism. Although most parasitic nematodes are capable of causing disease on plants by themselves they do operate in the soil where the damaged plant roots are constantly surrounded by fungi and bacteria. Many of these bacteria/fungi can cause plant diseases, and in many cases this association is never made.

Isolation of nematodes from the soil or even from infected plant material is based on a series of filtering and/or sieving methods to allow the nematodes to migrate to an area (usually towards the bottom layer) where they are concentrated and collected for examination under the microscope. Sometimes a sugar flotation and gradient method is used under centrifugation to achieve a larger concentrated layer of nematodes (Horst 2008).

Nematode infection of plants results in the appearance of visible areas and even lesions, or reduce growth:

(a) **Roots** – symptoms may appear as root knots, root galls, lesions, excessive root branching, injured root tips and occasionally root rots; the latter is most likely a secondary effect.
(b) **Above-ground plant** – mostly non-characteristic symptoms but primarily reduced growth, symptoms analogous to nutrient deficiency like yellow foliage, excessive wilting, reduced yield and poor quality.

Direct mechanical injury by nematodes while feeding only slightly damage plants. Most of the damage is caused by a secretion of saliva injected into the plant cells. Some nematodes feed rapidly within seconds and then quickly move on, while others can take several hours. In either case the feeding process results in dead or damaged plant cells, especially at the growing tips of roots where lesions and tissue degradation due to the digestive enzymes and toxins is evident. In some cases this is accompanied by stimulation of cell division uncontrollably and the formation of galls.

3.4.2 Important Diseases

The more important nematode diseases of crop plants are summarised in Table 6.4 and Strange and Scott (2005):

(a) **Root-Knot Nematode (*Meloidogyme*)**
Root-knot nematodes occur throughout the world, but are more prevalent in warm or hot climates with short winters; but can also be widespread in greenhouse plants where growth conditions are ideal. Root-knot nematodes can be easily controlled with steam pasteurisation of soil or growing media. There are also nematicides that have been successful, but are usually expensive to use, and in

the field soil fumigation with chemicals is a more practical and a cheaper method. Damage to plants is usually by lowering crop production, but can also disfigure and reduce market value of crops. This group of nematodes attack over 2,000 species of plants in cultivation and reduces crop production by on average 5%, but can be much higher during heavy infestation.

(b) **Cyst Nematodes (*Heterodera/Globerella*)**
A diverse group of nematodes mostly in temperate parts of the world. Some can have a limited region for infection and some attack only a few crops. Most susceptible plants are tomato, potato and eggplant (for *Globerella*). *Heterodera* on the other hand are most important in cereals, soybean, sugar beet, spinach and crucifers. Soil fumigation is the only practical method of control but may not be economically viable for all crops, and as well nematodes cannot be totally eliminated in most cases. Soybean are the most susceptible crop in North America, Europe, Middle East and Australia where in heavy infested fields it can cause between 25% and 50% loss.

(c) **Citrus Nematode (*Tylenchulus*)**
This nematode is present and common in nearly all parts of the world where citrus is cultivated. In some areas different strains or races are also capable of attacking other crops like grapes and olives in close proximity. Infection results in slow decline in trees, leaves can turn yellow many drop leaves early before some die-back symptoms appear. Citrus nematodes are easily transferred from soil to soil and control of citrus nematode is mostly based on prevention and avoiding re-introduction. Nursery stocks are treated with hot water or a combination of hot water and nematicides before propagation for new plantings.

(d) **Lesion Nematodes (*Pradylenchus*)**
Lesion nematodes occur in all parts of the world where they can attack most field crops, vegetables, fruit trees and many ornamental plants. Plants grow poorly due to root damage and produce lower yields; many plants after some time can die completely. Control is by treatment of the soil with nematicides where control is good, but often not complete. Eventually crop rotation will be necessary.

(e) **Burrowing Nematode (*Rodopholus*)**
Most of these nematodes occur in tropical and sub-tropical regions of the world. Banana plants are particularly susceptible and the disease is called by a few names; banana root rot, blackhead toppling disease or decline of banana. Banana plants are so badly affected where the profitable life of infected banana can be as short as 1 year. The nematodes can also affect severely avocado, tea, black pepper, coconut, coffee, sugar cane, corn, citrus, vegetables, fruit and even ornamental plants. Control can only be achieved by nematode-free plantlets and soil fumigation.

(f) **Stem and Bulb Nematode (*Ditylenchus*)**
This nematode occurs world-wide but is at its most destructive in temperate climates. It can be one of the most destructive of nematodes attacking large numbers of plants including onion, hyacinth, tulip, oat, alfalfa and strawberry.

The nematode is so destructive that it can enter growing young roots after germination at or near the root growing tips (cap), as well as later in growing plantlets via the stomata. Control can be affected through long rotation strategies of crops (at least 2–3 years) and/or fumigation and formaldehyde treatment.

(g) **Seed Gall Nematode (*Aguira*)**
Wheat seed gall nematode is the best known and is prevalent in Europe, Asia, Africa and Australia. The nematode is quite large (3 mm long), overwinters in infected plants or seeds, and galls fall to the soil for re-infection. Fields infested should not be planted with wheat or rye for at least 1 year afterwards.

(h) **Foliar Nematodes (*Aphelenchoides*)**
Nematodes that can feed both ectoparasitically or endoparasitically on above ground parts of plants. Nematodes are most prevalent in ornamental plants like chrysanthemum, aster, dahlia, zinnia and strawberry. Defoliation can occur and if so flowers are affected by reduction in size and quality. Several sanitary practices are required for control, including keeping stems dry and propagation cuttings being treated with hot water.

(i) **Stubby Root Nematodes (*Paratrichedus/Trichedus*)**
Nematodes that occur world-wide and attack cereals, vegetables, shrubs and trees. They can cause stunting, reduced growth, reduced yield and poor quality, but rarely or seldom cause death of plants. Control is mainly through the application of nematicides, but these chemicals are rarely very effective.

(j) **Pine Wilt and Palm Red Ring Disease (*Burapheluchus*)**
Nematode can cause severe wilt of several tree species, but the nematode has developed a special symbiotic relationship with insects, mainly beetles and weevils which it uses as vectors. Pine wilt can be severe in *Pinus* species and can affect different parts of the tree. Pine nematode vectors reside or burrow into the wood and can overwinter there; new nematodes develop in early spring. Control may involve an insecticide to control the insect vectors, burying any dead wood, however these measures are sometimes very difficult to introduce in pine plantation forests.

3.4.3 Control Measures

Several methods of effective control of nematodes are available, but expense and type of crop may influence the control methods chosen. Control is usually affected through a number of methods, these include cultural practices (ie crop rotation, fallow and cover crops), resistant varieties to nematodes, physical agents like heat treatment and flooding, antagonistic bacteria and fungi and chemicals of various types in the nematocide group. Cultural practices have been successful for decades against nematodes, and as in all cases of pathogen control, exclusion of the nematodes and not introducing them into an area is the best option (Agrios 1997).

3.5 Protozoa and Insects

3.5.1 Protozoa

Certain types of flagellate protozoa (*Phytomonas*) have been associated with plant diseases. The taxonomy of the protozoa that have been associated with plant diseases is rather complex except that most, if not all inhabit the phloem sieve tubes of their hosts. It is thought that parasitic protozoa inhibit phloem function, and in this way cause decreased growth, but the mechanism of action is not clear (Agrios 1997). There are four important diseases in this group which do affect some commercially important crops:

(a) **Phloem Necrosis of Coffee** – in Malaya, Guyana, Brazil, San Salvadore and Columbia. The flagellate pathogen is *Phytomonas*, which can be traced from the roots to the trunk where it appears to migrate vertically in the phloem and spreads vertically through the sieve plates.
(b) **Hartrot of Coconut Palm** – in Malaya, Colombia, Ecuador and Trinidad-Tobago. The pathogen is a flagellate *Phytomonas* and is present in the sieve elements of younger leaves, plugging the sieve plates as the elements mature. In this case insects appear to be the vectors for the protozoa.
(c) **Sudden Wilt of Oil Palm** – in South America, especially in Columbia and can spread rapidly in plantations; and is caused by *Phytomonas*. The pathogen is similar to Coconut Hartrot and an insect appears also to be a vector for spread.
(d) **Empty Root of Cassava** – especially prevalent in Brazil where the root system develops poorly, roots remain small and slender. The organism can be transmitted during grafting and spreads rapidly in the field; probably also by an insect vector.

3.5.2 Insects

(a) **Introduction**
Insects are important pests of many plants world-wide because of the damage inflicted by direct feeding, but also because they are vectors or provide infection sites for plant pathogens. The relative importance of insects compared to other biotic stresses (ie pathogens or weeds) depends greatly on the crop and its location. Tropical crops usually can suffer more direct feeding damage from a wider variety of insect pests than the same crops in cold seasonal climates (Berg 1991a). However crops grown in temperate areas or in cold climates are more likely to be attacked by aphids and the viruses they transmit (Heie 1994). The economic importance and damage of insect herbivore will also greatly depend on the life stage and tissues of the plant they attack, and their method of feeding. Insects can feed and damage plants by:

1. Damaging seeds in store or in the field.
2. Damage pods and flowers.
3. Damage leaves and/or may also be sap feeding.

(b) **Defence**

Most plants rely on a suite of defences for protection against insect pests; such as:

1. Accumulation of anti-nutritional compounds during germination (eg lectins).
2. Chemical defence localisation or translocated throughout the plant.
3. Structural defence including simple and glandular hairs (ie trichomes).

All of these defences can act directly on the herbivour pests themselves by:

1. Deterring herbivour feeding (antiserosis).
2. Suppressing herbivour growth and development (antibiosis).
3. Minimising damage symptoms (tolerance).
4. Increase herbivour mortality, directly or indirectly (toxins).

Many of the mechanisms important to insect defence have been bred out of many cultivated crops, mainly because they also detract from the taste and texture of edible plants (Clement 2002). Breeders are only now realising this mistake and are deliberately trying to re-introduce some of these defences in many crops. But achieving pest resistance without reducing agronomic quality has been a huge problem to overcome. An exception to this has been breeding resistance to aphids in alfalfa/lucerne, in part due to the simple and dominant inheritance pattern that many aphid resistance genes have (Edwards and Singh 2006).

(c) **Signalling Pathways**

Similar to plant-pathogen interactions, where defences are induced to improve insect resistance in the plant. However our knowledge of these signalling pathways and downstream changes is not well developed. The best known are chewing insects where potential elicitors have been identified. These include lytic enzymes like β-glucosidase and glycine oxidase, and fatty acid-amino acid conjugates (Kessler and Baldwin 2002). Genomic approaches are being used to study plant responses to chewing insects and primarily have involved clear overlap with pathogen induced and wound induced responses, but some specific effects caused by the insects themselves have been described (Ferry et al. 2004). Voelckel et al. (2004) compared changes brought about by simple, sequential and simultaneous insect attack. They found quite different profiles of metabolites obtained following these different types of attacks, which makes studies of pathways complex.

While concentrating on the plants themselves, additional interactions also open important research areas of what the insects themselves are doing to try to overcome plant resistance (Edwards and Singh 2006). These include insect developed mechanisms like:

1. Contain potent proteinase inhibitors and actively up-regulate proteases.
2. Detoxifying enzymes such as glutathione-S-transferase and cytochrome P450.
3. Minimise and regulate the signals that are part of the plants defence response.

3.6 Higher Plant Taxa (Weeds and Parasitic Plants)

3.6.1 Introduction

Weeds have major economic, environmental and social impacts causing damage to agriculture, natural landscape, waterways and coastal areas. Weeds can impact severely on agriculture by competing with production, contaminating produce and poisoning livestock. Some weeds cause severe allergenic reactions in people. It has been estimated that the cost of weeds to Australian agriculture and horticulture alone is in the vicinity of $4 billion per year. Worldwide weed plants make-up more than one third of the costs to agricultural products (US$350 billion per year) (Pimentel 2002). Once established, weeds pose an ongoing challenge and cost to government, agriculture and the community. Weeds can be imported or enter a country or area by increase of international movements of goods and produce, and this can be legal, illegal or even inadvertently. After establishment in an area the spread or control of this spread can be a major issue to address. Weeds are resilient and very successful in spreading, and establish easily by their very nature. They often respond to changes in environmental conditions very quickly and the causes of these changes in conditions can be varied; including a number of factors such as:

(a) Pathogens and vectors
(b) Pollinators
(c) Storm damage and floods
(d) Fire and frequency of burning
(e) Vegetation clearing
(f) Changes in land-use pattern.

3.6.2 Herbicides

Total reliance on herbicides for weed control is unsustainable with the spread of herbicide resistance and environmental need to reduce the use of pesticides. Herbicide resistance can be a major limitation to crop production around the world, and has spread rapidly to a broad range of weed species (Heap 2004). This rapid development of resistance may have been helped by recent agricultural practices, planting semi-dwarf varieties, poor weed competitive cultivars and a high dependence on herbicides for control. All of these factors are forcing plant breeders to think about different strategies to combat weeds, and even breed for more weed competitiveness in future. Coleman et al. (2001) found that the heritability of important traits for more weed competitiveness was low due to large ‘genotype X year’ interactions, making genetic gains through phenotypic selection difficult.

However advances are being made in the breeding of strongly competitive crops that have high tolerance to weed pressure and therefore one can maintain high yields in the presence of weeds, and this can be a low cost option. In this regard the newly developed Incremental Crop Tolerance (ICT) measure proposed by Lemerle et al. (2006)

is an interesting tool for breeders. ICT can be applied to existing and new cultivars to reflect increased yield differences between genotypes associated with tolerance to weeds, over and above differences in the underlying yield potential.

3.6.3 Biological Control

In Europe a recent report has concluded that research in biological control of weeds was lagging well behind other countries such as North America, Australia, New Zealand and even South Africa (Sheppard et al. 2006). Classic biological control over the last 200 years or so has in many countries slowly but surely proven itself to be the only low cost, low risk and viable long term control of weeds; but it was not always reliable as a long-term weed control method in the past. Classic biological control uses a co-evolved and specific antagonist of the weed from its native range, which is then screened for effects to any participating non-target native or commercially important species in the new area. The antagonists are then released into the weed population for control. This approach has a long history compared with many other approaches to active pest management (ie agrochemicals).

Classic biological control has had a historically tarnished reputation, largely due to early unscientific and uncontrolled release of mainly invertebrate predators, to the detriment and even decimate native species. In recent cases as well, insect biological control agents have spread beyond their intended targets (Roy et al. 2005). Biological control of weeds now adopt a precautionary approach using the most specific antagonistic invertebrate organisms or even microorganisms against the target. They follow best practice scientific risk analysis and regulator approval before any release is approved (Sheppard et al. 2003). When conditions above are met and practiced, negative effects have proven to be predictable and releases have recently not caused problems.

3.6.4 Parasitic Higher Plants

More than 2,500 species of higher plants are known to live parasitically on other plants. Relatively few of these cause important diseases or loss in production on agricultural crops. However there are a few pests:

(a) **Dodders** – widely distributed in Europe and North America, and crops that suffer losses include alfalfa, corn, sugar beet, several ornamentals and potato. Dodders encircle plant stems and lower leaves and can easily elongate to adjacent plants where they send haustoria within the stem tissue. Infected host plants become weakened, vigour declines, produce poor yields and may kill plants. Prevention is mainly by not introducing dodder seeds and plants into fields, cleaning equipment and tools; however drastic measures like cutting and slashing or burning to kill both the host and dodder may be required. Frequent tilling and herbicides are other methods, especially the use of pre-emergent herbicides to kill dodder seeds.

(b) **Witchweed** – a serious parasite weed in Africa, Asia and Australia, where it can parasatise important economic plants like corn, sugarcane, rice, cowpeas, tobacco and other small grains. Affected plants remain stunted, wilt and turn yellow; death may follow. Witchweed seeds germinate near roots of the host plant and haustoria attack the host roots, and then produces the above ground part of the witchweed plant. Witchweed is difficult to control and best to avoid its introduction at all cost. Plowing material under and use of herbicides are possible, but with variable effects, and the use of resistant cultivars is an additional tool.

(c) **Broomrapes** – occur mainly in warm and dry regions world-wide. They can attack many species of herbaceous dicotyledon crop plants. In some areas up to 70% of crops are lost, but the losses are usually lower. Seeds of broomrapes germinate and grow towards the roots of the host plant and attach to them. Apressoria can grow and extend to the xylem of the roots absorbing nutrients and water, weakening the plant considerably. Control of broomrapes mainly depends on preventing the introduction of seeds into an area.

4 Conclusions/Future Directions

Predicted growth in world population and the likely effects of climate change will pose a serious challenge to crop production and food security, particularly in developing countries. Conventional plant breeding has proven to be effective for the improvement of many traits of economic importance, especially disease resistance. Plant breeders have extended the range of adaptation in crops and improved agronomic traits (eg seed yield potential). Molecular plant breeding techniques have proven to be valuable tools for the improvement of quantitative inherited traits and traits that do not allow phenotypic selection; and some have already proven to be effective for marker assisted selection (MAS) of biotic related stress. Therefore screening of genetic resources coupled with conventional breeding still offers high discovery potential, especially if researchers take full advantage of the landraces and wild species available world-wide. It is therefore clear that an integrated approach involving research involving both the plant and the biotic stress organism, and their biological interactions are still good approaches.

The use of MAS for some complex biotic traits will require a markedly better understanding of the genetic basis of expression of these traits. Genomic mapping of the traits should be a considerable help in the future, as well as a better understanding of the basis for genotype-environment interactions. A reliable, easy and efficient transformation system needs to be further developed. Traits already using genetic manipulation (GM) technology (such as herbicide and insect tolerance) have the potential to lower the cost of crop production and reduce land and soil degradation. At the moment only *Agrobacterium*-mediated and particle bombardment can be considered to be routine. Gene targeting for example is still an elusive goal for the plant biotechnologist. In this regard the ability of a

plant to defend against pathogens is important, and contributing to this successful defence are the role of protein kinases and phosphatases (signalling proteins), and the recent development of proteomics which is likely to increase our knowledge of plant-pathogen-signal interactions.

The area of complex metabolic engineering and gene modification is still less successful than conventional and molecular plant breeding. Nevertheless the combination of plant breeding and targeting genes in a biotechnological sense can contribute substantially to human well-being by providing renewable sources of food, feed, chemicals, pharmaceuticals, energy, and for the creation of a sustainable environment for the developed, as well as the developing world. During the last two decades the development and adoption of transgenic technology has progressed rapidly to the point that almost every significant food crop has been successfully transformed. However, the issue of consumer acceptance of such biotech crops should be seriously taken into consideration before development and release of transgenic crops. Finally, a number of important food crops are produced and consumed in developing countries. This means that breeding programs, especially those in developing countries need access to knowledge and methodology that will ensure the use of these enabling technologies of molecular plant breeding in developing countries.

Future impacts of biotechnology in crop production is predicted to be in the areas of:- (i) developing new hybrid crops based on genetic male-sterility, (ii) exploiting transgenic apomixes to fix hybrid vigour in inbred crops, (iii) increase resistance to insect pests, diseases, and abiotic stress, (iv) improve effectiveness of bio-control agents, (v) enhance nutritional value (vitamin A, zinc and iron) of crops and post-harvest quality, (vi) increase efficiency of soil phosphorus uptake and nitrogen fixation, (vii) improve adaptation to soil salinity and metal toxicity, (viii) understanding the nature of gene action and metabolic pathways, (ix) increase photosynthetic activity, sugar and starch production, and (x) production of pharmaceuticals and vaccines.

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Chapter 7
Breeding Wheat for Salt Tolerance and Stem Rust Resistance

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Contents

Abstract Fast and effective hydroponics screening technique that could identify physiological variation in salinity tolerance of wheat was applied. A set of 442, previously unexplored wheat varieties/lines representing a wide range of genetic diversity was planted as control in ½ strength Hoagland's nutrient media, whereas two sets of the same material were exposed to salt (NaCl) application under two treatments i.e., 10 dS m^{-1} and 20 dS m^{-1} for the first 2 years (2003–04 and 2004–05).

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For the third year (2005–06), more intensive stress was applied with salinity levels of 12.5 dS m^{-1} and 25 dS m^{-1}. Salinity tolerance was defined as differences in biomass (root-shoot ratio) production in saline versus non-saline conditions over prolonged periods, of 3–4 weeks (seedling to pre booting stage). For this purpose parameters like shoot length, root length, shoot weight and root weight alongwith their relative ratios were measured. As a result of 3 year study 11 common salt tolerant varieties/lines were identified including Pasban-90, LU 26S, V-01078 (Seher 06) and V-01180. Under saline field conditions some of these lines like Gamdow-6, BAV 92//SAP/MON and Lakata-1 produced higher grain yield, whereas, Uqab-2000 was the best yielding in saline field conditions, although it was found non tolerant in hydroponic studies. Line Ning 8319 showed good resistance for stem rust comparable to Parula and Pavon. These selected lines are being used in specific breeding programs for the development of high yielding wheat varieties having high degree of salt tolerance and stem rust resistance.

Keywords Spring wheat • Salinity • Root-shoot ratio and hydroponics • Stem rust

1 Introduction

Vast areas of the world including Pakistan are rendered agriculturally unproductive due to high concentration of salts in the root zone. Out of 20.2 million hectares of cultivated land in Pakistan, 6.8 million hectares are affected with salinity and 2.67 million hectares of the affected area is located in Punjab. The salinity area has been categorized into four major classes (Table 7.1). It has been reported that 1390.03 thousands hectares were strongly saline lands, 804.8 thousands hectares moderately saline, while 472.4 thousands hectares saline areas in the world (Anonymous 2002).

Root zone salinity affects growth of wheat and reduces the number of fertile spikes and spike-bearing tillers (Steppuhn and Wall 1997). The reduced number of tillers, plant height and reduced root growth ultimately affect the yield. A high salt level interferes with the germination of new seeds. Salinity acts like drought on plants, preventing roots from performing their osmotic activity where water and nutrients move from an area of low concentration into an area of high concentration (Greenway and Munns 1980; Munns 1993). Therefore, because of the salt levels in the soil, water and nutrients cannot move into the plant roots.

Salt tolerance is usually defined as the percent biomass production in saline medium relative to plants in non-saline medium, after growth for an extended period of time. As soil salinity levels increase, the stress on germinating seedlings also increases. Perennial plants seem to handle salinity better than annual plants like wheat. In some cases, salinity also has a toxic effect on plants because of the high concentration of certain salts in the soil. Salinity prevents the plants from taking up the proper balance of nutrients they require for healthy growth. Ultimately, salt tolerance of

Table 7.1 Soil salinity rating and electrical conductivity value

Soil depth	Non-saline	Weakly saline	Moderately saline	Strongly saline	Very strongly saline
0–60 cm	<2 dS m^{-1}	2–4 dS m^{-1}	4–8 dS m^{-1}	8–16 dS m^{-1}	>16 dS m^{-1}
60–120 cm	<4 dS m^{-1}	4–8 dS m^{-1}	8–16 dS m^{-1}	16–24 dS m^{-1}	>24 dS m^{-1}

wheat is tested as yield from farmer's fields. However, evaluating field performance under saline conditions is notoriously difficult because of the variability of salinity within fields (Daniells et al. 2001). To address this problem, a rapid and more reliable hydroponics screening program was developed (Iqbal 2003). The germplasm was screened and tolerant lines were evaluated in the field.

A recent outbreak of new wheat stem rust race (Ug99), capable of parasitizing many commercial cultivars has threatened local and world wheat production and potentially 25% of the World's wheat crop is thought to be at risk (Reynolds and Borlaug 2006; Ayliffe et al. 2008). The rust has spread from African nations to Middle East. From where it is expected to reach the wheat growing regions of Asia, raising the possibility of a major epidemic (Stokstad 2007). Due to urgency of stem rust problem, salt tolerant material was got screened from Kenya and a breeding program was initiated to address these problems simultaneously for the development of high yielding wheat varieties.

2 Materials and Methods

2.1 Screening in Hydroponic Culture

Research Site

The research was conducted in the glasshouse of Wheat Research Institute, Faisalabad during the years 2003–04, 2004–05 and 2005–06.

Seed Material

For the year 2003–04, 442 wheat varieties/lines were tested, whereas in the year 2004–05, 496 varieties/lines were included in the salinity screening program. This material includes past and present wheat cultivars of Indo-Pak Subcontinent, promising lines (MTWV and NUWYT), and exotic germplasm (CIMMYT and ICARDA).

Screening Technique

Seed was cultured in sand filled pots. At two leaf stage, four seedlings of each genotype were transferred to thermopore sheets and plugged in holes especially made to hold the seedlings so that they do not drop down in the growth media. Special steel tubs (1 × 1 m) were made having 300-l capacity. Half strength Hoagland's solution (Hoagland and Arnon 1950) was used as nutrient medium. Sodium Chloride (NaCl) was used as a source of salinity.

Electrical Conductivity

Salt tolerance is mostly measured in terms of the stage of plant growth over a range of electrical conductivity (EC) levels. Three treatments with two salinity levels i.e. control, 10 dS m^{-1} and 20 dS m^{-1} (Iqbal et al. 1998) were applied to the wheat seedlings for the first 2 years (2003–04 and 2004–05). For the third year (2005–06), a more intensive selection process was adopted with salinity levels of 12.5 dS m^{-1} and 25 dS m^{-1}. Air pumps were used for proper root aeration and growth. Tap water (EC ≤ 2 dS m^{-1}) was used as a main component of the hydroponic medium.

After 3–4 weeks of seedling transplantation (Munns and Richard 2003), plants were plugged out of the thermopore sheets and data for shoot and root lenths and fresh and dry weights were taken for the following parameters:

Statistical Analysis

Data for all the above-mentioned parameters were taken from all four plants/genotypes and mean values calculated. Relative ratios were computed for shoot length, root length, shoot weight, root weight with the following formula (Zulfiqar et al. 2002).

$$\text{Relative salt tolerance} = \frac{\text{Mean performance in stress}}{\text{Mean performance in control}}$$

To calculate relative ratio of shoot length at 10 dS m^{-1} salinity level, shoot length of a genotype at 10 dS m^{-1} of salt was divided by shoot length of the same genotype under control. Similarly, to calculate relative performance of the same genotype at 20 dS m^{-1} salinity level the shoot length of the said genotype was divided by the performance under control. Same calculations were done with all other genotypes. Salt tolerant lines were selected on the basis of their mean relative ratios near or equal to unity (1.0). Genotypes deviating sharply from unity were rejected as they failed to show synchronized performance for the above mentioned parameters.

Frequency distribution of each parameter at two levels of salinity was calculated and population curves of each character were also plotted to study the population dynamics at different levels of salinity.

2.2 Field Trial

Sixteen wheat varieties/lines found tolerant in the hydroponic experiment along with four checks, i.e., Inqilab-91, Uqab-2000, AS-2002 and V-02192 were assessed for salt tolerance under field conditions at Biosaline Research Station, Pacca Anna, District T.T. Singh. Randomized Complete Block Design (RCBD) was used having six replications. Plot size was kept at 1.8 m × 3 m (5.4 m^2). There were six rows per plot each having 3 m length and row to row distance of 0.30 m. Soil type was sandy loam. Ground water was applied having EC of 4.8 dS m^{-1}, RSC 21.8 me L^{-1} and

SAR 40 me L^{-1}, whereas soil EC (upper 15 cm) was recorded as 11.0 dS m^{-1} at sowing and 12 dS m^{-1} at harvest. Sowing was done in dry soil with the help of single row cotton drill and then irrigation applied afterwards. The crop was sown on 15th of November 2006 and harvested on 20th of April 2006 and threshed a week after. Grain yield per plot (g) was recorded. Analysis of variance (ANOVA) was performed using MSTATC (4.1) computer software.

2.2.1 Screening for Stem Rust

The salt tolerant material along with some other lines was sent to KARI, Njoro, Kenya for screening against stem rust race Ug99 through the Wheat Coordinator, NARC, Islamabad during 2006 and 2007 and for its utilization in some breeding programs.

3 Results

The results of Hydroponics experiment and field trial are discussed below:

3.1 Hydroponics Experiment

Data for the characters under study was recorded from all the entries in all the three treatments. The data was compared and relative salt tolerance ratios were calculated for the characters which are discussed character wise as follow:

Shoot Length (cm)
In the first year of screening (2003–04) normal population curve (Fig. 7.1a) was observed at 10 dS m^{-1} salinity level for this character, whereas at 20 dS m^{-1} of salinity the population showed abnormal behavior. Highest frequency of 121 was found at the relative ratio of 0.6–0.9 for 10 dS m^{-1} salinity level. For 20 dS m^{-1} salinity level, highest frequency of 123 was observed in the relative ratio range of 0.3–0.6.

For the second year of screening (2004–05) normal population behavior (Fig. 7.1b) was observed at 10 dS m^{-1} salinity, whereas at 20 dS m^{-1} salinity more abnormal population behavior was recorded. The highest frequency i.e. 185 was observed at the range of 0.6–0.9 at 10 dS m^{-1} salinity, whereas highest frequency of 210 fell in the range of 0.3–0.6 at 20 dS m^{-1}.

For the third year more intense selection criteria were adopted with salinity levels of 12.5 dS m^{-1} and 25 dS m^{-1} to further refine our results. Normal population curve was observed (Fig. 7.1c) for the parameters under study at 12.5 dS m^{-1}, whereas at 25 dS m^{-1} salinity the population curve showed abnormal behavior as it peaked at a value much lower than unity (1.0). Highest frequency of genotypes fell

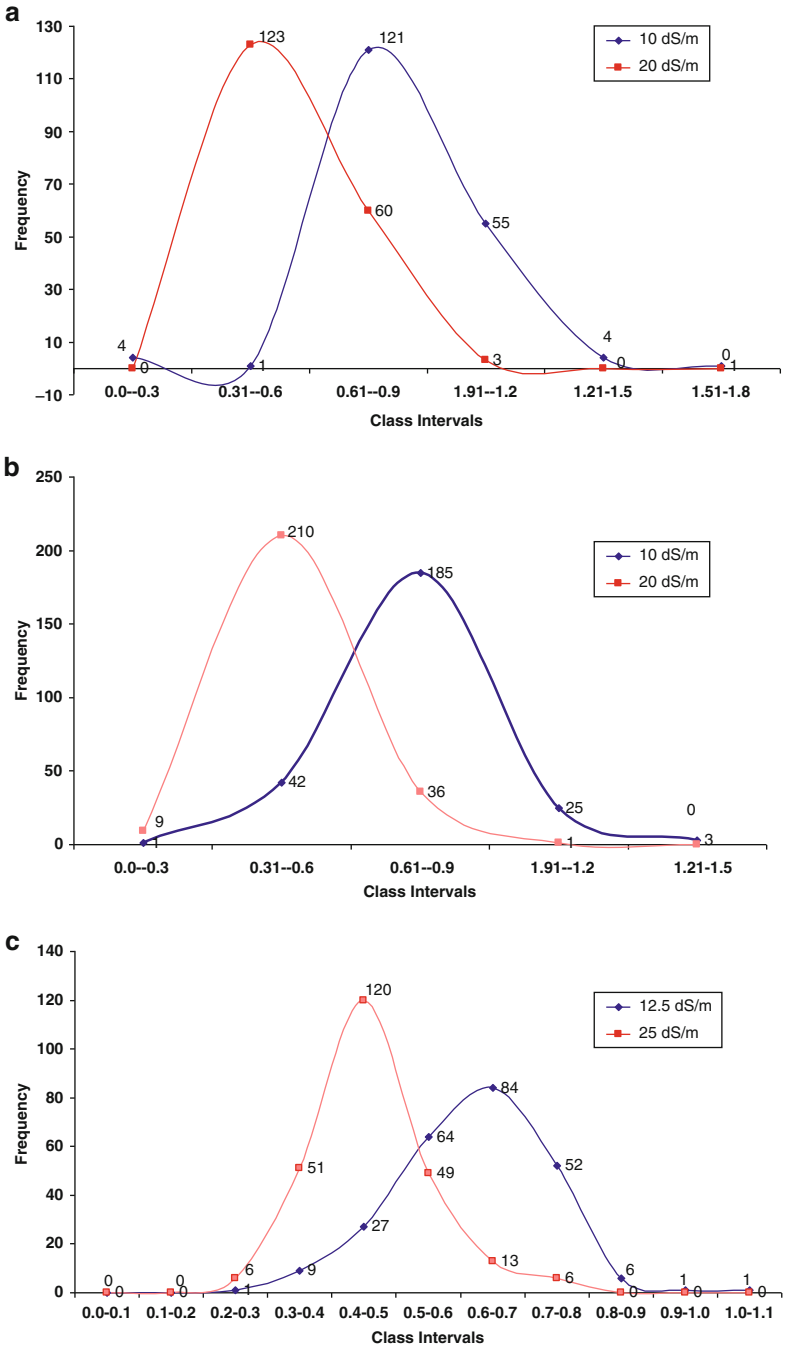

Fig. 7.1 (**a**) Frequency distribution of shoot length at 10 and 20 dS m^{-1} (2003–04). (**b**) Frequency distribution of shoot length at 10 and 20 dS m^{-1} (2004–05). (**c**) Frequency distribution of shoot length at 12.5 and 25 dS m^{-1} (2005–06)

in the range of 0.6–0.7 relative ratio at 12.5 dS m^{-1}. However, at 25 dS m^{-1} salinity, the highest frequency i.e. 120 was observed at much lower range of 0.4–0.5. At the highest salinity level of 25 dS m^{-1}, 7 lines fell in the range of 0.7–1.0 (which is near or equal to unity). These results are strongly in accordance with Cicek and Cacirlar (2002) who also reported that salinity reduced shoot length.

Root Length (cm)

In the first year of screening (2003–04) normal curve for population was observed at 10 dS m^{-1} salinity, whereas at 20 dS m^{-1} abnormal population curve was observed (Fig. 7.2a). Highest frequency of 94 was observed at the range 0.6–0.9 at 10 dS m^{-1} salinity, whereas at 20 dS m^{-1} salinity, the highest frequency of 116 was recorded at the range of 0.3–0.6. For the second year of screening (2004–05), abnormal population curve (Fig. 7.2b) for both salinity levels (10 dS m^{-1} and 20 dS m^{-1}) was observed. Highest frequencies of 152 and 175 were recorded at the range of 0.3–0.6 at salt levels of 10 and 20 dS m^{-1}, respectively.

In the third year of screening (2005–06), genotypes were screened at 12.5 and 25 dS m^{-1} salinity. Wheat genotypes showed abnormal curves at both salinity levels for root length (Fig. 7.2c). Highest frequency (75) was observed for 12.5 dS m^{-1} in the range of 0.8–1.0. In contrast, at 25 dS m^{-1}, highest frequency of 99 was recorded at the range of 0.4–0.6.

Shoot Weight (g)

For the first year (2003–04) of screening, abnormal population behavior at both salinity levels was observed (Fig. 7.3a). 77 and 113 were highest frequencies observed at the range of 0.3–0.6 at 10 and 20 dS m^{-1} respectively. At 10 dS m^{-1} salinity, frequency was distributed between the range of 0.0–0.3. However for 20 dS m^{-1}, the distribution of frequency was in much shorter range of 0.0–2 for this character.

For second year (2004–05) of screening abnormal population curves were observed (Fig. 7.3b) at both salinity levels. Highest frequency of 50 was recorded at the range of 0.4–0.5 at 10 dS m^{-1} salinity. At 20 dS m^{-1} salinity, the highest frequency of 77 was observed at very low range of 0.1–0.2. At 10 dS m^{-1} salinity, the frequency was more evenly distributed within the range of 0.0–2. However at 20 dS m^{-1}, population was unevenly distributed among ranges of 0.0–1.7.

Abnormal population behavior was observed (Fig. 7.3c) at both salt levels of 12.5 and 25 dS m^{-1} for third year (2005–06). At 12.5 dS m^{-1} Salinity, 54 was the highest frequency recorded at the range of 0.2–0.3. Whereas at 25 dS m^{-1}, highest frequency of 99 peaked at much earlier range (0.1–0.2). Frequencies were more evenly distributed among different ranges at 12.5 dS m^{-1}, whereas at 25 dS m^{-1} all the frequencies were unevenly distributed among a lower range of 0.0–1.3. Eleven lines were found in the near to unity range (0.7–1.0) which is considered as selection criteria for salt tolerance.

Root Weight (g)

In the first year of screening (2003–2004) abnormal but almost similar population behavior was observed for both salinity levels (10 and 20 dS m^{-1}) as shown in

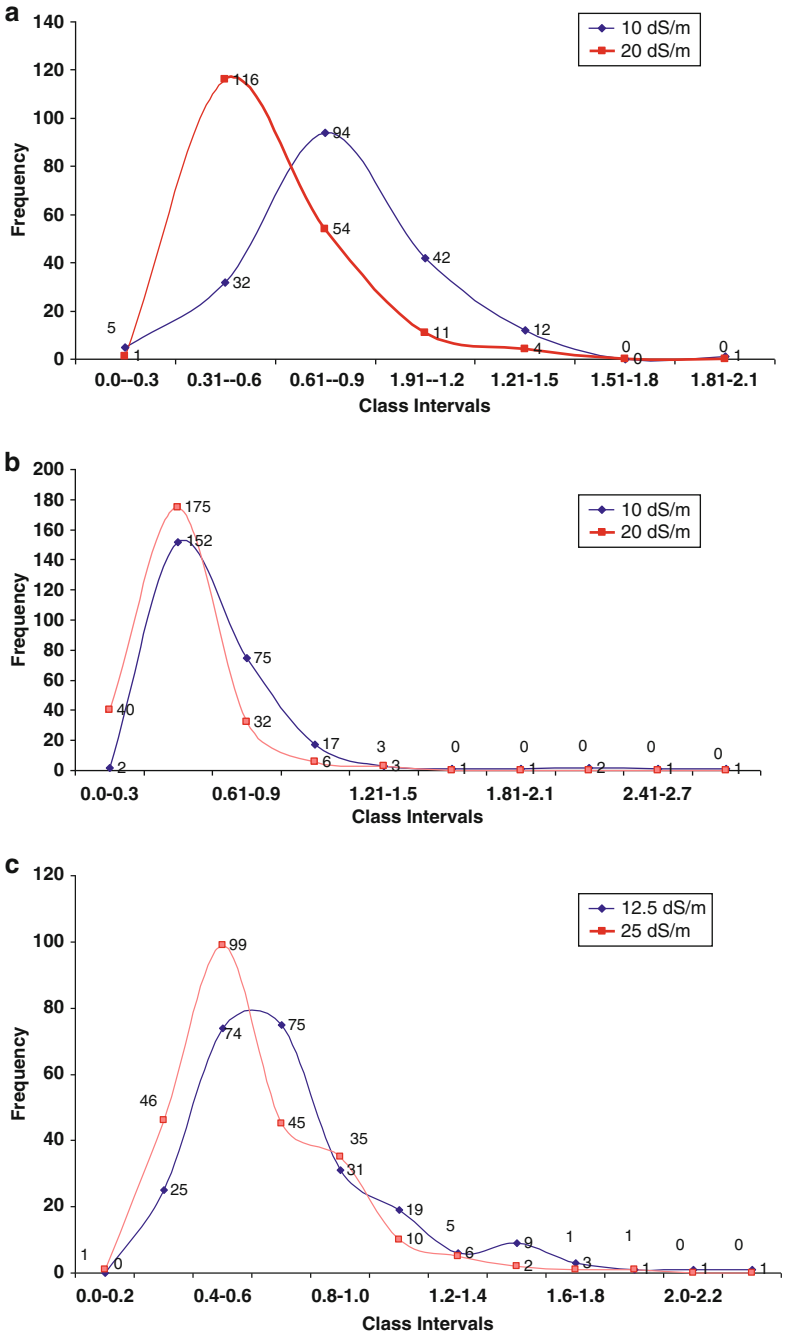

Fig. 7.2 (**a**) Frequency distribution of root length at 10 and 20 dS m^{-1} (2003–04). (**b**) Frequency distribution of root length at 10 and 20 dS m^{-1} (2004–05). (**c**) Frequency distribution of root length at 12.5 and 25 dS m^{-1} (2005–06)

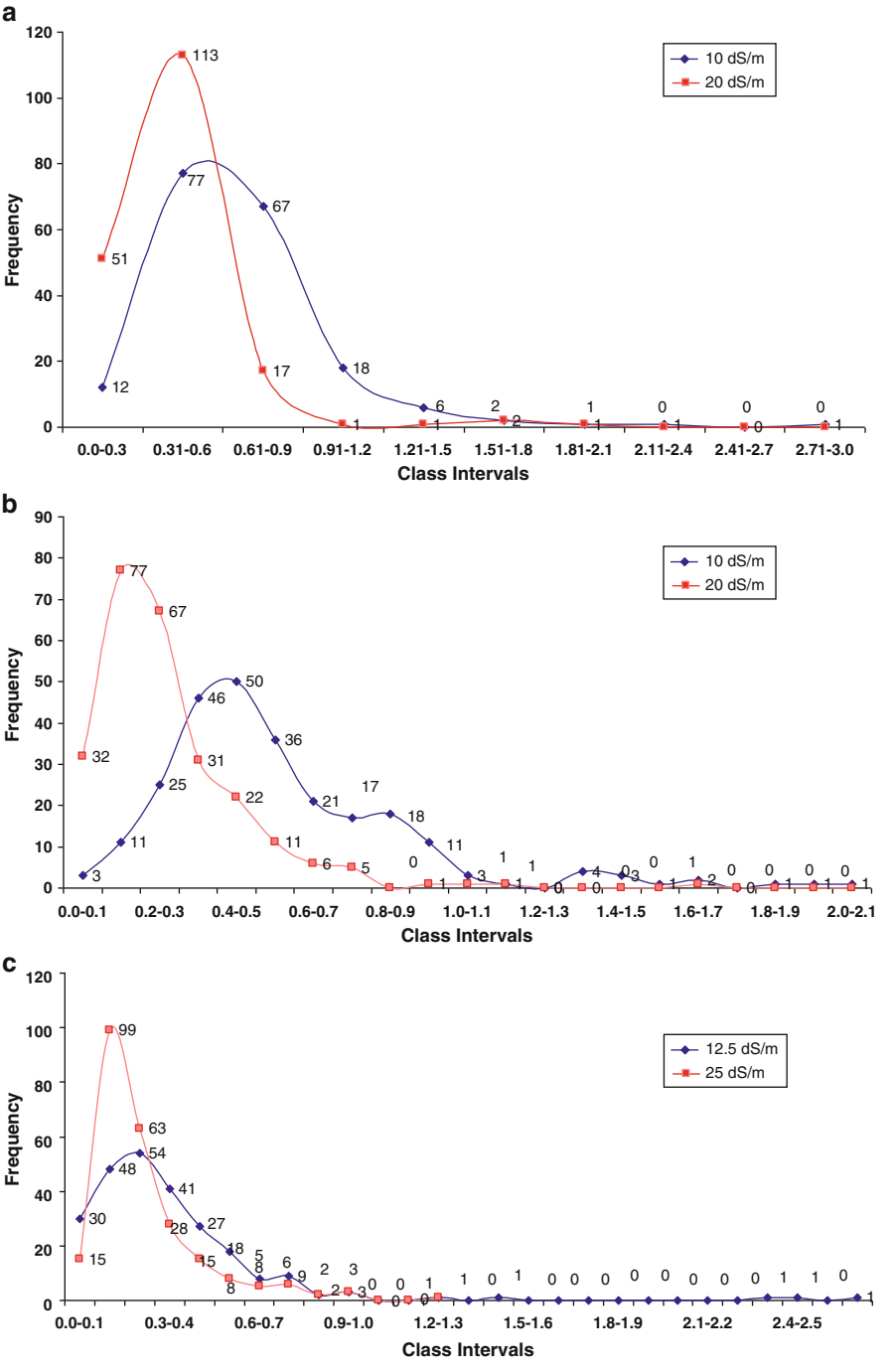

Fig. 7.3 (**a**) Frequency distribution of shoot weight at 10 and 20 dS m^{-1} (2003–04). (**b**) Frequency distribution of shoot weight at 10 and 20 dS m^{-1} (2004–05). (**c**) Frequency distribution of shoot weight at 12.5 and 25 dS m^{-1} (2005–06)

Fig. 7.4a. The highest frequencies of 68 and 72 fell in the range of 0.6–0.9 at both salinity levels (10 and 20 dS m^{-1} respectively).

For the second year (2004–05) of screening again abnormal population curve was noticed at both salt treatments for root weight (Fig. 7.4b). At 10 dS m^{-1}, the frequencies were more evenly distributed with high frequency reaching up to 71 at the range of 0.6–0.9. On the other hand at 20 dS m^{-1}, the frequencies were more unevenly distributed among different class intervals and highest frequency of 98 peaked at relatively lower range of 0.3–0.6.

Like previous 2 years, abnormal population behavior was recorded (Fig. 7.4c) for the third year (2005–06) at both salt levels (12.5 and 25 dS m^{-1}). At the range of 0.4–0.6 the highest frequency (51 genotypes) was recorded at 12.5 dS m^{-1}, whereas at 25 dS m^{-1} frequency of 42 and 44 were closely clustered at the ranges of 0.4–0.6 and 0.8–1.0. At top salinity of 25 dS m^{-1}, 64 lines were found in the 0.7–1.0 range. At 25 dS m^{-1} salinity, relatively less root weight reduction was recorded as compared to lower salinity level of 12.5 dS m^{-1}. Similar findings have also been reported by Grewal et al. (2004).

Salt Tolerant Lines

Eleven common salt tolerant lines were identified from 3 years study. Common salt tolerant lines and their pedigree/parentage are given in Table 7.2. The mean relative ratios of parameters studied were near to unity for these lines as minimum salinity effects were observed for all the above mentioned characters. The relative ratios for root length, shoot length, root weight and shoot weight for these lines were in the range of 0.8–1.

3.2 Field Trial

Analysis of variance (Table 7.3) showed that all replications have non significant differences, whereas highly significant differences were found among wheat genotypes for salinity tolerance. Table 7.4 indicates that most of the varieties/lines showed significant differences ($P \geq 0.05$), when subjected to salt affected field. According to Table 7.4, Uqab-2000 out-performed all other genotypes with respect to yield under salt stress. Whereas lines Gamdow-6, V-01171 and LAKATA-1 were ranked after Uqab-2000 as most salt tolerant varieties and these varieties also showed significant higher yield ($P \geq 0.05$) than the other lines including Inqilab-91 under salinity stress. Line FRET-1 was ranked 5th with line VEE/PJN//2*KAUZ at 6th position. Our check Inq-91 was ranked at 7th position with respect to performance in salt stress. Known salt tolerant varieties Pasban-90 and LU26'S were ranked at 17th and 19th positions respectively. These lines are recommended for wheat breeding for salt tolerance.

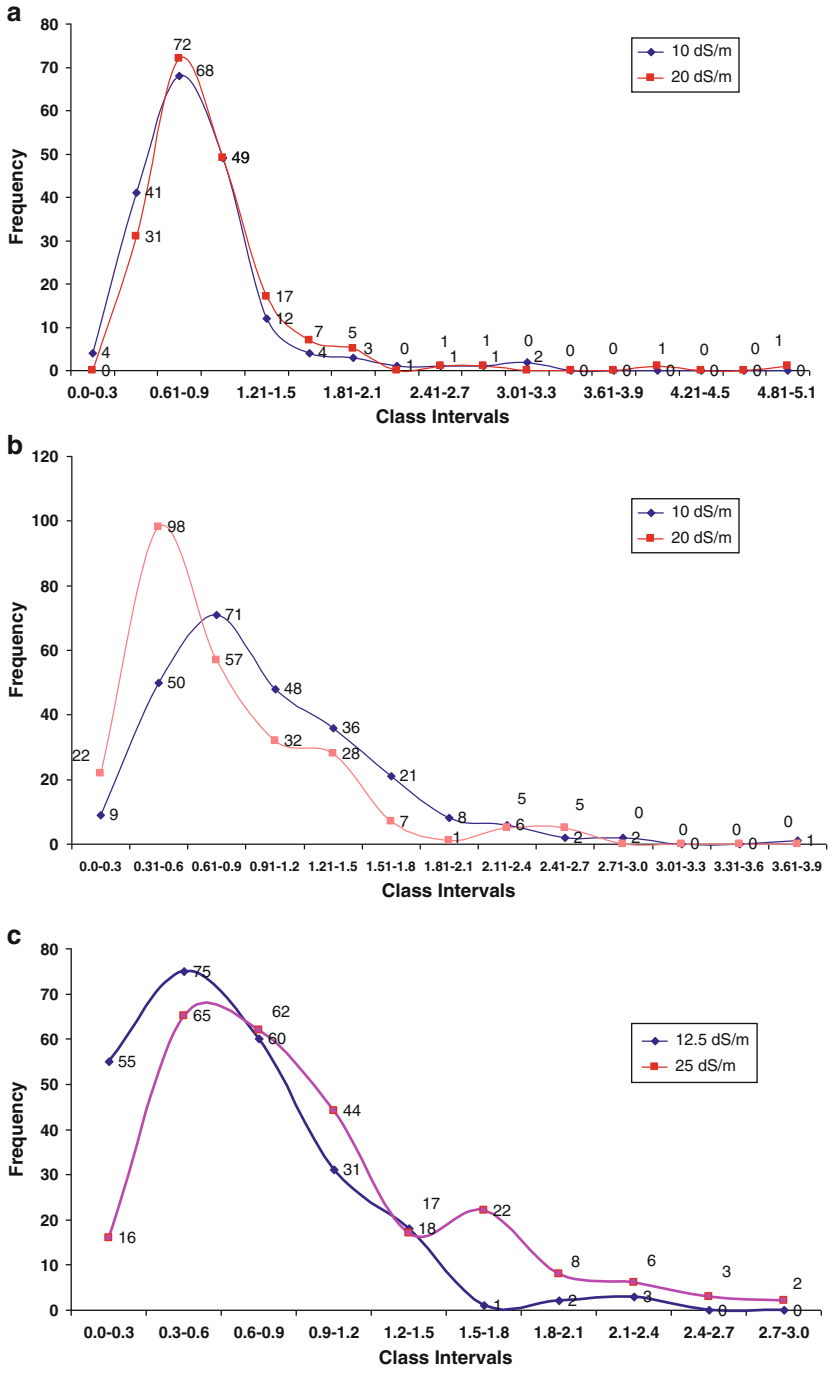

Fig. 7.4 (**a**) Frequency distribution of root weight at 10 and 20 dS m^{-1} (2003–04). (**b**) Frequency distribution of root weight at 10 and 20 dS m^{-1} (2004–05). (**c**) Frequency distribution of root weight at 12.5 and 25 dS m^{-1} (2005–06)

Table 7.2 Salt tolerance lines during 3 years study (2003–06)

*C.B. #	Pedigree/parentage
44	KANCHAN
	OBGD
53	LU 26
	BLS/KHUSHAL 69
72	PASBAN 90
	INIA 66/A. DISTT//INIA 66/3/GEN 81
172	GAMDOW-6
	CM79515-044Y-----
173	LAKTA-1
	ICW91-0233-------
232	PF 70402/ALD'S'//PAT 72/160//ALD'S'/3/PEW'S'
	CM 70474-(1-1)M-1013Y-19M-2Y-1B-0Y
311	NING 8319
414	CHIL/2*STAR=V-98124
	CM 112793-0TOY-22M-20Y-1M-3Y-0M
430	PB-96/87094//MH-97
	V01180
439	V-01171
	BAV92//SAP/MON
450	V-01078
	CHIL/2*STAR/4/BOW/CROW//BUC/PVN/3/…

*CB # according to 05–06 data book

Table 7.3 Analysis of variance for salt tolerance yield trial conducted at Pacca Anna

S.O.V	d.f	Sum of squares	Mean square	F-value	F-tab
Replication	5	1412649.52	282529.90	2.25^{ns}	0.056
Varieties	19	5606269.76	295066.83	2.35**	0.004
Error	95	11949830.41	125787.69		
Total	119	18968749.69			

**Significant at $p \leq 0.01$; ns non significant

3.2.1 Screening for Stem Rust

According to the results obtained (Table 7.5), of the salt tolerant lines screened, line NING 8319 showed desirable level of stem rust resistance like other stem rust resistant lines, Parula and Pavon.

3.2.2 Utilization in Breeding Program

Salt tolerant lines have been utilized in breeding program for the development of salt tolerant material. During 2007–08, 60 crosses have been developed using local salt tolerant lines PASBAN 90, LU 26'S' NING 8319, and some other good local varieties and stem rust resistant lines. During the crop year (2006–07), 40 crosses were developed.

Table 7.4 Field evaluation of the selected lines for salt tolerance at Pacca Anna

Line/variety	Parentage	Ave. yield
Uqab-02	CROW'S'/NAC//BOW'S'	1537.08 a
C.B 172	GAMDOW-6	1325.65 ab
V-01171	BAV 92//SAP/MON	1317.93 ab
C.B 173	LAKATA-1	1313.31 ab
C.B 147	FRET-1	1253.12 abc
C.B 159	VEE/PJN//2*KAUZ	1194.47 abcd
Inq-91		1049.41 bcde
V-98124	CHIL/2* STAR	1040.15 bcdef
V-01180	PB-96/87094//MH-97	1015.46 bcdef
V-01078	CHIL/2*STAR/4/BOW/CROW//BUC/PVN/3	1002.19 bcdef
C.B 226	ESDA 'S'	993.85 bcdef
C.B 330	TRAP # 1/YACO//BAV 92	955.27 bcdef
C.B 232	PF 70402/ALD'S'//PAT 72/160//ALD'S'/3/PEW'S'	939.84 bcdef
V-96015	INQ.91/TANGER	930.58 bcdef
V-02192	SHL88/87094//MH97	896.63 cdef
C.B.44	Kanchan	862.68 cdef
Pasban-90	INIA 66/A. DISTT//INIA 66/3/GEN 81	848.79 cdef
C.B 311	NING 8319	845.70 def
LU26'S	BLS/KHUSHAL 69	787.06 ef
AS-02	WD-97603	638.90 f

LSD value: 406.5 at $P < 0.05$

Table 7.5 Stem rust reactions of selected salt tolerant lines along with some other lines at Njoro, Kenya

C.B.#	Variety/line	2006	2007
44	KANCHAN	80S	–
172	GAMDOW	40S	–
173	LAKATA 1	50S	–
232	PF70402/ALD'S'S//PAD72/160//ALDS/3/PEW'S'	40S	–
311	NING 8319	20M	10MR
330	BAV92/SAP//MON	60S	–
430	V-01180	60S	–
414	V 98124	60S	–
–	BB#2/PT//CC/NIA/3/ALD'S'	60S	70S
450	SEHER 06 (V-01078)	60S	–
–	PARULA	30MS	1MSS
–	PAVON	30MSS	–
2	AUQAB	40S	
72	PASBAN90	60S	–
53	LU26'S	70S	70S

C.B. #=Crossing block entry number

4 Discussion

Hydroponic experiment revealed that salt stress reduced the shoot length and the effect becomes more pronounced with the increase in salt concentration. At 10 and 20 dS m^{-1}, normal population behavior was observed. At 20 and 25 dS m^{-1}, the population curve was abnormal which indicated that higher salt concentration reduced the shoot length. Root length also decreased under salt stress. Abnormal population curves indicated that root length was more drastically affected by salt stress as compared to shoot length. This type of behaviors in wheat has previously been reported by Iqbal et al. (1998) and Cicek and Cakirlar (2002).

Salinity stress reduced the shoot weight and shoot weight was severely decreased at higher salinity levels. Only 11 lines were found at the relative value range of 0.7–1.0. This decrease in shoot weight was due to decreased water uptake, reduced photosynthesis and toxicity of sodium and chloride in the shoot cell (Ali et al. 2005), whereas salinity stress increased the root weight of many genotypes as more lines had shown relative ratio near to unity at higher salinity level as compared to lower salinity level. Munns and Termatt (1986); Marcum and Murdoch (1992) also reported increased root weight in plants under salinity stress. The lines that showed relative ratios near to unity for all the characters studied were subjected to field testing in salt affected field along with some other high yielding varieties/lines.

In the field trial, non significant replication mean squares indicated homogenous field conditions. Significant varietal differences for yield performance under saline conditions indicated the presence of genetic variability for grain yield in salt affected fields. Uqab-2000 was the highest yielding line in field conditions, whereas it was not among the best performing lines in aquaculture. Similarly some lines, like KANCHAN, which showed tolerance in lab conditions, were not good as for yield performance in saline field conditions is concerned. Differences in varietal/ lines behavior in the field and aquaculture conditions have been reported in literature (Reynolds and Borlauge 2006). However, lines like GAMDOW-6, V-01171, LAKATA-1, FRET-1 and VEE/PJN//2*KAUZ proved tolerance in laboratory conditions and also produced higher grain yield in saline fields. These lines were sent to Kenya for screening against stem rust. One line (NING 8319) showed good resistance level comparable to known stem rust resistance lines like PARULA and PAVON. These lines were used in the specific breeding programs and the crosses will be further evaluated following shuttle breeding approach. The F2 generation will be sown at Kaghan in summer and F3 in a salt affected field at Faisalabad in normal season. Again F4 at Kaghan and F5 at salt affected field in Faisalabad. The material will be enhanced following modified bulk method. From F5 single heads will be taken and the material will be further evaluated in the normal field in F6 and in the next generation in normal as well as salt affected fields to see their adoptability to normal and saline conditions.

5 Conclusion

Rapid and specific screening methods were adopted for screening wheat germplasm for 3 years and it was concluded that in general terms salinity reduces the biomass of wheat plant but when it reaches a particular level wheat plant increases its root weight to better cope with high salt concentration in the root zone and also to uptake water and nutrients more efficiently. This study helped in selecting 11 salt tolerant varieties/lines on the basis of better and consistent performance for 3 years. Field studies also revealed the superiority of some of these lines for grain yield potential over Inqulab-91, the most popular variety of Pakistan. These genotypes are valuable asset for the wheat breeders and can be used in hybridization program or as commercial cultivars for salt affected areas of Pakistan. Screening for stem rust revealed that NING 8319, PARULA and PAVON can be used as parents for stem rust resistance. The crosses developed during the previous 2 years will be utilized for the development of salt tolerant and stem rust resistant material.

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Chapter 8
The Potential of Breeding Okra (*Abelmoschus esculentus* L.) for Water Stress Tolerance

Abdul Naveed, Asif Ali Khan, and Saeed Rauf

Contents

Abstract Okra plant grows well but produces a few pods under water limited conditions. A linear relationship between okra production and the amount of water supplied is known to exist. Review of previous literature has shown that drought cause damage to okra plant of varying degree during its ontogeny, the reproductive phase being the most prone to the detrimental effects of drought. More specifically, the highest effect on yield was recorded when drought was found to occur during flowering and pod formation. Existence of genetic variability among genotypes is

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the most important component for the success of a breeding programme for increased drought resistance. Previous reports show the existence of a substantial amount of variability among okra genotypes. However, most of the traits related to drought tolerance are quantitative in nature. Therefore, estimation of type and amount of total genetic variability associated with the target traits is equally important. Determination of the type of genetic variability may help in the formulation of comprehensive breeding programme regarding the further improvement of the target trait. It is also important to find out the target trait that is related to drought tolerance and also shows good relationship with yield. Many traits are measured with complex and time-consuming techniques that are unsuitable for screening large numbers of progeny in breeding programmes. As a result, breeders tend to measure them in small populations. In addition, the stage at which trait allows the evaluation of a plant would also predict its suitability. A trait, which is evaluated earlier in the plant growth and shows freedom of its interaction with growth stages, will speed up the breeding programme. Breeding efforts at molecular level are usually intense for finding drought related molecular markers which helped us in the screening of large population without actually been exposed to drought stress. Furthermore, development in the reverse genetics has led us to isolation of novel drought tolerant genes synthesized by cDNA obtained form mRNA expressed in drought stress and non stress plants. An integrated approach has been presented here for the development of drought tolerance in okra. The increasing strengths in genomics, proteomics and molecular marker systems should be made use of to facilitate breeding of okra genotypes for water stress tolerance.

Keywords Drought tolerance • Genetic variability • Molecular markers • Okra • Susceptibility index

1 Introduction

Okra (*Abelmoschus esculentus* L.) is the only vegetable crop of significance in the Malvaceae family and it is very popular in the Indo-Pak subcontinent. In India, it ranks number one in its consumption but its original home is Ethiopia and Sudan. It is one of the oldest cultivated crops and presently grown in many countries and is widely distributed from Africa to Asia, southern Europe and America (Oyenuga 1969; Hamon 1991; Ariyo 1993; Oyelade et al. 2003). It is a tropical to subtropical crop and is sensitive to frost, low temperature, waterlogging and drought conditions, and the cultivars from different countries have certain adapted distinguishing characteristics specific to the country to which they belong (Siemonsma 1982).

It is an oligo purpose crop, but it is usually consumed for its green tender fruits as a vegetable in a variety of ways. These fruits are rich in vitamins, calcium, potassium and other mineral matters (Camciuc et al. 1981). The mature okra seed is a good source of oil and protein (Karakoltsidis and Constantinidesm 1975; Martin and Ruberte 1979; Oyelade et al. 2003) and has been known to have superior nutritional

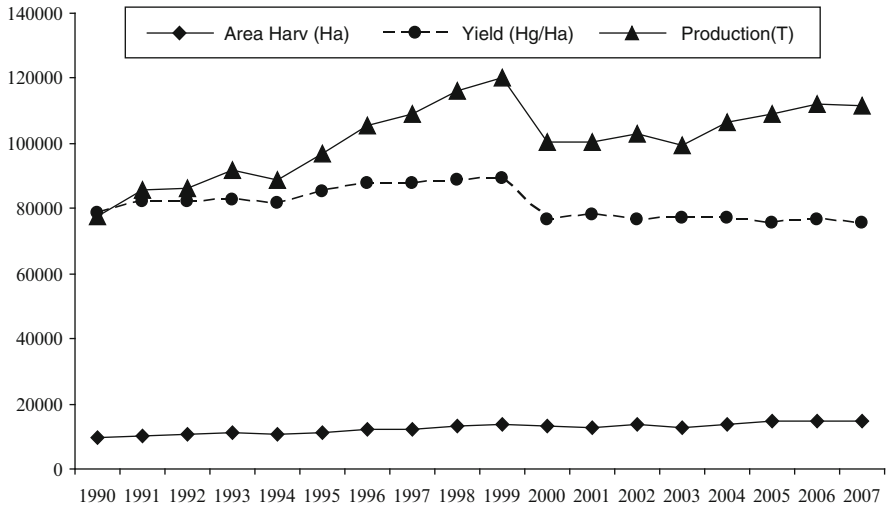

Fig. 8.1 Area (ha), production (Mt) and yield (kg ha^{-1}) of Okra in Pakistan (from 1990 to 2007)

quality. Okra seed oil is rich in unsaturated fatty acids such as linoleic acid (Savello et al. 1980), which is essential for human nutrition. Its mature fruit and stems contain crude fibre, which is used in the paper industry.

In Pakistan, okra is a favourite vegetable cuisine, which resulted in a progressive increase in the area of okra but the last 5-year statistics show that there was a decreasing trend in yield per unit area and production as compared to the last 10 years (Fig. 8.1). A number of factors are considered to cause reduction in yield and production but it is argued that the limiting availability of irrigational water has been a key factor for its reduction.

The rainfall data of 1995–2007 presented in Fig. 8.2 indicates that during the years 1999–2005, Pakistan had been under severe drought. The percentage of rainfall decreased to 66% in Sindh, 52% in Balochistan, 20% in NWFP and 13% in Punjab. In addition, the underground water and water storage reservoirs are rapidly depleting due to a nominal recharging by the rainfalls and continuous silting in the dams. As a result national economy suffered a loss of about $ 2 billion due to water shortage (MinFAL 2006).

In order to face the calamity it is necessary to devise strategies that may improve water use efficiency of crops. There are two strategies to manage drought stressed environment. The first strategy includes the drought stress management by means of agronomic practices i.e. improved soil and water conservation practices associated with the tillage system, weed control, fertility management, optimized plant population, improved forage/ livestock/grains integration and rotation, avoidance of mono-cropping and the diversification of farming (Blum 2005).

The second strategy is to improve the crop drought tolerance. It is the most efficient, cheapest and long-term strategy to manage drought stress (Ludlow 1989) and once it is introduced in a population it will be permanent source to endure stress.

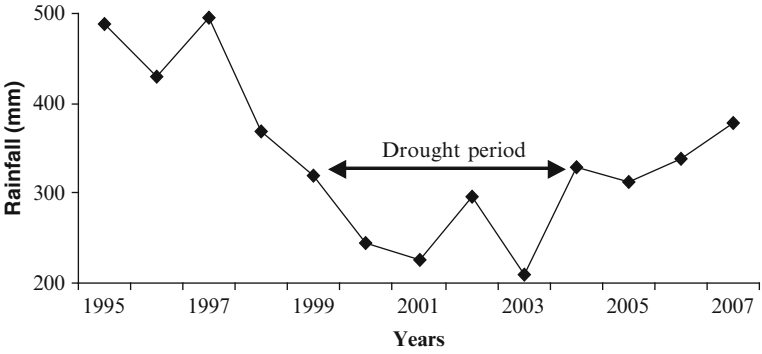

Fig. 8.2 Average annual rainfall (mm) in Pakistan from year (1995–2007)

The progress in plant breeding for drought tolerance has been limited by at least two major problems. First, drought resistance is a complex attribute of a crop community (Blum 1979) and it is not clear how specific mechanisms at the tissue or organ level are integrated in determining yield under water stress (Hsiao and Bradford 1983). The genetics of drought tolerance is complex and not adequately understood (Osmanzai et al. 1987). The complexity arises because of the number of morphological and biochemical systems within a plant, which are related to drought tolerance. Compensation of one system for another and interaction with environment make it even more difficult to correlate physiological and morphological traits with yield under drought conditions.

The importance of okra vegetable in respect of its nutritional value and area under cultivation in Pakistan, and alarming condition of occurrence of drought demands a comprehensive review of the genetic, molecular, physiological and breeding work to facilitate the formulation of a strategy for the development of drought tolerant cultivars in okra.

2 Drought and Its Types

Drought is a condition where soil moisture contents are too low or tightly attracted to soil particles (due to lower osmotic potential) and thus a plant cannot absorb water or quantity of the absorbed water cannot meet its transpiration demands. A plant faces different types of drought stress depending upon its growth stage (Seghatoleslami et al. 2007; Sinaki et al. 2007; Kron et al. 2008). The stress that occurs at seedling stage or during development phase may be called as early drought. Such type of drought usually reduces the crop stand and as a result it damages yield due to lower than optimum plant population (Ashraf et al. 2005). However, farmers usually respond to this drought by replanting their crops (Muasya and Diallo 2001). This type of stress has been proved lethal for early maturing varieties (Muasya and Diallo 2001), which seldom recover from the stress while late maturing varieties have enough time to recover.

Drought occurring during the vegetative growth period is called as vegetative phase drought. This type of drought affects plant assimilatory organs, which usually decrease in number and size, resulting in lower photosynthate production (Kaiser 1987; Chaves 1991; Larcher 1995; Chaves et al. 2002). Consequently the yield decreases due to less amount of assimilates available to the developing pods.

The third type of stress develops during bud formation, flowering and grain filling period. It reduces yield due to abortion of ovule, embryo and sterility of pollen. Review of previous literature shows that this type of drought has the highest detrimental effects on pod yield (Ahmad et al. 2003; Sawadogo et al. 2006; Seghatoleslami et al. 2007). However, the highest effect on yield was recorded when drought was found to occur during flowering and pod formation. Mbagwu and Adesipe (1987) found the greatest reduction (70%) in yield of okra when stress was imposed at flowering and pod filling stage. Ahmad et al. (2003) studied three treatments of drought, (water stress at pentafoliate and bud formation, flowering and pod formation, and seeding and maturity). They recorded maximum reduction in yield when water stress (three consecutive irrigations withheld) was imposed at the flowering and pod formation stages. Sawadogo et al. (2006) observed two types of flowering phases of the okra plant i.e. the period which intervenes during the accelerated growth phase of the main stem (type 1) and the one which starts once the growth of main stem begins to decline (type 2). It was also observed that water deficit during the budding phase causes earlier flowering of type 2 plants and leads to delayed flowering of the type 1 plant.

3 Water Availability and Okra Plant

Although okra is a drought tolerant plant, the availability of water has a significant impact on okra production. It has been found that there was a linear relationship between okra production and the amount of water supplied (Batra et al. 2000; Ahmad et al. 2003). In each season, okra requires supplemental water of 24-acre inch. In okra different studies were carried out on the basis of irrigation regime using ID/CPE ratio where ID is irrigation depth, and CPE is cumulative evaporation from a USWB Class-A pan (Singh and Singh 2005). Batra et al. (2000) used three levels of irrigation based on ID/CPE ratio of 0.6, 0.9 and 1.2 and concluded that irrigation of 1.2 and 0.9 ID/CPE resulted in higher DM production than 0.6 ID/CPE. Verma and Batra (2001) showed that the highest fruit yield could be ensured with moderate intensity of irrigation (ID/CPE 0.9).

4 Plant Adaptation to Drought

Okra is considered to grow well under drought conditions, although plant has shown reduction in yield under drought stress (Ahmad et al. 2003). The drought tolerance of a species may be considered due to its better crop stand rather than yield under drought conditions. However, for sustainable agriculture, better crop stand alone

means nothing to farmers until it produces an acceptable yield under drought stress. Therefore drought tolerance may be defined on the basis of yield rather than plant survivability.

Plants have four adaptive mechanisms for drought (Levitt 1972; Mitra 2001) that must be reviewed and subsequently incorporated according to the species intensity and timing of drought. First of them is drought escape. This type of drought tolerance mechanism is achieved by reducing the growth cycle of the crop. Moisture stress occurs at the end of growth cycle and plants usually complete the growth cycle well before the onset of moisture stress (Turner 1986). Early maturing varieties have been equipped with this adaptive mechanism. On the other hand, stress may coincide with the flowering or the grain filling period in case of a late maturing variety. As a result late maturing variety may show reduction in yield. However, this type of mechanism has not been found successful and popular among the breeders due to its negative correlation with yield. The second type of drought resistance mechanism is drought avoidance. In this type a plant does not show the symptoms of drought and usually maintains higher degree of moisture contents through out the crop growth cycle. Several types of traits have been found to be involved in this type of mechanism such as lower transpiration rate, small leaf area, leaf hairs, high root-to-shoot ratio which help the plant to use water slowly and efficiently (Passioura 1976; Turner 1986). Such types of plants are called as water savers. This type mechanism has not been found successful due to its negative correlation with yield. The third mechanism is drought tolerance. This mechanism may be defined as the ability of a genotype to produce higher yield when compared with other genotypes and treated with similar intensity of moisture stress. This type of mechanism is based on the traits such as drought susceptibility index, root length, relative water contents, turgor pressure, and osmotic adjustment that help the plant to absorb more water and from the deeper soil profiles. This type of mechanism has been proved most successful due to its positive correlation with the traits associated with yield. The fourth type is drought recovery. This type of mechanism allows the plant to recover from the deleterious effects of drought when stress occurs during plant development (Kholodova et al. 2007). Different types of traits such as stay green and leaf retention allow the plant to recover after drought (Oosterom et al. 1999; Lenis et al. 2006). Leaf retention and stay green plant traits are indicators of normal functioning of the leaves thus helping the plants to recover from drought by producing more assimilates.

5 Breeding for Drought Tolerance

The genetic improvement of crop plants for drought tolerance, through breeding and selection, involves a favourable combination of plant traits. The success of a drought tolerance breeding programme depends on the following four important features:

1. Presence of sizeable genetic variability for target traits
2. Type of genetic variability associated with the selected traits

3. Relationship of the target traits with yield
4. Cost, stage and speed of measurement
5. Genetic variability for target traits
6. Genetic variability

Success of a drought tolerance breeding programme depends on the existence of genetic variability for the target traits. Genetic variability arises as a result of mutation, inter-variety or inter-specific genetic recombination. These variations tended to accumulate in the germplasm that has been under selection pressure by the target environment. Therefore, local germplasm evolved under target environments would provide the desired variability for selection. However, target traits are to be integrated in superior high yielding cultivars, since land races and wild species have some undesirable features i.e. introgression of target traits may result in deterioration of yield and quality due to linkage drag phenomenon. The basic approach for development of drought tolerant genotypes is to select locally adapted germplasm containing genetic variability for high yield potential and drought adaptive traits (Beck et al. 1990; Vasal et al. 1997). Furthermore, the unpredictable nature of drought dictates that improved genotypes must perform well in both favourable and stressed environments. Thus, combination of stressed and unstressed environments may be used in the selection of genotypes for drought stressed areas.

Existence of genetic variability for drought resistance among the genotype is the most important factor for the success of drought resistance breeding programme. Previous reports showed the existence of a substantial amount of variability among okra genotypes in dry-land for harvest index. This variation in harvest index was due to differences in leaf area, biomass accumulation, osmotic adjustment, photosynthetic efficiency and distribution and in root water extraction (Fereres et al. 1986; Gimenez and Fereres 1986).

In okra variability among the cultivated genotypes for different traits has been reported by Gill et al. (1997). They found considerable variation with respect to vegetative, floral and fruit characters and reaction to diseases and pests. A few distinguishing characters were identified in each variety. Chehda and Fatokun (1991) evaluated the exotic and locally collected germplasm for genetic variability. Significant differences were found between exotic and local material. Three agronomic types (A, B and C) were identified based on flowering date, plant size and fruit yield. Local germplasm belonging to types B (Soudanian or *Abelmoschus esculentus*) and C (Guinean or *A. caillei*) were crossed with a view to transfer drought resistance from type C to B by backcrossing.

Ahmad et al. (2003) screened six okra varieties and showed significant genetic variability in yield and its components and also revealed drought tolerance on the basis of yield in a few varieties. Similarly, Sawadogo et al. (2006) conducted a greenhouse experiment to assess tolerance to water deficit among six okra ecotypes evolved under different conditions. Among these ecotypes, three were chosen for using in okra breeding programmes for tolerance to water deficit.

6 Genotype × Environment Interactions

Progress in developing high-yielding, drought-tolerant cultivars by conventional breeding has been slow, because of difficulties in defining the target environment and complex interactions of drought tolerance with environments (Cooper et al. 1999; Wade et al. 1999; Pantuwan et al. 2002). Breeders appears to have different views that whether selection for drought yield should be carried out under optimum conditions or under targeted drought environment. However, a large number of breeders have shown that drought tolerant genotypes should be selected under a target drought environment (Ceccarelli 1987; Din et al. 1992; Chapman et al. 1997). Ceccarelli (1987) emphasized that selection under stress conditions is expected to be more efficient than selection under favourable conditions when dry areas are the target environment (Ceccarelli 1987). Similarly, Chapman et al. (1997) and Din et al. (1992) concluded that the yield gains under drought would have been unlikely to occur if selection had been done only in well-watered environments.

7 Types of Genetic Variability

Since most of the traits related to drought tolerance are quantitative in nature, therefore, estimation of type and amount of genetic variability associated with the target traits is equally important. Determination of the type of genetic variability may help us in the formulation of comprehensive breeding programme regarding further improvement of the target trait. Additive or non-additive type of genetic variability has been found to be associated with any trait. Additive type of genetic variability arises as a result of cumulative effect of minor alleles while non-additive type of genetic variability arises as a result of dominance and epistasis. Dominance arises as a result of intragenic interaction while epistasis due to intergenic interaction. In addition, these intergenic or intragenic interactions have also been found to be affected by the external stimuli such as drought (Rauf et al. 2007, 2008; Khan et al. 2007; Rauf and Sadaqat 2008a, b).

Due to their quantitative nature, drought related traits couldn't be studied in simpler ways. Specialized biometrical techniques are required to work out the type of genetic variability associated with the traits. These biometrical techniques are dependent on different mating designs such as diallel, line × tester, North Carolina design and generation mean analysis for the estimation of type of genetic variability. Among these mating designs, generation mean analysis has been the most powerful biometrical analysis since it gives additional information about the epistatic interactions. It is based on the mean of five generations i.e. parental, F_1, F_2, BC_1 and BC_2. Information derived from these analyses can be further utilized for the formulation of an effective breeding strategy. A population with preponderance of additive genetic variability can be easily managed and selected through progeny rows or pedigree method. The additive genetic variability has also been used for the

estimation of narrow sense heritability (ratio of additive genetic variability to the total genetic variability) and genetic advance. The heritability and genetic advance provide further information on the proportion of genetic variability, which can actually be selected and how much improvement can be brought through selection. Panda and Singh (1997) obtained information on genotypic coefficients of variation, heritability and genetic advance from data of pod yield and seven other traits in 40 F_1s progeny of okra (*Abelmoschus esculentus*) grown at Varanasi under two sowing dates. Number of branches, number of pods and total pod yield per plant had higher genotypic and phenotypic coefficients of variation in all optimum environments. All the characters under study except days to first flower appearance and girth of pod were highly heritable. High heritability coupled with high genetic advance was observed for plant height, number of pods and total pod yield per plant, indicating that these traits are suitable for improvement through selection.

A population high in non-additive genetic variability is difficult to manage and further improvement in trait will be slow. Recurrent selection has been recommended for the population high in non-additive genetic variability in order to break the intergenic or intragenic interaction.

In okra, previous literature has not been able to show a single report on inheritance characteristics of drought related traits. Most of the work was available under optimum conditions. Deo et al. (1997) developed different generations to estimate type of gene action for five quantitative characters, namely number of pods/plant, length of pod, girth of pod, number of seeds/pod and pod yield/plant. They concluded that all type of genetic variability i.e. additive, dominance and epistasis was significant for all traits. However, few reports are available for yield and its related traits, which showed the prevalence of additive type of gene action under drought stress (Ahmad et al. 2004). Ahmad et al. (2004) studied the type of genetic variability in morphological traits through 6×6 diallel technique under normal and drought conditions. They reported additive gene action for fresh fruit yield per plant, days to first flower and seed yield per plant under drought conditions, which suggested selection to be effective in early segregating generations for these traits.

Reports on the inheritance of drought related traits are available in other crop species. Dhanda and Sethi (1998) conducted an experiment on the F1 generation from a half-diallel set of crosses involving two drought tolerant, two moderately tolerant and two sensitive varieties. Additive gene action, in general, played a major role in determining the inheritance of relative water contents. General combining ability (GCA) was the main source of genetic variation among crosses, while specific combining ability (SCA) was negligible. Strong phenotypic correlations existed between *per se* performance and GCA effects in majority of cases, but heterosis was not found important. Genotype-environment interactions and/or differential gene expression appeared to account for different results found between environments and growth stages, respectively. Similarly, Rebetzke et al. (2003) estimated leaf stomatal conductance prior to anthesis on irrigated plants representing different generations of crosses between the low conductance parent and three high conductance varieties. They obtained significant genetic differences between generation means for conductance measured in different crosses and on different days. Gene

action was complex with both additive and non-additive (dominance and additive-based epistasis) genetic effects important for expression of leaf conductance. Family-mean and heritability varied, depending on cross and time of sampling. It was suggested that breeders selecting for altered leaf conductance maximise genetic gain by delaying screening of populations until later in the day, and repeat measurements across a minimum of 2 days.

8 Ideal Trait for Evaluation of Drought Tolerance

It is important to find out the target trait that is related to drought tolerance and also shows good relationship with yield. Any traits that improve the drought tolerance by improving its yield may be considered ideal. It may be a physiological, morphological or biochemical trait. Previously, plant breeders have focused on the traits that were related to plant survival, i.e. lower leaf area, lower transpiration, etc. As a result plant breeders continued to evolve varieties that had good crop stand but poor yield in the field (Rauf and Sadaqat 2008a) and these varieties ultimately turned towards failure. Richard (1996) showed that large number of criteria that have been proposed to increase drought resistance of our crops had little impact on improving crop yields in dry environments. They concluded that there were several reasons for this lack of success. Some of these were: (i) criteria proposed have been related more to survival mechanisms under drought than to productivity, (ii) criteria are inappropriate to the target environment, and (iii) criteria are temporal and are therefore likely to have minimal impact on growth and yield over the entire life cycle. Another important reason is that the breeders have never been convinced that the proposed criteria will be successful, as they are too difficult to measure. Therefore it is important to find out the ideal traits for selection. Some of the traits are discussed below:

8.1 Pod Yield

Okra plant is usually grown for its green tender pods that are harvested over multiple times. Therefore, fresh pod yield may be considered as an ideal candidate for selection. However, it has been found in a number of studies that selection through yield and yield components under drought conditions was slow. This may be due to very low heritability of the yield and yield components under drought stress (Quisenberry et al. 1980; Blum 1988; Ntare and Williams 1998; Collaku 1994; Szilagyi 2003). The decrease in the heritability estimates was due to increase in error variance under stress (Hulmel et al. 2005). It was concluded that direct selection for yield and yield components was difficult. Annicchiarico and Pecetti (1998) showed that selection indices based on heading displacement and kernels per spike, was on the average, 20% and 11% more efficient than yield-based selection. Furthermore, genotypes

selected under drought conditions on the basis of yield could not take advantage of non-stress year and did not produce any higher yield. Since drought stress is an unpredictable feature, therefore ideal genotypes should produce high pod yield under non-stress conditions while comparable yield under drought stress condition. However, few reports have shown that the most effective selection criterion, among various morphological, physiological, phenological, yield, and yield related traits, for identifying drought resistant genotypes was mean seed yield under drought stress and non stress environments (White et al. 1994; Abebe et al. 1998; Ramırez-Vallejo and Kelly 1998).

8.2 Drought Susceptibility Index

Drought susceptibility index is calculated on the basis of differences between pod yield under non-stress conditions to the stress conditions. Drought susceptibility index can be calculated as under adopting Fischer and Maurer (1978).

$$S = \frac{1 - Y/Y_p}{1 - X_d/X_p}$$

Where Y is the green pod yield per plant of a given genotype under drought, Y_p is the green yield per plant of the same genotype under irrigation, X_d is the mean green yield of all genotypes within group under drought, and Xp is the green yield per plant of all genotypes within group under irrigation. A genotype with higher susceptibility index has been considered as susceptible genotype while genotype with low index has been considered as tolerant (Fischer and Maurer 1978). Drought susceptibility index is calculated on the basis of pod yield thus it reflects yield at whole plant level. Different studies have shown significant relationship of drought susceptibility index with pod yield, thus genotype showing lower susceptibility index may also yield better under drought stress conditions (Fereres et al. 1986; Rauf and Sadaqat 2007a, b). However, in these studies drought susceptibility index was unable to show relationship with yield under non-stress conditions. Thus, selection for lower drought susceptibility index may improve drought tolerance without affecting yield under non-stress conditions. As a result, the genotype may be able to take benefit of non stress years and lower drought susceptibility index may act as a buffer for yield reduction during stress years.

8.3 Root Length

Root length is an important plant trait for the evaluation of genotypes under drought stress, since roots are in direct contact with soil moisture contents and first to be affected by the drought and other abiotic stresses aluminium toxicity (Khan and McNeilly 1998) and soil salinity (Khan et al. 2003). A longer downwards root

growth may enable the plant to extract water from deeper profile, while lateral root growth enable it to extract water from its surroundings. However, measuring root length is a difficult and laborious job. Different studies have shown a significant relationship of different traits with root length (Rauf and Sadaqat 2007a, b, 2008a). For example, Rauf and Sadaqat (2008a, b) evaluated sunflower genotypes under drought stress and showed a significant relationship of osmotic adjustment with root length. Therefore, increased osmotic adjustment may be an index of higher root length. Similarly, a positive correlation between root and shoot has been obtained in number of studies. Thus a higher shoot length will be an indication of higher root length. Furthermore root length itself is a morphological trait that depends on the pattern of plant dry matter partitioning. Higher allocation of dry matter to the roots depends upon the level of different of plant growth regulators such as absicisic acid (ABA). It has been found that under drought stress level of ABA sharply increases which triggers the accumulation of dry matter to the roots (Quarrie 1981).

8.4 Osmotic Adjustment

Osmotic adjustment (OA) has shown its promise in plant adaptation to drought stress. Many reports have shown a significant relationship of AO with yield. Osmotic adjustment is the ability of a genotype to produce certain osmolytes such as sugars, proline, K^+, Ca^{2+} etc. that are essentially non-toxic and have the ability to reduce the osmotic potential of the plant so that the plant can absorb more water from the soil. Due to higher osmotic adjustment ability, the plants were able to show more root growth, increased translocation of stem reserves to the reproductive parts, thus showing higher harvest index and yield under drought (Ludlow et al. 1990; Moinuddin 2004; Silva et al. 2006). However, a few studies have also shown negative effects of osmotic adjustment on yield and some others have shown no significant effect on yield (Turner et al. 2007). Therefore, beneficial effects of osmotic adjustment have been found to be dependent on the type of crop species or genotype, and specific and environment. Turner et al. (2007) evaluated yield and osmotic adjustment in the F_8 progeny under drought in Australia and India. They found that differences in osmotic adjustment among lines and parents varied from year to year and did not consistently benefit yield when measured in the field under terminal drought. In Australia, differences in osmotic adjustment were not associated with any yield benefit in any year, while in India early flowering resulted in higher yields at three of the four sites, and osmotic adjustment had an inconsistent effect on seed yields. In okra, different studies have shown significant genetic variability among the okra cultivars for osmotic adjustment (Wullschleger and Oosterhuis 1991; Ashraf et al. 2002). Ashraf et al. (2002) studied the osmotic adjustment ability of two cultivars i.e. Sabzpari and Chinese-red, after subjecting them to drought for 30 days. They found significant differences in cultivars for their osmotic adjustment ability. The leaf osmotic adjustment was much lower in Chinese-red than that in Sabzpari. However, overall growth of the two okra cultivars was negatively correlated with the osmotic adjustment.

Different methods have been devised to determine the osmotic adjustment ability of plants. However, simplest method was based on the difference of osmotic potential under controlled conditions to the drought condition at full turgor or after irrigating the field. Babu et al. (1999) compared four different methods for measuring osmotic adjustment in plants. The four methods were: (i) derivation of osmotic adjustment from regressions of leaf relative water content (RWC) on leaf osmotic potential (OP); (ii) estimation of osmotic adjustment from osmotic potential of stressed plants calculated to rehydrated state; (iii) estimation of osmotic adjustment from osmotic potential of stressed plants that have been rehydrated; and (iv) estimation (from data used in Method 1) of osmotic adjustment capacity by the sustained RWC at given osmotic potential of −3.5 MPa. They hypothesized that method 1 was a priori considered as the best estimate. However, they concluded that methods 2 and 3 were less demanding in terms of labour and plant materials than Methods 1 and 4. The results support the use of method 3 (the "rehydration method") as a faster and an economical replacement of method 1.

8.5 *Leaf Relative Water Contents*

Leaf relative water content is a simple technique that can easily determine the water status of the plant. On the basis of relative water contents, plants can easily be discriminated into tolerant or susceptible types. Bhatt and Rao (2005) estimated relative water contents in the irrigated plants of okra, the relative water content (RWC) varied from 72% to 82%, while in the stressed plants, it decreased to 66–75%. The highest RWC was recorded in cv. BO-1 during the stress. Relative water contents have also shown significant relationship with yield. Tahara et al. (1990) studied genetic relationship between grain yield and relative water contents in a random group of plants, selected from an F2 population having the pedigree, TAM W-101/Sturdy. A positive relationship was observed between grain yield and RWC measured during anthesis and mid-grain fill, as the high-yield selections maintained a significantly higher RWC than the low-yield selections. Grain yield and RWC were also positively associated among random selections segregating for both traits. In addition, different studies have suggested that relative water contents can be utilized as a physiological marker of other traits. Sawadogo et al. (2006) also concluded that the best variables for discriminating okra ecotypes during water deficit were the relative water content, and the length and the number of fruits.

8.6 *Stomatal Conductance*

Selection for stomatal conductance has the potential to increase the yield under both irrigated and drought conditions (Rebetzke et al. 2003; Khan et al. 2007). Under irrigated conditions, higher stomatal conductance has been found to be associated

with the maintenance of leaf or canopy temperature (Lu et al. 1998) while higher stomatal conductance under water deficit conditions was an indication of some drought tolerance mechanisms that allow the plant to extract more water and transpire (Sashidhar et al. 2000). Stomatal conductance has shown a positive relationship with yield and has been used to evolve high yielding genotypes (Lu et al. 1998). Lu et al. (1998) has shown a significant improvement in the yield of cotton when plants were selected for higher yield. Two types of plant drought tolerance mechanism were observed on the basis of stomatal conductance (Sashidhar et al. 2000). The first mechanism allows lower stomatal conductance with warmer canopies. The lower stomatal conductance inhibits water losses, thus helping the plants to cope with the drought in better way. A term 'water saviours' has been given to these types of plants. However, lower stomatal conductance inhibits the CO_2 fixation, resulting in lower productivity (Jones and Corlett 1992; Cornic 2000; Chaves et al. 2002). The plants showing higher stomatal conductance with cooler bodies form another group (Sashidhar et al. 2000). Higher stomatal conductance under drought stress depends on different mechanisms such as root elongation that allows extraction of more water from the soil. This type of mechanism has shown a significant relationship with yield under stress conditions. Sashidhar et al (2000) evaluated the advantages and disadvantages of both mechanisms. They found that first mechanism resulted in the termination of growth while second mechanism has two disadvantages (a) high demand of energy for root growth (b) danger for the moisture contents to be exhausted quickly. It was concluded that presence of both mechanisms in low intensity i.e. drop in the stomatal conductance by at least 25–30% and uptake of water by lower roots in contact with moist soil help the plants to cope with drought more successfully.

8.7 Cost, Speed and Stage of Measurement

Many traits are measured with complex, time-consuming techniques that are unsuitable for screening large numbers of progeny in breeding programmes (Khan et al. 2001). As a result, breeders tend to measure them in small populations. In addition, the stage at which trait allows the evaluation of plants would also predict its suitability. A trait, which is evaluated earlier in the plant growth and shows freedom of its interaction with growth stages, will speed up the breeding programme.

Although morphological traits such as yield contributing traits and reduced plant height are evenly important. However, these traits may be only measured at plant maturity. On the other hand, some of the physiological traits i.e. relative water contents have the potential to evaluate large number of populations during earlier plant growth. While other more complicated and time-consuming techniques such as osmotic adjustment, root length and dry matter partitioning are only useful for screening a small number of genotypes for use as parents.

9 Conclusions and Future Prospects

Stress tolerance in general and drought tolerance in particular is a complex phenomenon, which is affected by many factors and characters and varies in different plant species. Further complexity in breeding crop plants against drought stress arises because of the quantitative nature of the multiple contributing traits. There are some common and specific responses across stresses and plant species (Khan et al. 2000). A common response to stresses is thought to be the relaxation of epigenetic regulation, leading to activation of suppressed sequences and secondary effects as regulatory systems attempt to re-establish genomic order (Madlung and Comai 2004).

Unlike other abiotic stresses, drought is a relatively temporary condition in terms of time, space and plant growth stage. During some years it occurs severely but may be another year there is no drought on account of rainfall in a particular area or in the water catchment areas of a river supporting the irrigating system of the area. Therefore, drought tolerance of a species may be enhanced by selecting traits related to drought tolerance and essentially positively correlated with yield. Independence of correlation with yield under non-stress conditions would help to take advantage of non-stress years. However, the trait(s) under selection should be easily scoreable to handle large segregating population at early growth phase. The molecular markers provide a high throughput system to identify and keep track of the potential trait(s) in large segregating populations. The review has considered the role of various physiological and morphological traits as selection criteria under drought stress. Among the traits leaf relative water contents and osmotic adjustment appeared to have potential to be used as selection criteria for breeding okra genotypes for low moisture conditions. The presence of additive genetic variability and high narrow sense heritability for these traits would increase their responsiveness to the selection. Furthermore, the increasing strengths in genomics, proteomics and molecular marker systems should be integrated in the drought tolerance breeding programmes.

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Part II
Biotechnology, Molecular Biology and Genetics

Chapter 9
Biotechnology as an Aid for Crop Improvement to Overcome Food Shortage

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Abstract World's population has crossed 6.5 billion with majority of human beings living in developing or under developing countries. Clearly, food security in such countries will be a primary concern over the next few decades. However, options for increased food production to meet this population pressure are limited because most arable land is already under cultivation, and in many areas land use cannot be further intensified without a risk to the long-term productivity. Agricultural land use has been especially intense in recent years because of rapid urbanization and increasing environmental pollution. The ultimate need is to use newer technologies which could help us to curb this food insecurity. Biotechnology is globally recognized as a rapidly emerging, complex and far reaching new technology. It has revolutionized all the fields of life. Recent discoveries and technical innovations in the field of genomics and biotechnology are revealing the full complement of genes in crops, the ability to define genetic variation and use DNA markers to follow chromosome segments with known functions through breeding programmes are leading to new efficiencies in breeding. The ability to isolate and redesign genes and transfer them into different plants also offers the breeder solutions to several key limitations. The convergence of advances in biology-genomics, proteomics, bioinformatics and information technologies is driving the emergence of a new bio-economy. By the usage of this technology we have achieved remarkable success in increasing crop productivity, improving crop quality as well as overcoming food shortage. Additionally the genetically engineered crops have shown a remarkable potential to tackle some of the world's most challenging socioeconomic problems which are more prevalent in the developing world than in the industrialized nations.

Keywords Biotechnology • Food security • Molecular markers • Transgenics • Proteomics • Nanobiotechnology

1 Introduction

The world population has increased by 2.3 billion people in the past 40 years, and by the year 2040, an additional 3.6 billion will be added to it. In fact, in every earth hour about 13,000 new human beings are added up to this globe. Most of this increase is in the developing countries where already one billion people go hungry every day and live in dismal poverty. The African nations of Ethiopia, Nigeria or Egypt each add more people than all of Western Europe combined (World Watch Institute, Washington, DC). India's population is around 1.14 billion with an annual per capita income of less than US $ 1,043 and its population is projected to continue to increase to a total of 1.5 billion by the year 2030 (FAO 2008). The population increase in developing countries constitutes 97% of the global increase (Swaminathan 1995). It is therefore, an intimidating task to feed the ever increasing population in the resource-poor countries where agriculture is already constrained by lack of new arable land, small sized farms, and certain destructive agricultural practices contributing to soil degradation, salinization and ultimately the desertification.

Agriculture is an essential component of societal well-being. It occupies 40% of the land surface, consumes 70% of global water resources and manages biodiversity at genetic, species and ecosystem levels. Intensive use of inorganic fertilizers and pesticides, expansion of irrigation, and capital-intensive farm management has resulted in an unparalleled increase in global agricultural productivity since 1950s. Agriculture, therefore, is and will continue to be central to all strategies for planned socio-economic development of the countries. Despite major advances in agriculture and strong growth in food production in the latter part of the twentieth century, food security for the masses continues to be an area of concern. The application of biotechnological techniques in the agriculture sector can potentially improve food security by raising crop tolerance to adverse weather and soil conditions, by enhancing adaptability of crops to different climates and by improving yields, pest resistance and nutrition, particularly of staple food crops. Biotechnology can, over the next two decades, deliver the next wave of technological change that can be as fundamental and invasive as that brought about by information technology.

Recently the World Food Program (WFP) and the Food and Agriculture Organization (FAO) -reported that 22 countries are experiencing protracted food insecurity, and 17 of these countries are in Africa. There has been a remarkable increase in total grain production between 1950 and 1980, but only a marginal increase has been realized during 1980–1990 (Myers 1999; Ozturk and Uzonur 2004). This increase in grain production has mostly resulted from an increase in area under cultivation, irrigation, better agronomic practices, and improved cultivars. Yields of several crops have already reached a plateau in developed countries, and therefore, most of the productivity gains in the future will have to be achieved in developing countries through better natural resources management and crop improvement. Productivity gains are essential for long-term economic growth, but in the short-term, these are even more important for maintaining adequate food supplies for the growing world population. It is in this context that biotechnology will play an important role in food production during next few decades.

In the present review we attempt to take a practical look at the prospects and constraints of various types of biotechnologies and their application for increasing crop production and improving nutritional quality. Under this context, we also address the important issues of biosafety and impact of the genetically engineered crops on the environment.

2 Biotechnology in Agriculture

The Convention on Biological Diversity (CBD) defines biotechnology as: “any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use”. This technology has been incorporated in every part of living system, from disease cure to crop improvement in which new traits are being either introduced or old ones modified to yield better system. Genetic modification of crops is one such method allowing

individual characters (gene, factor or trait) to be transferred into crop plants. With the advent of genetic modification through genetic engineering in early 1980s, the natural barrier of only intra-specific exchange of characters was removed and scientists were able to identify and transfer specific genes associated with desirable traits from one organism to the organism of other species that otherwise cannot breed naturally (Ozturk and Uzonur 2004; Shigeto et al. 2006). With these techniques genes from varied class of organisms like bacteria, virus or even animal may be transferred into plants to develop genetically modified plants having exclusively changed characteristics controlled by the specific gene. This gives scientists/ breeders a broader access to desirable traits from any living organism and its possibility of transferring it with much faster rate and greater precision. There are numerous biotechnological approaches through which we can develop effective changes in agricultural fields and yield better crops in terms of their productivity as well as quality. Future impacts of biotechnology in crop production will be in the areas of:

1. Developing new hybrid crops based on genetic male-sterility,
2. Exploit transgenic apomixes to fix hybrid vigour in inbred crops,
3. Increased resistance to insect pests, diseases, and abiotic stress factors,
4. Improved effectiveness of bio-control agents,
5. Enhance nutritional value (vitamin A and iron) of crops and post-harvest quality,
6. Increase efficiency of soil phosphorus and essential micronutrients uptake especially Zn uptake and its translocation in plants,
7. Increase the nitrogen fixation capacity of legumes,
8. Improve adaptation to soil salinity and heavy metal toxicity,
9. Understanding the nature of gene action and metabolic pathways,
10. Increase photosynthetic activity, sugar and starch production, and
11. Production of pharmaceuticals and vaccines in suitable pants (biofarming).

Further understanding the biochemical process(s) at molecular scales and changes in expression levels could be an enormous help with which biotechnology proves beneficial.

3 Fertilizer Usage and Biotechnology

Increase in agricultural productivity during green revolution is largely associated with the augmentation of fertilizers (particularly N-based fertilizers). It is estimated that around 50% of the human population relies on nitrogen (N) fertilizer for food production globally (Pathak et al. 2008). The N fertilizer consumption has grown dramatically in Asia, about 17-fold in the last 40 years (Dobermann and Cassman 2004). However it is surprising to know that only 50% or less of the applied nitrogen is used for producing the aboveground biomass of cereals. The other 50% or more gets dississipated in the wider environment by volatilization, leaching, surface runoff and denitrification (Jeffrey et al. 2002), resulting in the major detrimental impacts

on environment, such as eutrophication of fresh water and marine ecosystems (Beman et al. 2005), gaseous emission of oxides reacting with the stratospheric ozone, and the emission of toxic ammonia (Stulen et al. 1998) into the atmosphere. Despite these hazardous impacts on the biosphere the use of N fertilizers has increased 100-folds over the last 100 years, as it is known that the cereal yield and the fertilizer N consumption have increased in a linear fashion during the past 40 years and both are highly correlated (Ladha 2005). At this juncture, the use of nitrogenous fertilizers cannot be reduced because of the pressure of more and more food production to feed the teeming population. Development of crop varieties that can grow and yield well at low nitrogen condition can be a solution to the problem. Tolerance of a crop to low N conditions is a highly desired characteristic for sustainable crop production. Many approaches such as optimal time, rate, and methods of application for matching N supply with crop demand; the use of specially formulated forms of fertilizer, including those with urease and nitrification inhibitors; the integrated use of fertilizers, manures, and/or crop residues; and optimizing irrigation management have been suggested for increasing nitrogen use efficiency (Abrol et al. 1999; Abdin et al. 2005; Raghuram et al. 2006). But their adoption at the farm level has been limited for various reasons in developing countries. Progress has been made in genetic and molecular analysis of low N tolerance and breeding crops for low N conditions. To develop varieties with improved nitrogen use efficiency it is necessary to have high level of genetic diversity for N uptake efficiency. Lian et al. (2005) analyzed the genetic components associated with low N tolerance in rice at the seedling stage. So, through biotechnological approaches we can work out the molecular mechanism of nutrient assimilation in crop plants which will help us to manipulate the important genes (s) which may affect nutrient use efficiency and hence crop yield.

4 Engineering Plants with Other Gene Systems Regulating N Metabolism

Studies with transgenic plants overexpressing genes affecting the N metabolism pathway suggest it is possible to improve or manipulate N metabolism and the growth phenotype of plants, which can improve the nitrogen use efficiency (NEU) of crop plants. In order to identify and understand the regulation of the genes involved in enhancing NUE, proper evaluation of the combined genetic and transgenic approaches to improving NUE are needed as a component of any crop improvement program. The benefits of growing NUE-efficient crops will not be realized until breeders evaluate N metabolism and nitrogen use efficiency in economically important crop plants.

In higher plants, the expression of the NR genes is influenced by several external and endogenous factors and is highly regulated at the transcriptional as well as post-translational levels. The overexpression of either the NR or the NiR gene in plants increases mRNA levels and often affects N uptake. However, the increased uptake

of N does not seem to increase the yield or growth of plants, regardless of the N source (Andrews et al. 2004). This is believed to be due, in part, to the complex regulation of both NR and the pathway as a whole. Lea et al. (2006) demonstrated that post-translational regulation of NR strongly affects the levels of free amino acids, ammonium, and nitrate, whereas transcriptional regulation has only minor influence. Plants expressing fully unregulated NR accumulate high concentrations of asparagine and glutamine in leaves; however these transgenic plants grow and develop normally, despite having an NR enzyme that is active during both light and dark periods (Good et al. 2004; Shrawat and Good 2008). Mutants or transgenic plants with altered levels of GS/GOGAT are used to determine the effects of these proteins on plant development and to study the expression of the different members of the GS multigene family. Although several studies demonstrate that an increase in GS activity in transgenic plants has no effect on the phenotype, many researchers show a direct correlation between an enhanced GS activity in transgenic plants and an increase in biomass or yield, upon incorporating a novel *gs1* construct. Transgenic tobacco plants enriched or reduced in plastid glutamine synthetase (GS2, a key enzyme in photorespiration) have been developed (Kozaki and Takeba 1996). Those transgenic plants having twice the normal amount of GS2 had an improved capacity for photorespiration and an increased tolerance to high-intensity light, whereas those with a reduced amount of GS2 had a diminished capacity for photorespiration and were photo-inhibited more severely by high-intensity light compared with the control plants. Ectopic expression of GS1 has been shown to alter plant growth (Oliveira et al. 2002) and the over expression of GS1 in transgenic plants could cause the enhancement of photosynthetic rates, higher rates of photorespiration and enhanced resistance to water stress (El-Khatib et al. 2004). The overexpression of soybean cytosolic GS1 in the shoots of *Lotus corniculatus* was reported to accelerate plant development, leading to early senescence and premature flowering, particularly when plants were grown under conditions of high ammonium (Vincent et al. 1997). Man et al. (2005) provided additional empirical evidence for enhanced nitrogen-assimilation efficiency in GS1 transgenic lines. However, differences in the degree of ectopic GS1 expression have been reported and attributed to positional effects, effectiveness of chimeric constructs, or differences in growth conditions. These differences could account for the lack of correlation between the enhanced expression of GS1 and concomitant growth (Vincent et al. 1997; Ortega et al. 2001). Interestingly, the differences are more striking at a low nitrate concentration. In addition, higher rates of N incorporation into the transgenic plants further demonstrate that the transformed plants have increased NUE (Man et al. 2005).

In comparison to GS, few reports have described the production of transgenic plants overexpressing *GOGAT* genes. Transgenic overexpression and antisense technology have been employed recently to modulate the expression of NADH-GOGAT in alfalfa and rice plants (Schoenbeck et al. 2000; Yamaya et al. 2002). The studies on transgenic rice plants expressing antisense RNA for either GS1 or NADH – GOGAT point towards the possible involvement of GS1 in the export of N via phloem in senescing leaves. On the other hand, in case of developing leaf blades and spikelets, NADH-GOGAT was implicated in the utilization of glutamine transported

from senescing organs (Yamaya 2003). While these genes appear to be good candidates for improving NUE in the short run, the degree of improvement may vary with the crop and cropping conditions. Therefore, the utility of transgenic over-expression of N-assimilatory genes for major improvements of NUE remains uncertain, though the possibility that different crops respond differently cannot be ruled out yet.

5 Signalling and Regulation of Nitrogen Metabolism

It is a well known concept in signal transduction that whenever multiple genes are subject to transcriptional regulation by a common signal, it is mediated through a regulatory sequence that exists in all the genes that respond to the signal. These signature sequences, commonly known as response elements, are identified by mutations that abolish their function, and their conserved nature as revealed by homology comparisons. Early experiments in transgenic *Nicotiana* plants using GUS gene fused to NR and NiR promoter sequences clearly demonstrated for the first time that nitrate induction of gene expression requires some sequence(s) associated with the NR and NiR promoters (Rastogi et al. 1993; Quesada et al. 1997). Subsequent studies in transgenic tobacco incorporating the 5′ flanking regions of the two *Arabidopsis thaliana* nitrate reductase genes *NR1* and *NR2* (designated *NP1* and *NP2*) demonstrated that 238 and 330 bp of *NP1*and *NP2* respectively are sufficient for nitrate-dependent transcription (Lin et al. 2005; Lea et al. 2006). These nitrate-responsive elements (NREs) are composed of several copies of a core A[G/C]TCA sequence motif preceded by an ~7-bp AT-rich sequence present in the 5′flanking regions of nitrate reductase (*NR1* and *NR2*) genes. This particular sequence motif was also found to be very well conserved in the 5′ flanking regions of NR and NiR genes from eight other plants (Hwang et al. 1997). Sarkar (2003) compared the flanking sequences of all available plant nitrate responsive genes and found that the NRE core sequence (A[C/G]TCA) was present in multiple copies on both strands in all the known nitrate-responsive genes in many dicots, monocots and cyanobacteria. Though most of the NREs examined contained both the core sequence and a proceeding AT rich sequence, there were some cases which had GC rich regions or did not reveal any AT/GC bias. A more detailed bioinformatic analysis of the entire *Arabidopsis* genome revealed that the proposed NREs are randomly distributed, with no difference between nitrate responsive genes and the presumably nonresponsive genes and intergenic regions in the rest of the genome (Kang et al. 2004; Raghuram et al. 2006). These findings raise doubts on the validity of the proposed NRE as comprising of (A[C/G]TCA) elements preceded by AT-rich sequence. Further work in this area will need a combination of bioinformatic and experimental approaches to redefine the NREs that mediate the expression of all nitrate responsive genes in all plants. The discovery of NREs is important, as it provides an end point for nitrate signal transduction.

For the regulation of nitrate uptake, signals are derived from nitrate, which are involved in triggering widespread changes in gene expression; resulting in

reprogramming of N metabolism to facilitate the uptake and assimilation of nitrate and its incorporation into amino acids. The nitrate assimilatory pathway is under tight regulation by the available nitrate and reduced N. In strawberry, increasing external nitrate concentration from 0 to 4 mM markedly increased the cumulative nitrate uptake (Taghavi and Babalar 2007). Several of the LATS- and HATS-related genes, apart from being root specific, are also inducible by nitrate and there is evidence that at least one HATS-related gene, *NpNrt2:1* is also repressible by reduced nitrogen (Quesada et al. 1997). In barley and white spruce, cHATS provides a high affinity, low capacity pathway for nitrate entry in uninduced plants. Nevertheless, cHATS activity is up regulated (approximately three folds) by exposure to nitrate (Trueman et al. 1996). In barley, the fully induced iHATS flux was approximately 30 times higher than that resulting from the cHATS (Quesada et al. 1997). The increase in transcript is accompanied by increased rates of nitrate uptake (Imsande and Touraine 1994). The results on citrus seedlings suggest that LATS is under feedback control by the N status of plant. A decline in uptake rate by the addition of amino acids (Glu, Asp, Asn, Gln) to the external solution has been reported (Cerezo et al. 2000). The use of chemical inhibitors in physiological studies has suggested that protein synthesis is important for nitrate uptake (Aguera et al. 1990) and the transporters may turn over relatively slow. A degradation mechanism for transporter protein in *Arabidopsis* (*AtNrt2:1)* has been suggested (Cerezo et al. 2001). The presence of a number of conserved protein kinase C recognition motifs in the N and C domains of *HvNRT2:1* (Forde 2000) suggests that phosphorylation events are involved in regulating *AtNrt2:1* activity in response to environmental cues. Remans et al. (2006) found that under N-limited conditions, *AtNrt2:1* played a key role as a major NO_3^- uptake system and coordinated lateral root initiation and development with external NO_3^- availability.

The precise mechanism of nitrate sensing and signalling is not yet fully understood. Post-translational regulation of some of the nitrate-responsive enzymes is brought about by 14-3-3 proteins, though they mainly mediate the effect of light and other signals, rather than nitrate. A few elements possibly associated with nitrate signaling are Ca^{2+} and protein kinases/ phosphatases; these have been implicated in mediating the nitrate signal for the expression of NR, NiR and GS2 m RNAs (Sakakibara et al. 1997). In addition to the kinases, Hartwell et al. (1999) described a Ca^{2+} independent PEPCase protein kinase, which is a novel member of the Ca^{2+} calmodulin regulated group of protein kinases. Krapp et al. (2002) described their specific roles in mediating nitrate and other interacting signals. A better understanding of the nitrate-signaling cascade might emerge from the study of mutants related to the signal-transfer cascade from nitrate to the NR gene (Ogawa et al. 2000), revealing more intermediate and potential sites for the manipulation of NUE. Light is an additional signal that regulates the expression of many nitrate-responsive genes, though it has been studied in depth in only a few of them. The role of light in regulation of NR gene expression has often been reviewed (Raghuram and Sopory 1995; Lillo and Appenroth 2001). The effects of light in green plants are signalling and N-use efficiency probably mediated more indirectly, through photosynthesis and sugars (Lillo and Appenroth 2001). At the post-translational level, light acts by modulating the phosphorylation status of the enzyme, in conjunction with 14-3-3 proteins.

Transcriptional regulation of several hundreds of nitrate-responsive genes by nitrate as a signal requires cis-acting regulatory sequences or nitrate responsive elements (Raghuram et al. 2006). ANR1, a putative signalling and N-use efficiency transcription factor homologous to the MADS box family has been reported in *Arabidopsis thaliana* (Zhang and Forde 1998). ANR1 is nitrate inducible and root-specific, and has been shown to be involved in nitrate-dependent stimulation of lateral-root proliferation in transgenic plants (Forde 2002). However, this root-specific transcription factor does not account for the transcription of all the known nitrate responses even in the root, besides being irrelevant for nitrate-responsive gene expression in the shoots. In terms of finding a global target for manipulation of NUE, the successful manipulation of N content by overexpression of the Dof1 transcription factor indicates that unravelling the signalling mechanisms that bring about their coordinated expression of nitrate-responsive genes by N and C metabolites could reveal new targets and approaches for future metabolic-engineering efforts (Lochab et al. 2007). Yanagisawa et al. (2004) generated transgenic *Arabidopsis* lines over expressing Dof1, a maize protein that belongs to Dof family of plant-specific transcription factors known to activate the expression of several C-metabolizing genes associated with organic-acid metabolism. The transformants showed up to 30% higher levels of mRNA and enzyme activities for PEP carboxylase and pyruvate kinase, without any reduction of NR, GS, and GOGAT RNAs. If Dof1 is not nitrate inducible, it means that multiple transcription factors may be involved in the coordinated expression of N and C metabolizing genes.

6 Marker Assisted Selection of High Yielding Varieties

Marker-assisted selection and DNA fingerprinting allow a faster and much more targeted development of improved genotypes for all living species. They also provide new research methods which can assist in the conservation and characterization of biodiversity. The new techniques will enable scientists to recognize and target quantitative trait loci and thus increase the efficiency of breeding for some traditionally intractable agronomic problems such as drought resistance and improved root systems. A genetic marker is a measurable character with Mendelian inheritance. The advancement in DNA marker technology has facilitated in genome analysis and rapid development of many high-density linkage, physical and consensus maps in crops of interest (Gupta and Varshney 2000; Lörz and Wenzel 2005). The most commonly employed markers in crop plants are random amplified polymorphic DNA (RAPD), intersimple sequence repeat (ISSR) markers and amplified fragment length polymorphism (AFLP). These are the markers of choice for crops with inadequate genomic resources, do not require prior sequence information and scan the genome including the repetitive sequences. In fact, the RELP (restriction fragment length polymorphism) approach has been used successfully to identify genetic markers in plants, including rice (Tanksley et al. 1989: Wang et al. 1994). However, the RFLP technique needs specific probes for the target DNA sequences, and use of radioactive elements makes it more costly and tedious. The development

of PCR technique has offered a good alternative to the RFLP analysis. The PCR-based RAPD approach using single 10-mer arbitrary primers requires much less DNA, and is technically simple and cheaper compared to the RFLP (Williams et al. 1990). In maize, 42 pairs of proteins showed a 1:2:1 segregation in the F2 population indicative for a monogenic inheritance. Two linkage maps were constructed from RFLP and position-shift loci, which revealed that protein markers were interdispersed between the RFLP markers on all chromosomes (De Vienne et al. 1996). In many cases position-shift variants correspond to the same protein as shown by micro-sequencing (Touzet et al. 1995; Plomion et al. 1997). It is expected that the maps of expressed genes obtained by 2-Dimentional Electrophoresis (2-DE) will be crucial for the candidate gene strategy of quantitative trait loci (QTL) characterization (Thiellement et al. 1999). QTL analysis has been applied to map genes controlling protein quantity for spots on 2-D gels (Touzet et al. 1995) and the loci have been termed PQL for protein-quantity loci (Thiellement et al. 1999). Co-localization of a protein-quantity locus (PQL) and its protein-coding locus would indicate that expression level of the protein is a consequence of allelic differences, whereas co-localization between a PQL and a QTL for a different trait would point to an association of a candidate gene and the variation observed for a trait (Zivy and de Vienne 2000; Thiellement et al. 1999; Consoli et al. 2002; Lian et al. 2005). The level of molecular polymorphism in wheat has been found to be low as compared to many other species (Prasad et al. 2000; Song et al. 2002), thereby limiting studies on variability and diversity using molecular markers. Among different classes of molecular markers, simple sequence repeat (SSR) markers are short (1–6 bp long) tandemly repeated DNA sequences that are highly polymorphic and are useful for a variety of applications in molecular breeding due to its reproducibility, multi-allelic nature, co-dominant inheritance, abundance and high polymorphic information content (PIC), and thus have recently been used to study the genetic variability based on DNA polymorphism in a number of crop species (Morgante and Oliveri 1993; Powell et al. 1996; Gupta et al. 1996; Gupta and Varshney 2000; Prasad et al. 2000; Agarwal et al. 2008). It has also been demonstrated that even limited numbers of SSR markers were adequate to discriminate closely related wheat and barley varieties (Russel et al. 1997; Prasad et al. 2000). The genomes of all eukaryotes contain a class of sequences termed microsatellites (Litt and Luty 1989) or simple sequence repeats (SSRs) (Tautz et al. 1986). Microsattelite are short tandem repeat of 1–6 bp that can repeat up to 100 times (Schloetterer et al. 1991), have emerged as an important source of ubiquitous genetic markers for many eukaryotic genomes (Wang et al. 1994). In plants, it has been demonstrated that microsatellites are highly informative, locus-specific markers in many species (Liu et al. 1996; Moerchen et al. 1996; Smulders et al. 1997), because they are multiallelic, microsatellites have high potential for use in evolutionary studies (Schloetterer et al. 1991; Buchanan et al. 1994) and studies regarding genetic relationships. SSRs show a much higher level of polymorphisms and are informative in hexaploid bread wheat than any other marker system (Ma et al. 1996; Bryan et al. 1997). They can be utilized for several

applications in plant genetics mapping, cultivars discrimination and detection of genetic diversity (Gupta and Varshney 2000). SSRs provide an efficient means of detecting genetic diversity as they can detect high number of alleles per assay (Powell et al. 1996).

7 Development of Transgenic Crops

To improve both productivity and sustainability of agriculture, new crop varieties have been introduced by transgenic approach. Over the years, considerable progress has been made in developing transgenic plants in which agriculturally important crops have been improved upon by the incorporation of gene from similar or another species. Much emphasis has been give to develop insect-resistant transgenic crops as the world annual losses from plant pathogens alone are estimated to be 12% (Cook 2006). Transgenic insecticidal crop cultivars are in the process of revolutionizing agriculture and are likely to become a major insect management tactic worldwide. Introducing novel resistant genes into economically important crops can develop insect-resistant crops. This tactic has a potentially key role in integrated pest management of several important pests (Gatehouse and Gatehouse 1998). *Bacillus thuringiensis* (Bt)-, a soil-borne bacterium, based transgenic cultivars have been produced for Cotton (*Gossypium hirsutum*), Potato (*Solanum tuberosum*), maize (*Zea mays*), rice (*Oriza sativa*), tomato (*Lycoperscion esculentum*), alfalfa (*Medicago sativa*) and numerous other crop plants (Hilder and Boulter 1999). The *bar* gene conferring herbicide tolerance was introduced in 1-month-old wheat calli employing both particle bombardment and *Agrobacterium*-mediated transformation strategies (Chugh and Khurana 2003). Herbicide-tolerant soybean was the dominant transgenic crop grown commercially in the USA, Argentina, Canada, South Africa, Romania and Uruguay, occupying 33.3 million hectares in 2001. The current efforts to improve plant stress tolerance by gene transformation have resulted in important achievements. Present engineering strategies rely on the transfer of one or several genes that are either involved in signaling and regulatory pathways, or that encode enzymes present in pathways leading to the synthesis of functional and structural protectants, such as osmolytes and antioxidants, or that encode stress-tolerance-conferring proteins. Transgenic system in indica rice and wheat has been developed. Rice has been transformed with codA/COR47, AtHSP100 and PDC gene while wheat transformed with *hva1* to confer stress resistance. All the transgenics have been characterized at the molecular level for gene integration. Studies have been conducted to transform indica rice plants with *p5cs* gene to make it more tolerant to salinity. The assessment of transformed plants harboring this gene as a single copy showed promising results and further efforts are on to pyramid one more gene for higher tolerance to salinity.

8 Modern Biotechnological Tools and Crop Productivity

During the advent of post genomic era we came to know the newer branches of biotechnology which provides us new insights in understanding the different process(s) and mechanisms that ultimately leads to crop improvement.

8.1 Genomic Approach for Crop Improvement

Genomics research has provided breeders new tools, such as functional molecular markers and informatics, as well as new knowledge about statistics and inheritance phenomena that could increase the efficiency and precision of crop improvement. Currently an impressive number of advances in genetics and genomics have given us enhanced understanding of structural and functional aspects of plant genomes and basic knowledge have been integrated in such a way which can enhance the ability of plant breeders to improve crop plants for our benefit. In the last decade the whole genome sequencing of model plant *Arabidopsis thaliana*, rice, *Sorghum bicolor*, *Medicago truncatula*, *Musa* spp., grape, apple and recently draft sequence of wheat have become available (http://www.ncbi.nlm.nih.gov/genomes/).

The first complete plant genome to be sequenced was that of *Arabidopsis*. The sequenced regions cover 115.4 Mb of the 125-Mb genome and extend into centromeric regions. The genome contains 25,498 genes encoding proteins from 11,000 families (The Arabidopsis Genome Initiative, 2000). *Arabidopsis* contains many families of new proteins but also lacks several common protein families. The complete genome sequence provides the foundation for more comprehensive comparison of conserved processes in all eukaryotes, identifying a wide range of plant-specific gene functions and establishing rapid systematic methods of identifying genes for crop improvement (Tacchini et al. 1995; Varshney et al. 2009; Thakur and Varshney 2010).

Among the most important food crops, rice has the smallest genome (389 Mb) and wheat the largest (15,966 Mb). Arumuganathan and Earle (1991) have grouped other crops into seven classes: *Musa*, cowpea and yam (873 Mb); sorghum, bean, chickpea and pigeonpea (673–818 Mb); soybean (1,115 Mb); potato and sweet potato (1,597–1,862 Mb); maize, pearl millet and groundnut (2,352–2,813 Mb); pea and barley (4,397–5,361 Mb); and oat (11,315 Mb). Genome size is often correlated with plant growth and ecology. The diverse cellular and physiological effects of large genomes may be a function of selection of the major components that contribute to genome size such as transposable elements and gene duplication. The recent advances in genome sequencing, through the development of second generation sequencing technologies and beyond, provide opportunities to develop millions of novel markers, in non-model crop species, as well as identification of genes of agronomic importance. Identification of all genes within a species permits an understanding of how important agronomic traits are controlled, knowledge of

which can be directly translated into crop improvement (Chia and Ware 2011). This systematic whole genome sequencing will provide critical information on gene and genome organization and function, which will revolutionize our understanding of crop production and the ability to manipulate those traits contributing to high crop productivity (Pereira 2000).

The use of whole genome information and high-throughout tools has opened up a new field of research called functional genomics. Among its subdisciplines, transcriptomics (the complete set of transcripts produced in a cell) (Zimmerli and Somerville 2004), proteomics (the complete set of proteins produced in a cell) (Roberts 2002) and metabolomics (the complete set of metabolites expressed in a cell) (Stitt and Fernie 2003) have been used by the plant science community.

Recent advances in microarray technology will allow the simultaneous expression and analysis of vast numbers of genes that will elucidate gene function, and the complex multifaceted interactions between genes that result in different phenotypes under varying environmental conditions. These high-throughput or large-scale experimental methodologies combined with statistical and computational analysis (bioinformatics) will give the detailed information about specific gene/genes function linked to specific characters required for crop improvement. These studies will be augmented by more specific investigations based on gene suppression, co-suppression or anti-sensing of a defined sequence (Jain and Barr 2010). Advances in these areas will fuel the mapping of QTL (quantitative trait loci) underlying agronomic traits in less studied crops. The use of QTL markers in crop improvement promises rapid and efficient utilization of novel traits from closely related wild species (Varshney et al. 2011). These new information provided by all the omics disciplines will lead the plant science community to *in silico* simulations of plant growth, development and response to environmental change.

The recent addition of the high quality draft genome of the soybean in the rapidly growing list of crops will not only help the breeders to improve soybean varieties in terms of protein and oil content but will also help to improve nitrogen fixation capacity of many important legumes used for human and animal nutrition. Schmutz et al. 2010 report that the 1.1-gigabase soybean genome-the largest shotgunsequenced plant genome-is predicted to encode 46,000 genes. Two genome duplication events are likely to account for the observation that ~75% of these genes are found in multiple copies. Although the importance of soybean as a source of protein and oil alone testifies to the potential implications of understanding its genetic makeup, this genome will also serve as the reference for ~20,000 leguminous species that play a critical ecological role through their unique ability to fix nitrogen with the help of rhizobial bacteria. Availability of the genome should accelerate the association of quantitative trait loci of nutritional, economic and ecologically important traits with the causal DNA sequences from soybean in the near future. In the longer term, the genome will likely also be leveraged to improve the way in which a range of leguminous subsistence crops are used to both replenish soil nitrogen through crop rotation and meet the expanding needs of developing nations for protein and energy.

Crop improvement can also be carried out by engineering novel RNA interference (RNAi) pathways that create small RNA molecules to alter gene expression in crops and can generate new crop quality traits or provide protection against insects, nematodes and pathogens without introducing new proteins into food and feed products (Auer and Frederick 2009). Although miRNAs are relatively small, they play an important role in gene expression. miRNA is considered as one of the most important post-transcriptional gene regulators (Carrington and Ambros 2003) since it was originally recognized in 2001 (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001).

Recent studies have revealed powerful and unexpected roles for small interference RNAs (siRNAs) and microRNAs (miRNAs) in the control of plant growth by the silencing of native genes. Although both of these have pivotal roles in gene silencing, the actions of miRNAs are more extensive and remarkable. Several experiments have demonstrated that many miRNAs regulate almost every aspect of plant growth and development, including leaf morphogenesis and polarity, floral differentiation and development, root initiation and development, vascular development, and the transition from vegetative growth to reproductive growth (Jones-Rhoades et al. 2006; Chuck et al. 2009). Even more intriguingly, it has been discovered that miRNAs play a role in hormone signal transduction (Liu and Chen 2009), the response to environmental stress, and pathogen invasion (Chen et al. 2004; Sunkar et al. 2006). These regulatory miRNAs stimulated the idea of developing artificial miRNAs that can silence specific gene(s). Such targeted gene silencing could permit the direct molecular modulation of plant traits, which could in turn be applied to the breeding of crop species.

8.2 Proteomics and Crop Improvement

Proteomics is the fascinating field of research that attracts the thousands of scientific workers over the globe for a wide range of subjects (Tilleman et al. 2005; Bona et al. 2007). The term "Proteome" (PROTEins expressed by genOME) therefore is expected to represent a comprehensive survey of all proteins expressed at a given time, in given conditions. Moreover the expression levels in protein strongly depend on complex regulatory systems; unlike genome, the proteome is highly dynamic (similar to transcriptome). Proteomics is one of the fastest growing areas of biological research. At present, proteomic research aims both identifying new proteins in relation to their function and in unraveling how their expression is controlled within regulatory networks. In the past few years tremendous progress has been made in the field of crop plant proteomics. With the high advancement in technologies and latest bioinformatic tools, studying the plant proteome and its dynamic nature has become comparatively easy and very accurate. Plant physiologists now feel comfortable over this development as many unsolved mysteries about various physiological processes in the plants will now become easy to work out. From seed germination to fruit development/grain filling proteomics research is accelerating

by leaps and bounds throughout the globe. Expression of proteins regulating the processes of nutritional as well as hormonal balances has been providing the new insights towards understanding various metabolic processes fully. Plants like other organisms are always under the threat of various stresses. A remarkable achievement has been made in this direction by proteomicists by discovering the functions of various proteins and working out their expression levels.

All living organisms rely on the uptake of nutrients from the environment to sustain energy, metabolism and growth. They have, therefore, evolved numerous alternative programs to adapt to their permanently changing environment. Such programs involve instantaneous responses (changes in intracellular metabolites, activation/inhibition of enzymes by effectors and of proteins through post-translational modifications) as well as slower processes that affect the levels of macromolecules (transcription, translation, mRNA and protein degradation). The availability of complete genome sequences and technologies that allow comprehensive analysis of global mRNA profiles has greatly expanded the ability to monitor the transcriptional reprogramming of cells in response to their environment. However, further studies (often conducted with yeast) indicate that transcripts are imperfect indicators of protein levels and of *in vivo* fluxes (ter Schure et al. 2000; Griffin et al. 2002; Washburn et al. 2003; Daran-Lapujade et al. 2004; Wek et al. 2004; Kolkman et al. 2006), and therefore it brings limited understanding on whole biological systems. The implementation of sensitive and rapid methods for protein identification and the continuous technical improvement of the so far largely descriptive analysis of protein patterns by two-dimensional gel electrophoresis (2-DE) have transformed the combination of both techniques into a powerful tool for functional analysis now also more and more used in plant studies. Proteomics is proving an indispensable tool for examining alterations in the protein profile caused due to gene mutations, introduction or silencing of genes or in response to various stress stimuli in a relatively fast, sensitive and reproducible way. This science is becoming important for generation of information on physiological (e.g. regulatory behaviour and function), biochemical (e.g. metabolic and structural data), genetic (e.g. gene mapping and assigning of the structural gene to the 2D gel map) and architectural (e.g. location of the proteins in the cell) aspects. Proteomics-based approach is proving important for characterization of individuals or lines, estimation of genetic variability within and between different populations, establishment of genetic distances to be used in phylogenetic studies and characterization of mutants with localization of genes encoding revealed proteins (Thiellement et al. 1999). It is becoming a necessity in plant biology for deciphering the function and the role of genes in the on-going plant genome sequencing projects.

Analysis of the *Arabidopsis pasticcino* mutants by 2-DE revealed a considerable percentage of variable spots relative to wild-type controls; evaluation of responses to different hormone treatments indicated that the mutants were affected in cytokinin responses (Faure et al. 1998). A mutant was also used to study cytokinin effects on chloroplast division in the moss *Physcomitrella* at the protein level (Kasten et al. 1997). Comparison of protein patterns in leaves of the late flowering *Arabidopsis* mutant *fy* and wild-type demonstrated qualitative differences. Studies have also

demonstrated the capacity of 2-DE to document genetic variability and distinguish between lines and varieties, *e.g.*, when analyzing barley seed and malt (Ostergaard et al. 2002) or wheat grains (Skylas et al. 2005). Positional shifts of proteins were observed in 2-D gel analysis of segregating families of maize, barley, pea, and maritime pine (De Vienne et al. 1996).

Application of proteomics can enormously boost up agricultural production (Dhand 2000; Cánovas et al. 2004; Xu et al. 2006). It is the most promising technique to identify proteins that are induced, repressed, or post-transcriptionally modified during a developmental process as complex as senescence.

8.3 Nanobiotechnology and Crop Improvement

The term 'Nanobiotechnology' was used for the first time by Lynn W. Jelinski (a biophysicist at Cornell University, USA). It is an exciting and rapidly emerging technology allowing us to work, manipulate and create tools and materials at the molecular level that may be of great importance in our day-to-day life. Nature has been performing the "nanotechnological feats" for millions of years. This important technology has the potential to revolutionize the agricultural and food industry with new tools by enhancing the ability of plants to absorb nutrients etc. Smart sensors and smart delivery systems have a great potential to help the agricultural industry by combating viruses and other crop pathogens. Key advances have been made in the ability to make measurements at the sub-cellular level and in understanding the cell as a highly organized, self-repairing, self-replicating, information-rich molecular machine. Single-molecule measurements are shedding light on the dynamics and mechanistic properties of molecular biomachines, allowing the direct investigation of molecular motors, enzyme reactions, protein dynamics, DNA transcription and cell signaling. It has also been possible to measure the chemical composition within a single cell. Nanobiotechnological research and development is likely to facilitate and frame the next stage of development of genetically modified crops, animal production inputs, chemical pesticides and precision farming techniques. While nanochemical pesticides are already in use, other applications are still in their early stages, and it may be many years before they are commercialized. These applications are largely intended to address some of the limitations and challenges facing large-scale, chemical and capital intensive farming systems. This includes the fine-tuning and more precise micro-management of soils; the more efficient and targeted use of inputs; new toxin formulations for pest control; new crop and animal traits; and the diversification and differentiation of farming practices and products within the context of large-scale and highly uniform systems of production. Nanobiotechnology can have momentous application in plant sciences too for inducting foreign DNA in the cells, an improvement over the transgenics as the gene would not be fully incorporated thereby preventing unintended gene flow into the environment. Nanobiotechnology has made inroads into uncovering fundamental biological processes, including self-assembly, cellular processes, and systems

biology. It can also be used to enhance photosynthesis and improve soil management. Removal of heavy metal contamination can be achieved through intense sensing for precision farming. It has also enabled the development of biochips and has a role in green manufacturing. Major applications are in the design of sensors, biofluidics for handling DNA and other molecules, nano-filtration, bioprocessing and traceability of genetically modified food. Nanobiotechnology is evolving as a powerful tool as a result of cross talk between nano scientists and biologists. By operating in the nanoscale realm, at the molecular level, nanotechnology offers a wide range of tools, techniques and applications. Nano biotechnology can stimulate new technologies for studies in cell biology using nano tools, provide opportunities for early detection of diseases through *in-vivo* and *in-vitro* analysis using nano sensing structures with extra ordinary multi-function capabilities and targeted drug delivery. Some of the areas with research priority are: nanoparticles for biosynthesis of nanoparticles, biological templates for nanoparticle assembly, bionano composites, imaging/sensing of nanoparticles/biomolecules, tissue engineering, cell-cell interactions, mammalian/microbial cell development, nano-biosensors with multiple sensing capabilities. The development of genetically encoded molecular sensors, which transduce an interaction of the target molecule with a recognition element into a macroscopic observable, via allosteric regulation of one or more reporter elements, may provide us a chance to understand many physiological processes fully. The recognition element may simply bind the target, bind and enzymatically convert the target, or may serve as a substrate for the target, as in the use of a specific target sequence in the construction of a protease sensor (Nagai and Miyawaki 2004). The most common reporter element is a sterically separated donor-acceptor fluorescence resonance energy transfer (FRET) pair of spectral variants of the green fluorescent protein (GFP; Fehr et al. 2002), although single fluorescent proteins (Doi and Yanagawa 1999) or enzymes (Guntas et al. 2005) are viable as well. Some molecular sensors additionally employ a conformational actuator (most commonly a peptide which binds to one conformational state of the recognition element) to magnify the allosteric effect upon and resulting output of the reporter element.

9 Conclusion

Biotechnology has remarkably affected all the aspects of human life. In agricultural fields too, it has and will surely solve the problems of food insecurity. Understanding the important process(s) of crop production and manipulating the key steps of grain/fruit development have become easier and accurate by this wonderful technology. Developing stress tolerant, high yielding and nutrient efficient crop varieties are some attributes associated with biotechnological tools. One of the tremendous challenges before the breeders/scientists is food insecurity which needs to be addressed timely so as to feed the teeming population. Agricultural lands are vanishing due to the various processes like desertification; salination etc and just overflow of nutrient

fertilizers in these fields could not increase crop production as normally crop plants are not nutrient use efficient. Modern biotechnological approaches viz., proteomics, nanobiotechnology etc could also help us to understand the important process(s) of crop plants, thereby improvement in their production.

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Chapter 10
Plant Genetic Engineering: Problems and Applications

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Contents

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Abstract Introduction of Biotechnology has lead us to a stage where agricultural progress seems to be much different as before. It has introduced farmers to improve agricultural products while reducing the use of pesticides, and holds increased potential for pharmaceuticals and biomedical requirements. Technology has been improved to use the methods of transformation of agriculturally important crops by using different methods such as *Agrobacterium* and biolistic gun. Genetically engineered/modified crops, by *Agrobacterium*-mediated transformation system are becoming common in many countries. The health concerns on plants, animal and human life due to GM foods is unpredictable. However, plant genetic engineering can be considered either a progress or a threat to mankind depending on the way it is used.

Keywords Transgenic plants • Plant genetic transformation • Tissue culture • Transformation systems • Genetically modified crops • Plant biotechnology

1 Introduction

As the world population increasing, the agriculture sector has the priority to produce maximum foodstuff and other products with good yield in an environmentally sustainable way and cost-effective approach (Ferry et al. 2004). Agricultural production systems are dynamic and vary with time. The increasing awareness of the undesirable side effects of chemical pesticides and development of the insect resistance has forced scientists to look for alternative control measures. Improvements have been made in the crops for introduction of agronomic traits and other agriculturally important characteristics like yield, disease resistance and fibre quality by adopting conventional breeding techniques. This science has continued to offer the farmers with better environmentally friendly varieties. However, it is now improved by the introduction of technology as genetic engineering. Prospectively valuable and genetically superior characteristics known in different plants or yet in non-plants are sometimes unattainable due to the sexual hurdles in the crosses between distinct types. The improvement of technology to introduce and functionally express the foreign genes in plant cells has been adopted in less than two decades and leads to produce the transgenic plants. These transgenic plants show resistance against insects and diseases, better nutritional qualities in seeds and fruits and better adaptation of plants to undesirable environmental/ecological conditions (Danny et al. 1992). There is considerable interest in using transgenic approaches to produce resistance to different pathogens.

The genetically modified (GM) crops have been adapted commercially on an extensive scale since 1996; therefore they have been produced and became a reality in various countries (Dunwell 2000). The major countries initially growing Bt crops are USA, Canada, China, Australia, South Africa, and Mexico. It has been reported that in 2007, 22 countries grew Bt crops on a total area of 42.1 million hectares which accounted for the global area of 37% covered by GM crops (James 2007).

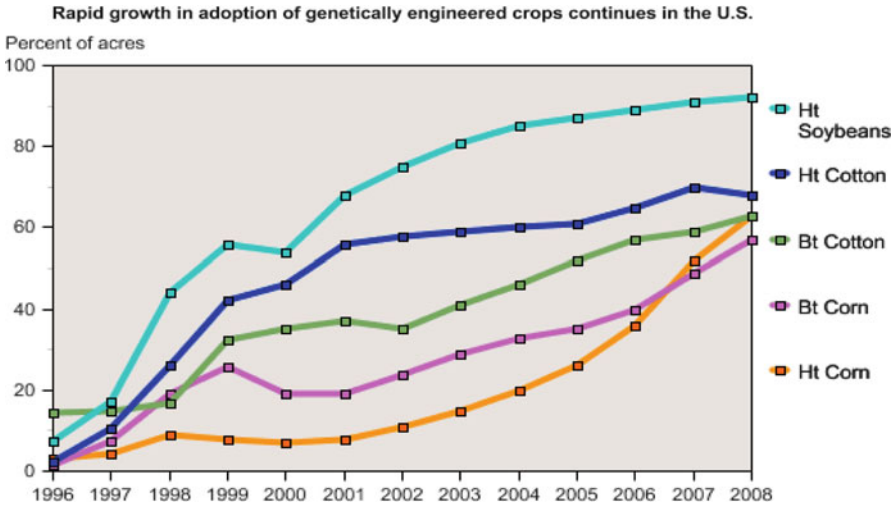

Fig. 10.1 Rapid growth in adoption of genetically engineered crops continues in the U.S. (Source: USDA, Economic Research Service)

Most important crops are; Cotton, Rice, Canola, Soybean, Potato, Corn and vegetables. The main important agronomic traits introduced into the transgenic crops include insect and herbicide resistance (Fig. 10.1). Advances have been made by using different transformation technologies like *Agrobacterium*-mediated transformation (Umbeck et al. 1987; Firoozabady 1989; Cousins et al. 1991; Gould and Magallanes 1998; Satyavathi et al. 2002; Shuangxia et al. 2005; Franklin et al. 2008), Gene Gun or combining both the methods together have also been reported (Firoozabady et al. 1987; Christou et al. 1989; Perlak et al. 1990; McCabe and Martinell 1993; Rajasekaran et al. 1996, 2000; Zapata et al. 1999; Taylor and Fauquet 2002). Sonication assisted transformation (Trick and Finer 1997, 1998; da-Silva and Fukai 2002; Solís et al. 2003; Jiang et al. 2004; Rashid et al. 2008) and Direct DNA uptake into protoplast (Khan and Maliga 1999; Daniell and Dhingra 2002) have also been used to which the researchers are required to get the reported literature/information. In the present review we have identified key issues to develop/adapt the transgenic plant technology and the main problems to be resolved in the practical application of this technology.

2 Confirmation of Transformation

The transformation of introduced genes involves stable integration and expression into the plant nuclear genome. Perlak et al. (1990) presented the idea that the successful integration and expression of a foreign gene in a plant could be predicted to be a result of many different factors. These factors may include the gene product

itself, a competent vector, suitable promoter, leader sequences, 3′ non-coding sequences, existence of prospective plant regulators, codon frequency and the secondary structure of the mRNA. Potrykus in 1991 offered an assessment that a combination of phenotypic, genetic and physical records is required for plant transformation technologies which can be based on a strict explanation of evidence of integrative transformation, but this arrangement was not practical to observe the confirmation of transformation in some plants. It is difficult to investigate the population of sexual progeny in some trees that grow and sexually reproduce slowly. The same is also complicated in sexually sterile varieties and some of the crops which are propagated vegetatively like sugarcane which has complex genetics such as polyploidy and aneuploidy.

Sometimes, transformants may have restricted copies of foreign introduced sequences, that would end up in the incorporation of numerous or repeatedly rearranged copies of the relocated DNA. Therefore, an association between substantial and phenotypic facts is not a really crucial attribute of gene transfer technique. Integrative transformation has yet not been clearly demonstrated in such type of cases (Birch 1997). Therefore as competitively commonly more appropriate criteria would be accepted as thorough confirmation of integrative transformation.

Southern blot is the DNA hybridization of various independent genetically modified plants. This method uses a probe for a foreign gene DNA digested with restriction enzymes which finally expect to produce hybridizing fragments of desirable size at required integration sites (Southern 1975). It has been reported that from the integration of T-DNA into plant cells, transforming vector border regions is deleted largely (Tinland 1996; Buck et al. 1999; Chen et al. 2003; Wang and To 2004). For transgenic lines, it is significant to verify that the size of hybridizing fragments including flanking DNA at each integration site should be reproducible within same transgenic line, but differ between independent lines.

This is well-known fact that most of the T-DNA transmited to the plant cell has not been integrated stably. Measurement of the comparative amounts of transient as well as stable expression of reporter gene activity in plant cells infected with *Agrobacterium* has proved this indirectly. Normally, the expression of stably integrated T-DNA is much lesser than the transient expression of T-DNA genes (Castle and Morris 1990; Janssen and Gardener 1990; Nam et al. 1997; Mysore et al. 1998). Single-stranded molecule of T-DNA enters the nucleus (Tinland and Hohn 1995; Yusibov et al. 1994) but it is not apparent if the T-DNA integrates by means of strand invasion of the nearby denatured plant DNA via single-stranded T-strand, followed by second strand synthesis (Tinland and Hohn 1995; Tinland et al. 1995; Rossi et al. 1996; Tinland 1996; Krizkova and Hrouda 1998) or might be the T-strand is changed to an extrachromosomal double-stranded appearance before integration (De-Neve et al. 1997). Switching of the T-strand into a transcriptional competent level involve the production of DNA strand complementary to the T-strand, therefore most of the extrachromosomal T-DNA that primarily enters the nucleus probably becomes double-stranded (Narasimhulu et al. 1996). Thus, for the specific integration on T-strand in the plant chromosome, *vir*D2 has a dynamic role. The energy included in its phosphodiester bond may be provided by the discharge

of *vir*D2 protein. This energy may be provided at the Tyr29 residue by means of the first nucleotide of T-strand provided that the 5′-end of the T-strand for ligation to the plant DNA. For nucleophilic 3′-OH from nicked plant DNA, the phosphodiester bond is capable to act as electrophilic substrate (Jayaram 1994). As soon as the mutant *vir*D2 protein attached to the T-strand is transferred, the nucleotides at the 5′-end of the T-strand is lost and the integration process starts to occur (Tinland et al. 1995).

Selection of transformed cells from a number of non-transformed ones is done by the marker genes and for visual selection of transformed tissues a reporter gene like the green fluorescent protein gene is required (Miki and McHugh 2004; Sripriya et al. 2008). Sometimes selectable marker gene affects the plant growth but an optimistic level of antibiotic is essential to reduce the false positives (Zapata et al. 1999; Zhang et al. 2001; Jin et al. 2005; Wu et al. 2006). However, the marker gene is not necessary after the selection of a transgenic plant. Various techniques even published after molecular proof could not be repeated or do not produce same results, therefore it is important that analysis of data at molecular, phenotypic, and genotypic level should be done in a more detailed manner on transformed lines produced for practical or commercial purposes.

The genetic transformation protocols differ from species to species and cultivar to cultivar. It is important to optimize the protocols required for successful transformation of species. There are several factors involved in this technology. First of all, standardization of cells taken from plant regenerable tissues and their interaction with the *Agrobacterium* and secondly, optimization of a suitable method for regeneration of plant organs from transformed cells.

3 Purpose of Plant Transformation

3.1 An Experimental Strategy for Plant Physiology

The advancement of plant genetic engineering technologies has allowed the introduction and functional expression of foreign genes in plant cells. This has further lead to the development of transgenic plants having superior characteristics like insect, disease and herbicide resistance. Plants have been produced for better adaptation to undesirable environmental circumstances and seeds or fruits with improved nutritional traits. Nutrition reduction and physiological injuries have occurred to the plants by sap sucking homopteran insect pests in the course of stylet probing and feeding. They also act as vectors to transmit viral diseases (Rao et al. 1998; Foissac et al. 2000). A homodimeric lectin, ASAL (mannose binding 25 kDa), is identified as a strong control agent against sap sucking insects and has been isolated from leaves of garlic (*Allium sativum*) (Bandyopadhyay et al. 2001; Banerjee et al. 2004; Majumder et al. 2004). It is reported that plants have the ability to adapt their gene expression pattern and activate resistance mechanism in response to control infection and transformation with *Agrobacterium* (Ditt et al. 2001, 2005, 2006). Recently, it

has been verified that the expression of *Agrobacterium vir* regulon could be shut down by salicylic acid- a plant signaling compound and it directly influences the infection development (Yuan et al. 2007). These reports present the existence of plant-microbe interaction for physiological improvements.

3.2 A Practical Strategy for Plant Improvement

Potential advantages of this technology include: increased yield, food security, less application of pesticides and practices to reduce soil erosion which ultimately would help out the small farmers for more saving and to improve quality of life and a technology easy to disseminate. Golden rice producers have recently been granted the patent licenses by Monsanto without any cost to meet the requirement of vitamin A in the human body. More crops such as wheat, rice and sugarcane having improved characteristics are now in different phases of inventory as engineering, testing and commercialization of salt-resistance. A large area of farmland presently inappropriate to produce crops will be used for cultivation by this technology. Fruit crops do not have significance as cereal or staple crops such as; wheat, corn, rice and soy in many countries. In Bangladesh papaya fruit as a crop has very essential part in their every day diet after rice. In 1990 Papaya ring-spot virus (PRSV) was menace to demolish the Hawaiian papaya. Therefore, GM papaya (PRSV-resistant cultivar) was produced and since 1998, it has been cultivated commercially very successfully.

4 Requirements for Genetic Transformation

Tissue culture is used in nearly all present practical transformation procedures. It is not a hypothetical requirement but without proper tissue culture procedure it is difficult to achieve an efficient gene transfer, selection and rejuvenation of putative transformants. Therefore improvement in tissue culture systems is desirable to provoke proficient transformation which should not be genotype dependent. Murashige and Skoog in 1962 discussed the comprehensive concerns, alternatives and standardization of tissue culture systems that are practical for plant genetic transformation. They further confer the detailed protocols and addressed the scope of this broad technology. There are advances which have been made by means of biotechnological applications such as ovule culture (McStewart and Hsu 1977; Thengane et al. 1986), protoplast culture (Peeters et al. 1994), somatic embryogenesis and regeneration (Shoemaker et al. 1986; Trolinder and Goodin 1988; Finer 1988; Kumria et al. 2003) and plant gene transfer (Firoozabady et al. 1987; Umbeck et al. 1987; McCabe and Martinell 1993). A number of crops have been transformed through *Agrobacterium* (Perlak et al. 1990; Bayley et al. 1992; Thomas et al. 1995), by gene gun method (Finer and McMullen 1990; Rajasekaran et al. 2000), or by

combining both the methods (Majeed et al. 2000). These crops contained different genes known for important agronomic traits. However, the most favorable means for gene delivery is through *Agrobacterium*. This is because it is simple, cost-effective and common for integration of single copy of gene into the host plant genome. It is strongly recommended to apply this system to all crops due to its access to an extensive selection of functional genes in the post-genomic time period. *Agrobacterium* infection diverges to a great extent for plant species, varieties, tissues and cells and amenability to this makes transformation either incompetent or not viable in recalcitrant plant species. Consequently, a plant-pathogen interaction study is needed to investigate the basis of plant recalcitrance towards this bacterium. A highly-efficient transformation procedure is required to develop which should be enormously concise, consistent, genotype independent. Moreover the method needs to be highly embryogenic, reduces the complexity of embryo germination, highly frequent for organogenesis, efficient in rooting of transgenic plantlets, and capable of hardening-off when shifted to soil pots.

5 Recalcitrance and Constraints

Many important plant species (legumes, cereals and woody plants) or best varieties of selected species, still remain recalcitrant to *Agrobacterium* method of transformation and unresponsive to many plant growth regulators (Choffe et al. 2000; Wang and To 2004). Sometimes regeneration is found restricted to only some of the cultivars (Firoozabady and DeBoer 1993; Zhang et al. 2000; Kumria et al. 2005). A number of reports have been published for transformation such as shoot apex using gene gun or *Agrobacterium* (Gould et al. 1991a, b; McCabe and Martinell 1993; Zapata et al. 1999). Particle bombardment of suspension cultures generally gave high-copy-number transformants, but is a time-consuming procedure (Finer and McMullen 1990; Rajasekaran et al. 2000). Therefore, most of the laboratories prefer the *Agrobacterium*-mediated method of genetic transformation followed by the formation of somatic embryos and then organogenesis (Firoozabady et al. 1987; Umbeck et al. 1987; Lyon et al. 1993; Thomas et al. 1995; Jin et al. 2005). But the limitations involved with this method are that: it is laborious, time-intensive procedure, which may take as long as 6–9 months to obtain embryogenic calli from transformed plant cells. It would lead to the development of a few embryos, problems of embryo germination and formation, low percentage and abnormal plantlets which ultimately result in low transformation efficiency and plantlet regeneration. Due to these reasons sometimes it takes 10–12 months to regenerate plantlets for even greatly receptive genotypes. There is evidence that *Agrobacterium* strain, co-cultivation time duration and temperature, bacterium optical density, addition of acetosyringone for the period of co-cultivation and embryogenic calli condition would obviously affect the transformation efficiency (Firoozabady et al. 1987; Fullner and Nester 1996; Li et al. 1996; Jin et al. 2005).

6 Steps to Achieve Transformation

There are many transgenic crops which have been released for commercial production due to considerable progress in plant genetic engineering technology. Progress in the technology has been made due to the improvement in a variety of appropriate tissue culture techniques for regenerating whole plant from single cell or tissues in a large number of plant species along with the development of improved or efficient transformation methods. Hence any type of totipotent plant tissue or the one containing the latent capacity is appropriate for genetic engineering (Sanford et al. 1987; Aragão 2002). Revealing the key events that could effectively be integrated into plant breeding program to develop commercially important transgenic crops, it is significantly important that recovery of fertile transgenic plants may be higher.

6.1 Tissue Culture

Production of virus-free germplasm through tissue culture using shoot meristem or apex cultures is an important discovery. The ornamental nursery industry became very popular after this discovery and fastly growing shoots of many virus infected clones produced the virus free plants (Morel and Martin 1952). Different scientists reported that MS basal medium directly regenerated the plants from inoculated shoots (Murashige and Skoog 1962; Shabde and Murashige 1977). The same medium has been used to regenerate other crops such as *Gossypium* (Gould et al. 1991a), maize (Gould et al. 1991b) and cereals (Hiei et al. 1997). Tissue culture is considered as an essential step for transformation of the majority of plant species, consequently higher priority is needed to establish research procedures that could reduce the somaclonal variation (Birch 1997). Somatic embryogenesis after callus formation would lead the somaclonal variations or genetic mutations but plants produced through shoot apex show very few somaclonal variations or genetic mutations. Because the shoot apex culture lacks the callus formation and somatic embryogenesis step (Gould and Magallanes-Cedeno 1998) which ultimately diminish the activation of retrotransposon activity in cultured plant tissues and avoid creating permanent mutations or somaclonal variations (Hirochika 1993).

Before plant transformation most important thing for the successful tissue culture systems is the accessibility of enough number of cells with latent capacity, because such cells are required to be easily available for genetic transformation treatment. Moreover they are expected to preserve the cell proliferation, regeneration capacity and selection treatment during or after transformation procedures. Somatic embryogenesis and establishment of successive regeneration of plantlets through callus derived lines is complicated and prolonged process. Limitation factor involved with the meristematic cell culture is that the cells are fewer in number and even within those the regenerable cells are very less. As a result the plantlets regenerated or the explants act in response to selection medium are less in quantity

(Firoozabady et al. 1987; Sunilkumar and Rathore 2001). However, once the development of an embryogenic calli from the preliminary material even from a single callus line in any commercial cultivar is established, then it may strengthen the corresponding, several transformation experiments (Rajasekaran et al. 2000; Leelavathi et al. 2004).

6.2 Gene Transfer

Plant biotechnology has enormous potentiality for genetic improvement. Accessibility of appropriate tissue culture system, correct selection of transformants and revival of transformed plants are the pre-requisites for the development of an efficient system for gene transfer technology. Presently the most extensively used techniques for plant genetic engineering or modifications are the *Agrobacterium*-based and particle bombardment through gene. In particle in the bombardment using metal beads as transporter, the DNA is directly incorporated into the host genome. This method has been employed for several years to transform many of the plant species related to cereals, legumes and woody plants (Genga et al. 1991; Aragão et al. 1992; McCabe and Martinell 1993; Zapata et al. 1999; Altpeter et al. 2000).

Dicot crops like cotton, maize have successfully been transformed for many years by applying *Agrobacterium*-mediated method of transformation, but by the passage of time, efficient procedure has been established for monocots (Yu et al. 2000; Tu et al. 2000). Generally, the *Agrobacterium*-based transformation system has benefits over gene gun particle bombardment as it has more chances for stable expression of transgene for next generation and larger insertion frequency for low copy of transgene (Dai et al. 2001; Shou et al. 2004; Travella et al. 2005).

Conventional transport of T-DNA has been involved in the process of *Agrobacterium* infection (Zambryski 1989; Hansen and Chilton 1999). VIP1 and VIP2 are known as the VirE2 interacting proteins (Tzfira and Citovsky 2000). Nuclear import of VirE2 has been promoted by VIP1, a bZIP protein has been expressed in yeast. VIP2 is a protein thought to mediate interaction between chromatin proteins and transcription factor's complex (Frolov et al. 1998; Tzfira et al. 2000) thus providing evidence that intranuclear transport or T-strand integration might be promoted by VIP2. Advanced evidences indicate, VirE3 is also exported into the host cell in addition to bacterial proteins (VirD2 and VirF) and T-DNA. A majority of these proteins are transported discretely from the T-DNA (Vergunst et al. 2000, 2003; Schrammeijer et al. 2003; Cascales and Christie 2004). These *Agrobacterium* proteins participate for key role inside the host cell during genetic transformation. Thus, the function of VirE3 demonstrates that bacterial virulence protein could be replaced by the cellular protein VIP1 inside the host cell. This will interact with and facilitate the nuclear import of VirE2 and this will lead to the successive expression of T-DNA through a-mediated pathway "karyopherin" (Lacroix et al. 2005).

During transformation premature expression of T-DNA corresponds to the stage that instantaneously pursues the nuclear import. Effectiveness of T-DNA expression has been confirmed by inoculating the leaf disks derived from the VIP1 antisense, wild-type, and VIP1 antisense/VirE3 double-transgenic plants transformed with *Agrobacterium* which carries its T-DNA a uidA gene encoding GUS reporter (Tzfira et al. 2001). Total numbers of GUS-expressing areas per transformed leaf disk are determined by the histochemical assay and enzymatic activity in the transformed tissues. The regulatory sequences for expression in bacteria are deficient in the *uid*A gene contained on the T-DNA (Janssen and Gardener 1990), therefore the T-DNA correspond to direct the GUS activity after it is transported to the plant cell other than or except its liable leaky expression in *Agrobacterium* (Tzfira et al. 2001). When comparison was made between the wild-type plants and VIP1 antisense *Agrobacterium* inoculated plants, more or less 25–35% GUS activity was exhibited by the inoculated plants. Encouraging or optimistic outcome has been obtained when the regenerating cells were estimated for transient assay after gene transfer. But based on negative results from transient assay, it is injudicious to anticipate too earlier. It is better to understand the restrictions distressing such type of expression. The level of expression of a transgene is variable and is subjective to various features, for example the site of integration (Matzke and Matzke 1998). Transgene stability also varies among transformants and some plants show a variety of instability even in subsequent generations (Datta et al. 2002).

6.3 Selection

A considerable number of true putative transformants are expected to be regenerated after transformation. For selection of transformants, genes confronting to a selective medium containing an antibiotic (Steinitz et al. 2002; Wang and Tu 2004), genes conferring a phenotype allowing visual or substantial selection (Jefferson 1987; Hadi et al. 1996; Christou 1997; Chen et al. 1998; Sunilkumar and Rathore 2001; Francois et al. 2002) or screening through molecular analyses like PCR to classify plants containing foreign genes (Datta et al. 2002; Jin et al. 2005; Chakraborti et al. 2008) can be used to select putative transformants.

It is reported that production of chimeric primary transformants comprised of transformed germline cells, like the intermediates in the development of homogeneous transformed R_1 progeny plants, the transformation systems usually involve the initial screening to a certain extent but not the toxic selection to get the primary transformants (Gould and Magallanes-Cedeno 1998; Zapata et al. 1999; Datta et al. 2002; Chakraborti et al. 2008). Important factor for *Agrobacterium* transformation in the inoculation of apical meristematic cells and explant is the selection or intensity of an antibiotic adapted to the activity level of promoter This would be helpful to avoid killing the transformed tissues. In R_0 the regenerated plants are ought to be chimeric and presence of transferred genes may be in less than single copy or they may be present as multiple insertion events. Progeny from R_1 or R_2 individuals of

self or cross pollinated plants are best to use or to confirm for Southern blot analysis. In this way the germ line revealing the transformation event that was present in the transgenic progeny without chimerism will be selected (Maqbool et al. 2002). In majority of the plant tissues, high level of expression during the selection or all through the life cycle has been produced by the selectable marker genes which are driven by the constitutive promoters. A good example for this is the promoter ASA2 as it drives adequate *hpt* expression which is strong enough for selection on hygromycin containing medium in soybean somatic embryogenic or callus cultures. Moreover, in most of the plant tissues extremely small level of expression is found but in mature maize seeds, there is no expression (Zernova et al. 2008).

A permissive protocol for selection has the key importance to perform the transformation procedures since the plant regeneration system is associated with the viability of the explant. Normally it is expected that, for selection, the promoter along with the *neo* selectable marker resistant gene is required to be active in the meristematic region of the explant. If the meristematic cells are dead or weak, the regeneration is stopped or the cells will not proliferate and the inheritance pattern is modified or come to an end. An optimistic level of kanamycin (10–30 mg/l) is anticipated to be used to select the inoculation of the small shoots for *neo* gene driven by the *nos* promoter in *nptII*. The *CaMV* 35 S promoter does not play dynamic role in the plant shoot apical meristematic cells. Yet if this promoter is used with the selectable marker gene, it will not defend these lively or active cells. Size or dimension and method to prepare the shoot apex explant is very important for the selection of the antibiotic concentration. Small amount of selective agent is suggested for the initial selection process or gradually enhance the selection level if the escapes are extensively produced.

6.4 Transgene Expression

Both the methods of transformation (*Agrobacterium*/gene gun) are known for the stable integration and expression of the foreign genes into the plant cells (Briddon et al. 1998; Finer et al. 1999; Aragão 2002). To observe the transient expression, tissues are bombarded/cocultivated and cultured for a specific duration, generally 24–48 h. Then the tissues are evaluated for the expression of transgene. Qualitative and quantitative measurement of transgene expression is done by reporter gene such as *uidA* (Jefferson 1987), luciferase (Millar et al. 1992) and GFP green fluorescent protein (Davis and Vierstra 1998). It is possible to determine the factors responsible for affecting the gene expression in various plant tissues. This possibility is effective by studying the outcome of mutations, role of introns, codon usage and effectiveness of promoters. Regulatory sequences are modified by deletions or mutations and bombarded into plant cells. Control elements are identified by dissection and promoters are studied for up or down-regulation under that specific physiological condition (Vain et al. 1996; Komari et al. 1998; Finer et al. 1999). Effectiveness of transgenes and promoters are estimated through transient expression assays before commencement of the plant genetic engineering (Jefferson 1987; de-la Riva et al. 1998).

For expression of Vir proteins, plasmid study is also important. Either one of the single plasmid, GFP-VirE3 or GFP-VirE3- mNLS12 from pEGFP-C1-VirE3 or pEGFP-C1-VirE3-mNLS12 were first cloned into the *Nco*I-*BamH*I sites of pRTL2-GUS as a replacement for GUS. After that 35 S promoter-GFP-VirE3-terminator or 35 S promoter-GFP-VirE3-mNLS12-terminator cassettes were moved into the *Sph*I site of pGDR, which already contained a DsRed2 gene driven by the 35 S promoter. This resulted in the construct pGDRGFP- VirE3 and pGDR-GFP-VirE3-mNLS12 (Citovsky et al. 1992; Goodin et al. 2002). VirE3-VirE2 correlation is significantly prone for infection of *Agrobacterium* because the VIP1 antisense plants significantly elevate the susceptibility of VirE3 for transgenic expression by *Agrobacterium* during genetic transformation. Therefore it has been proven that, the function of VIP1 throughout the *Agrobacterium* infection of plant cells is accomplished by VirE3 (Lacroix et al. 2005).

External factors such as temperature and pH also affect the activation of *vir* system. The *vir* genes are not expressed at higher temperatures, since the inactivation of its properties induces a conformational change in the folding of *vir*A (Jin et al. 1993). *Vir*Gc trigger the constitutive expression of *vir* genes. *Vir*Gc is a mutant form of *vir*G which suppress the effect of temperature on *vir*A. However, that temperature does not confer the virulence competence of the mutant for *Agrobacterium*. This may be due to the other proteins that effectively may participate in the transfer of T-DNA process undergoing the folding at high temperature (Fullner and Nester 1996).

Occurrence of gene silencing is a reasonably normal phenomenon for genetic engineering of plants which is wounded up by the presence of multiple copies within the plant genome. Function of cytosine methylation and cosuppression mutually is consequential for down-regulation of transgene expression which is sometimes very incompatible and irregular (Komari et al. 1998; Finer et al. 1999; Iyer et al. 2000; Dong and Li 2006). Methylation of promoter sequences mainly cause the gene silencing at transcriptional level and in this manner chromatin re-modeling proteins are attracted to these sites by interfering with assembly of the transcription factors (Meyer 2000; Wang and Waterhouse 2002). Double stranded RNA is produced by the activation of co-suppression at the RNA level. This would lead to commence the degradation of the target RNA, thus following the gene silencing (Vance and Vaucheret 2001). It is common problem in plant genetic transformation since it avoids sustainable expression of a desired phenotype within transgenic plants. However, while unraveling mechanisms underlying this phenomenon, it is realized that the down-regulation of native genes within the plants is possible with the increasing knowledge.

Recent studies demonstrated that most of the genome is found to be transcribed at individual or multiple phases of cell growth therefore overall patterns of gene expression are vastly active throughout the maturity of epidermal plant cell (Hovav et al. 2008). Advanced skill to collect the particular types of cells in plants and amplification of mRNA for expression profiling has directed for the transcriptome analysis at the cellular level (Galbraith and Birnbaum 2006). Isolating cells via sorting practices or laser microdissection and capture are among the recent technologies known for the determination of changes during differentiation under in vitro or natural systems (Birnbaum et al. 2003; Leonhardt et al. 2004; Casson et al. 2005).

Now microarray is a potent device offered for high throughput analysis of cell differentiation and progress and has made it possible to estimate the participation of thousands of genes in biological processes. Modern applications of this technology consist of wood-forming cambial meristematic tissues (Schrader et al. 2004), pollen transcriptome investigation of *Arabidopsis thaliana* (Pina et al. 2005), the quiescent-center cells of developing roots (Nawy et al. 2005), vascular tissue or epidermis cells of maize (Nakazono et al. 2003), and quickly growing cotton fiber preliminary cells (Wu et al. 2007).

6.5 Integration of Transgene

Genetic transformations of plants take place when tumor-inducing (Ti) plasmid transfer its single stranded copy of T-DNA into the host cell and then integrate that into the genome of the host cell. The current plant genetic engineering is based on this natural gene transfer principle. This phenomenon has been focused by taking out the tumor inducing genes from the bacterium. Then desired genetic sequences are substituted with most of the T-DNA region (Zupan and Zambryski 1997). Recently, using this concept the coat protein genes of several plant viruses have been transported into a number of plant species to develop resistance against viral infection. In this way the organisms engineered with genes derived from a pathogen may exhibit resistance against the pathogen itself (Vasudevan et al. 2008).

After particle bombardment integration of exogenous DNA takes place into plant's genome in the form of simple, single copy or complex multiple copies insertions (Christou 1997; Finer et al. 1999; Smith et al. 2001). This insertion of transgene process is considered to takes place in two-steps. Firstly, the exogenous DNA undergoes homologous recombination and then it is rearranged head to head and/or head to tail after the ligation. Fragmented plasmid and expression cassette's copies can also be interwoven collectively inside these concantamers. This recombination takes place within 30 min of the transformation process. Secondly, after rearrangement the DNA is introduced into the plant's genome. Integration of DNA is supposed to occur by the contribution of native DNA reparation along with the non-homologous recombination at sites of double stranded disruptions in plant's chromosome (Kohli et al. 1998; Puchta 1998).

Stable integration of introduced DNA, decreased somaclonal mutations and unforeseen segregation of transgene in successive generations are the important selection measures for an efficient transformation technology. Therefore, initially a large number of transformed plants should be produced or multiple genes should be introduced for testing or initial screening. Co-transformation technology assists the simultaneous introduction of important multiple genes. This is possible by using single selection marker gene (Francois et al. 2002; Sandhu and Altpeter 2008). Inheritance studies of the rice progeny produced with this technology showed that co-segregation of transgenes occurs altogether in the successive generation. This indicates integration had taken place at the same genetic locus (Taylor and Fauquet 2002). However if the marker and genes of interest are unlinked and they

integrate infrequently then this situation relies on the co-transformation methodology (Radhakrishnan and Srivastava 2005). Conversely, removal/modification in the DNA sequence existing in the host may happen by transposition phenomenon (Scholz et al. 2001).

Genes become silent at transcription stage if the same promoter and a polyadenylation signal for different marker genes have been used repetitively (Hohn et al. 2001). Hence it is proposed that the elimination of antibiotic resistant genes from transformed plants or selection for a distinct transgene insertion by segregating by the breeding technology will prove successful. For such situations tight association of the transgenes in the transgenic crops is disadvantageous. So the capacity to control the positions of multiple transgene integrations might be improved by employing the marker gene at restrained sites from the gene(s) of interest for this purpose. Thus, selectable marker gene elimination is considered to be critical for pyramiding of multiple characteristics in transgenic crop. Advances in the technology show that various approaches have effectively been used for exclusion of selectable marker gene from transgenic plants (Vergunst et al. 2000; Luo et al. 2000; Hoa et al. 2002; Zhang et al. 2003; Miki and McHugh 2004; Kopertekh et al. 2004; Kerbach et al. 2005; Radhakrishnan and Srivastava 2005; Sreekala et al. 2005; Cuellar et al. 2006; Darbani et al. 2007). Research in transforming plants need to concentrate on the complications related to the stable integration and consistent expression of the gene after the DNA has been integrated (Aragão 2002).

7 Constraints in Plant Transformation

7.1 Environment and Public

Genetically modified inventions in agriculture are not commercial goods marketed as such to the consumer. The research in advancements of genetically modified crops help out the researchers to yield these crops, perhaps along with novel genetic sequences and promoters and/or copyrights on upgraded research methodology. A commercial variety is then released in response to these transitional products (Pray et al. 2005). Since the commercialization of transgenic crops the cultivated land for GM crops has continuously been increasing. None of the negative affect has been observed on biodiversity, therefore many countries consider these crops safe for food and feed purpose. Though, new technologies in this area are in the process to produce the GM crops generating pharmaceutical agents or industrial raw materials. These products will be introduced to the market in near future. For such type of situations, crossing between genetically modified crops and commercial non GM crops is needed to be done under consistent protective processes.

Production of GM crops is one of the possible solutions for food scarcity among poor countries and presently a number of capable improvements are there. US universities and many biotech companies have declared not to ask for patent rights if the GM crop tools would be used to produce crops for developing or poor countries. The genetically modified crops with desirable improved characteristics will be having

simply smaller long-lasting impact. Providing farm financial assistance in the developed countries would persistently leave out farmers from the international market (Breithaupt 2003).

It is of enormous concern in crop biotechnology to avoid the flow of transgene from genetically engineered crops to other edible crops and their native species. Male sterility approach is being used for gene imprisonment in addition to decrease the consumers concerns of food purity (Konagaya et al. 2008). Issues relating to Biotech inventions are health (e.g. allergins, toxins, antibiotic resistance), environmental, social, ethical and religious concerns. Public is paying attention to the effects of agricultural applications on environment and human health. In agriculture sector, there is the development of regulatory and motivation programs which may influence the farming systems varying from the selection of agronomic applications to pest management.

The farming division is always being the key resource for improvement in the environment and food security technologies with these characteristics. The agricultural industry may perhaps preserve a small amount of economic profits out of these innovations. One way to do more with less is the theoretical approach to transfer the agricultural system technology from public to the private divisions. The key objectives here are: (i) to communicate the advantages of public research and development to prospective consumers, (ii) to discover the modern ways to execute the organization task in an era of comparatively limited assets, (iii) persuade to direct the technology improvement, (iv) attracting research resources through patent returns. These objectives may be achieved by direct contact between researchers and end users, sharing research findings through publications, utilizing patents and protecting technologies for copy rights and carrying out mutual scientific research (Rubenstein and Heisey 2005).

For recent advances, an online version of "Biosafety regulations of Asia-Pacific countries", published by the FAO, the Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB) and the Asia-Pacific Association of Agricultural Research Institutions (APAARI), is now available. The mechanisms for regulations associated to biosafety of agricultural and food biotechnological products offered in Asian and Pacific countries have been discussed in detail (http://www.apcoab.org/documents/bs_pub.pdf). The prospects of biotech crops seems to be hopeful as the number of countries adopting biotech crops and the area under cultivation, number of crops and the improved traits are estimated to grow twofold during 2006–2015 when large number of farmers will prefer to grow biotech crops and it may possibly be raised up to ten times to 100 million or more than that till 2015. Presently the major limitation to increase the crop production throughout the world is water shortage. By 2011, drought tolerance genes are likely to become accessible principally essential for developing countries suffering comparatively more from drought conditions (James 2007).

7.2 Intellectual Property Issues

Transgenic plants have been produced after the efforts of several years but following the overcome to technical limitations, marketable restrictions happen to be more severe

obstacle for utilization of genetic engineering. Cultivation and commercialization of transgenic crops in different countries has brought about the considerable economical benefits during the last decade. None of the serious concerns linked to biosafety, environment or pest resistance has been noticed. Modern biotechnology leads to the innovations in products, medicines, agriculture, food, soil rehabilitation, health, environment protection and industry etc. These innovations lead to the advances for inventions in the biotechnological field like: cDNA, mRNA, synthetic DNA, proteins, peptides, fragments, monoclonal antibodies, vaccines, vectors, microorganisms, host cells, cell lines, transgenic and cloned animals, laboratory apparatus, tissue engineering, production methods and gene therapy protocol. For biotechnological inventions patents should relate to the details of the process through which the biological material is prepared, processed or used. Biological material shall mean that the material having genetic sequence competent to reproduce itself or being reproduced while using in biological method.

A monopoly right granted to an inventor is a patent. This right enables the patentee to use his invention as he may use it by making and selling the product or he may authorize others to do so. A Patent remains valid for 20 years subject to renewal. It entails the creative activity and subjected to be used in any kind of industrial as well as agricultural relevance. Patent shall be granted for a new or novel invention and an invention is considered to be novel if it has not been publicized in the prior art. Patents provide benefits like (1) to encourage research and inventions, (2) to disclose new technological discoveries, (3) to disseminate technical information and know-how, (4) to make transfer of technology possible, (5) to create an atmosphere for investments. Policy formulation, administration and enforcement of intellectual property rights are improved by modernising the intellectual property right (IPR) legislation, creating more awareness of the role of IPR for economic growth and trade development and supporting the establishment of a local IPR Organisation. Industrial revolution has modernized the patent structure and modified the system to shelter the mechanical and chemical inventions. There is no management to offer the security to living organisms. In fact, living organisms are supposed to be the breakthrough by nature, but not the scientific innovations (http://www.opendemocracy.net/ecology-foodwithoutfrontiers/article_1817.jsp).

8 Future Needs for Research and Development in Plant Transformation

8.1 Transformation Efficiency

It has been well documented until now that proficient regeneration system is required for the transformed tissues, whatever the method of transformation is used either *Agrobacterium* or biolistic gun, these two process i.e. transformation and regeneration are closely intermingled. Although there are outstanding achievements and

novel practical progress has been made in plant biotechnology, but still there are shortcomings for the development of an efficient and genotype independent regeneration protocol to develop genetically engineered plants/crops. A number of factors can provide the boost to the transformation efficiency such as choosing the right strain of *Agrobacterium*, stimulation of *vir* genes with or without acetosyringione, temperature and duration of cocultivation and culture medium etc. (Gawel and Robacker 1990; Kumar et al. 1998; Wilkins et al. 2000; Popelka and Altpeter 2001; Sunilkumar and Rathore 2001; Mishra et al. 2003). Somatic embryogenesis would lead to the regeneration through a probable single-cell origin of the somatic embryo, therefore this method is favorite as compared to organogenesis, consequently reducing the chimerism (Merkle et al. 1995). Conversely the problems associated with regeneration via somatic embryogenesis are: time consuming, rate of recurrence of abnormal embryos is high, problems with embryo germination, problems associated with root and shoot development (Kumria et al. 2003).

The effectiveness of embryogenesis in a cultivar may be assured by adopting a stable and highly competent transformation system that can curtail the time of tissue culture. It was observed that chances of somatic embryogenesis of transformed cells may be increased by using embryogenic calli as explant propagated from a distinct somatic embryogenic callus. Thus it will increase the transformation efficiency and more number of transgenic plants will be produced in less time. Somatic embryogenic calli have effectively been used as explant in plant genetic transformation system (Scorza et al. 1996; Martinelli and Mandolino 2001; Wu et al. 2005). Moreover, stimulating the cell division by phytohormones at the time of cocultivation may enhance the *Agrobacterium* transformation in plant cell cycle at the particular phase (Gelvin 2000). Therefore, adoption of novel methods for cell and tissue culture and genetic transformation acquire considerable assurance to establish innovative genetic variability for progress of agriculturally important crops (Chahal and Gosal 2002; Rashid et al. 2004; Gill et al. 2006).

8.2 Genetic Damages

For the achievement of high number of transformants, an efficient transformation procedure that allows the desired integration of introduced genes and selection of events without severe somaclonal variation and unexpected segregation is needed. Traditional breeding techniques have been used since a long time to introduce the superb varieties of cotton. Nevertheless cotton breeding technology is restricted by being deficient in satisfactory genetic differences in the accessible germplasm collection (Wu et al. 2005) or the absence of various key characteristics like tolerance to insect pests and herbicides as in the genetic pools of many cultivars of sugarcane (Arencibia et al. 1997). Plant genetic engineering assists in the improvement of qualitative and quantitative characteristics and produces a number of agriculturally important crops with improved tolerance/resistance against insects, diseases, microbes, herbicides and abiotic stress. Similar molecular mechanism is employed

for the transfer of T-DNA to potential dicot and monocot crops in the presence of an appropriate transformation procedure. This capability is improved by insertion of a selectable marker gene at different position from the desirable gene(s), to control the integration transgene at multiple locations is consequently required to explore the plant biotechnology related research. After laboratory, it is important to investigate the performance of transgenic plants under open field conditions that include evaluating the affect of transgenic plants on other plants in the field and environmental conditions. Furthermore, it is also important to evaluate the stability of foreign genes and issues associated with their interaction with the complicated physiological performance of these transgenic plants.

8.3 Ideal Transformation Systems

Pre-requisite for the development of genetically modified plants is the potential for introduction of foreign DNA into the plant genome and after that restoration of fertile plants. It is also important that the transgene(s) may have inherited, integrated and expressed successively into the genome of progeny plants. Particle gun and Agrobacterium- are the most commonly used techniques for this purpose with varying level of accomplishment. Somatic embryogenesis leading the regeneration of transgenic plantlets is laborious and overwhelming which ultimately results for somaclonal variation. Another drawback is that there are insufficient cells for regeneration competence in the meristematic region. This will limit the number of cells to be selected against an antibiotic. Somaclonal variations may be reduced by maintaining the culture condition/time at different stages of the tissue culture procedure. This is possible by taking care of the appropriate phase of cell proliferation and subculturing at proper time. This procedure may be different for each crop or species. Consequently, putative transgenic plants must be selected with substantial efforts and incompetent/ non-integrated copies of the transgene(s) should be removed. Transgenic lines with stable inheritance, integration and expression of the foreign genes are to be screened for future breeding programs and commercialization of transgenic crop. In summary, preparing ourselves and selecting the proper methods would lead us to unravel the troubles involved to achieve the practical application of the aims and objectives.

8.4 Genetic Transformation vs Plant Breeding and Heritable Assortment

Plant transformation system independent of genotype is one of the key successes to promote the genetically modified crops technology. For academic purposes or fundamental comprehension related to the gene introduction, integration and expression of transgene in the transgenic plants, model plants of a particular genotype may be

used at laboratory level easy. These model plants are easy to study and understand the basic function of gene expression related studies.

As the Agrobacterium-mediated transformation method is species or genotype specific therefore, many issues are required to be considered for optimization of this procedure to establish the efficient transgene integration and expression in different species. These issues may be positive interaction of Agrobacterium and cells to be transformed and a proficient tissue culture procedure to restore the plants after transformation.

In the past, important crop varieties have been genetically modified with improved and desirable traits by conventional breeding programmes. But these conventional breeding techniques are time consuming, laborious and occasionally complicated to generate target tissues for diverse and considerable crops or their species. This is also true with some horticultural or vegetatively propagated plants like potato, cassava and banana to continue with conventional breeding. Single gene or oligogenic plant transformation system direct towards extreme genetic consistency in self as well as cross pollinated plants, but the conventional breeding technology involve the whole genome which is sometimes detrimental. There is need to develop de novo introduction and integration of the transgene for the essential cultivars (Taylor et al. 2002). Therefore it is important to remove undesirable sequences during genetic transformation of divergent genotypes with multiple genes within specie to introduce desirable physical characters. This will help to improve the genetically modified crop plants (Birch 1997). Genetic diversity and its intensity for certain crop specie most likely selected for breeding may be useful to study its evolutionary background (Zhou et al. 2008).

Recently, various agriculturally essential characters affecting the Oat crop were found to be correlated with at AFLP markers studies. This means that the comprehensive studies or investigations would be helpful to improve the genetic diversity. Ultimately marker assisted selections will confirm the selected traits in segregating populations (Achleitner et al. 2008). Further progress in this scenario is that to expand the new molecular markers, mapping of the regions of quantitative loci (QTL) has been proposed. It is documented that the outcome of single or joint QTL alleles is expected to be more perfect in an identical genetic background (Delourme et al. 2008; Holzapfel et al. 2008). Several alleles or quantitative trait alleles at each QTL affect to a particular attribute i.e. for every QTL known, one to three alleles affect that specific trait (Aitken et al. 2008). Moreover molecular marker assisted selection is a consistent technique to validate the additive effect of QTL for the development of the near isogenic lines (NILs) for quantitative trait loci (QTL).

8.5 Marketing of Genetically Modified Plants

Genetically modified plant technology has valuable impact on the recent plant improvement/breeding policies. Basic research involves the laboratory experiments and after that field trials are conducted. These field trials are only conducted for

research purpose. Independent homozygous or true to type lines of transgenic plants will be selected. Ultimately this would direct a new variety to public and commercialization. Applied research is must after the basic, to ensure the application of the inventions. Scientific data is mandatory to deal with the issues related to public and ethical matters. Solution of legal or regulatory matters will be helpful to extend the GM products and ultimately this will raise the productivity and improve the quality of foods. In this way people will be benefited all together by having the products which are modified with environmentally friendly traits.

9 Conclusion

The prospective for applications of plant biotechnology to agriculture sector are significant for the assistance to mankind. Presently the adoption of GM crops is recognized at international level. The first commercial transgenic crop was cotton which was engineered for resistance against insects or herbicide. It was first time released in 1996 for commercial production on only 12% of the area for cotton cultivation in United States. By 2007, the countries growing GM crops went up to 23, and among these 12 are developing and 11 developed countries. According to area these countries are in the sequence of USA, Argentina, Brazil, Canada, India, China, Paraguay, South Africa, Uruguay, Philippines, Australia, Spain, Mexico, Colombia, Chile, France, Honduras, Czech Republic, Portugal, Germany, Slovakia, Romania and Poland.

All problems related to food and crop production would not be solved by adoption of agricultural biotechnology. Indeed, these issues may be helped out by improving and adopting these technologies. Judicious classification of food allocation is desirable along with adoption of plant biotechnology which essentially can facilitate to integrated food security approach.

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Chapter 11
Transcriptomics and Proteomics Analysis of Root Nodules of Model Legume Plants

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Contents

Abstract Leguminosae is a large and economically important family of plants that, because of their capacity to fix atmospheric nitrogen in symbiosis with rhizobia, are essential components of agricultural ecosystems and an important source in the production of food, feed, forage and other compounds with strong industrial and commercial relevance. During the last decade, the 'omics' technologies of legumes especially the model legumes have provided and still provide unprecedented amount of molecular information that has to be understood in a physiological, developmental and organismal context. New technologies, for example high-throughput sequencing, high multiplexed mapping techniques and rapid development of new bioinformatics tools have provided new information about current model systems and emerging new

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models as well. The latest information on the status of development of genomic resources and of related information in model legumes and some other economically important legume crops are summarized in this review. The infection of the plant root by rhizobia triggers several important events in the root cell, resulting in the formation of a nodule – a nitrogen-fixing compartment. Some of the signal molecules involved in the communication between the symbiotic partners have been studied but little is known about the genes and proteins involved in membrane and protein trafficking and targeting towards the symbiosome-the cellular compartment containing the nitrogen-fixing bacteria in the nodule, which is one of the most important processes in nodule formation and development. The central point of this review is to summarize the recent developments in functional genome, transcriptome and proteome of model legume plants specially *Medicago truncatula*, Lotus japonicus and *Glycine max*. A multidisciplinary approach, including plant and bacterial genetics, molecular biology, live cell imaging, biophysics and bioinformatics has been taken to understand the plant-microbe interaction and the biogenesis of root nodules and the genes and proteins involved in nodule formation and efficient nitrogen fixation. Vesicular trafficking plays an important role in rhizobia- root interaction, infection thread formation and the development of root nodules. I have described the role of several small GTP binding proteins in symbiosome formation and root nodule development. Furthermore, there is an increasing need to efficiently convert scientific results into practical applications or products. The economic and environmental costs of the heavy use of chemical N fertilizers in agriculture are a global concern. For this reason legumes could be used as an alternative source for N fertilizers and this would help for cleaning up the environmental pollution caused by chemical fertilizers. Additionally nitrogen-fixing biological systems represent an economically attractive and ecologically sound means of reducing external inputs and improving internal resources. I hope the scientific information given in this review paper could not only be useful to the molecular geneticists and plant breeders but will also assist the agronomists to carry out their research efficiently.

Keywords Model legume plants • Genome • Transcriptome • Proteome • Root nodule • Symbiosis • Small GTP-binding proteins

1 Introduction

The legumes comprise the third largest family of flowering plants with one of the highest economical value in agriculture. Several members of this family, including soybean, alfalfa, peas and beans, have been fundamental to the development of modern agricultural systems. On a worldwide basis, this plant family contributes 33% of humankind's protein intake, while also serving as an important source of fodder and forage for animals, and of edible and industrial oils. Legumes are also distinguished by their unique property of symbiotic nitrogen fixation, providing one of the major sources of available nitrogen in the biosphere (Singh and Jauhar 2009).

It is estimated, that between 40 million and 60 million tons of nitrogen are fixed annually by cultivated legumes (Smil 1999) equivalent to about US$10 billion fertilizer (Graham and Vance 2003). Grain legumes (common bean, pea, lentil, chickpea, and others) are the major, if not the only, source of dietary protein for a large proportion of the population in South Asia, Middle East, North Africa, and other impoverished regions of the world (Singh and Jauhar 2009). Turkey is one of the major producers of grain and fodder legumes. Among the legumes produced in Turkey, chickpeas, lentils and beans hold the most important place and account for 44%, 35% and 18% respectively of the total legume production in Turkey (Anonymus 2000). Legumes also play a positive role in crop rotations with cereals and help replenish soil's nitrogen supply. Turkey is also one of the major gene centers of the legume crops especially for forage legumes (alfalfa and clover). These forage crops are grown as pastures in almost all regions, particularly in eastern and central Anatolia (Anonymus 2000).

Alfalfa has one of the highest nutritional quality as animal feed and contains between 15% and 22% crude protein. It serves as an excellent source of vitamins and minerals. Specifically, alfalfa contains vitamins A, D, E, K, U, C, B1, B2, B6, B12, niacin, panthothanic acid, inositol, biotin, and folic acid. Alfalfa is also high in minerals phosphorus, calcium, potassium, sodium, chlorine, sulfur, magnesium, copper, manganese, iron, cobalt, boron, and molybdenum, as well as trace elements such as nickel, lead, strontium, and palladium (Bickoff et al. 1972). The alfalfa sprouts are directly consumed by humans and its juice is also used in some health food products. Additionally alfalfa and other legumes are reported to be used as a biofuel for the production of ethanol and biodiesel. Alfalfa, clover and broad bean are found to be good candidates for the bioremediation of soils contaminated with high levels of organic compound especially petrol hydrocarbons (Cigdem et al. 2011). Alfalfa is also used for the production of industrial enzymes such as lignin peroxidase, alpha-amylase, cellulase, and phytase (Carroll and Somerville 2009).

2 Legume Genome Sequencing Strategies and Status

Soybean (*Glycine max*), bean (*Phaseolus vulgaris*), pea (*Pisum sativum*) and lentil (*Lens culinaris*) are the most important crop legumes in terms of food nutrition. They are difficult to study because of their large genome and low transformation capacities. The model legume species, *Lotus japonicus* and *Medicago truncatula*, are the accepted model legumes in which root-bacterial interaction, infection process, nodule formation and development and the genes and proteins involved in these processes are widely studied. Both plants have a small diploid genome (470–550 Mp) and provide a rich source of genetic and genomic information which could be utilized in understanding the genomic structure of other important legume crops. These plants have a short life cycle, are self-fertile and can be transformed easily (Cook 1999). *M. truncatula* is closely related to other temperate legumes, such as clover, pea, vetch, chickpea and lentil. *L japonicus* is more distantly related to tropical

legumes, such as soybean and bean (*Phaseolid clade*) (Ane et al. 2008). The Phaseoloids are the other major taxonomic group that contains many economically important legume crops including soybean, the legume of the greatest economic importance, for which extensive genomic information is available and is part of a genomic sequencing project (see http://soybase.agron.iastate.edu/). Soybean is proposed as a third model legume in addition to *M. truncatula* and *L. japonicus* because of its economic importance and the phylogenetic proximity to other major crops (Mathesius et al. 2008). In contrast to soybean, the development of the model legume systems in *Lotus* and *Medicago* were given more importance because of their small genome size and relatively easy to transform. *Medicago truncatula* and *Lotus japonicus* are also amenable to efficient molecular, genetic and reverse-genetic analyses, unlike the major crop legumes. This was driven by the discovery of several mutants that are defective in both mycorrhizal and rhizobial symbiosis (Duc et al. 1989; Marsh and Schultze 2001), so that the use of the genetic information from these model species facilitated the genomic sequence project of other economically important grain legumes. In fact this was the objective of the EU framework 6 funded project on 'Grain Legumes' (www.eugrainlegumes.org).

The *Medicago truncatula* sequencing project was first initiated by Samuel Roberts Noble Foundation affiliated with University of Oklahoma. The National Science Foundation and the European Union's Sixth Framework Programme provided funding in year 2003 to complete sequencing of the remaining euchromatic genespace. Six chromosomes are being sequenced by NSF project "Sequencing the Gene Space of the Model Legume, *Medicago truncatula*," and two are being sequenced by partners in Europe (Ane et al. 2008).

This project used a clone-by clone approach, in which BACs, with average insert size of approximately 120 kb, were sequenced and used to extend BAC-contig tiling paths to produce increasingly large sequence contigs. Contigs are anchor oriented using genetic markers developed from a large proportion of the BAC sequences. These BAC contigs and sequences cover approximately 60% of the major euchromatic regions of the *M. truncatula* genome (Young et al. 2003; Cannon et al. 2005; Young and Udvardi 2009).

A combination of cytogenetic and BAC sequence data show that the *M. truncatula* genome is organized into distinct gene-rich euchromatin separate from repeat-rich pericentromeric regions. This map-anchored, high-quality sequence is extremely valuable as a basis for genomic comparisons with other plant genomes, and laid a foundation for improving many crop and forage legumes. Nevin Young (University of Minnesota), Bruce Roe (ACGT, University of Oklahoma; (chromosomes 1, 4, 6, 8), and Chris Town (JCVI; chromosomes 2, 7) are principal investigators of the U.S. project. In Europe, collaborators include Giles Oldroyd (John Innes Center, UK.) coordinating sequencing of chromosome 3 at the Sanger Center, and Frederic Deballe (INRA-CNRS) coordinating sequencing of chromosome 5 at Genoscope, France. Genetic markers and maps were developed by collaboration between the University of California-Davis, the University of Minnesota, INRA-CNRS Toulouse France, and the Biological Research Center Institute of Genetics in Szeged Hungary. Cytogenetic analysis is underway at Wageningen University. Genome annotation is

Table 11.1 Web based genomic resources of Medicago and related legume species

Medicago genome sequence sites	
Medicago Genome Sequence Resources	http://www.medicago.org
TIGR *Medicago* Database	http://www.tigr.org/tdb/e2k1/mta1/
University of Oklahoma Sequencing Project	http://www.genome.ou.edu/medicago.html
European *Medicago* Genome Database	http://mips.gsf.de/proj/plant/jsf/medi/index.jsp
Medicago functional genomics sites	
Noble Foundation *Medicago* Gene Expression Atlas	http://bioinfo.noble.org/gene-atlas/
Noble Foundation Medicago Mutant Database	http://bioinfo4.noble.org/mutant/
Noble Foundation Medicago Metabolic Pathways	http://mediccyc.noble.org/
Medicago Handbook	http://www.noble.org/MedicagoHandbook/
Comparative legume genomics sites	
Legume Information System (LIS)	http://www.comparative-legumes.org/
INRA/CNRS Integrative Legume Resources	http://www.legoo.org/
Legume Genome Website Links at LIS	http://www.comparative-legumes.org/lis/lis_links.html
Other legume genome sites	
Lotus japonicus Genome Browser	http://www.kazusa.or.jp/lotus/
Lotus japonicus Legume Base	http://www.shigen.nig.ac.jp/bean/lotusjaponicus/top/top.jsp
Phytozome *Glycine max* Genome	http://www.phytozome.net/soybean
	http://www.soybeangenome.org/
	http://rsoy.psc.riken.jp/
	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=soybean
Soybase and Soybean Breeders Toolbox	http://soybase.org/
Phaseolus vulgaris (common bean)	http://phaseolus.genomics.purdue.edu/
	http://www.ccg.unam.mx/phaseolusest/
	http://www.phaseolus.net/
	http://beangenes.cws.ndsu.nodak.edu/
Pisum sativum (pea)	http://urgv.evry.inra.fr/UTILLdb
Vigna unguiculata (cowpea)	http://cowpeagenomics.med.virginia.edu/CGKB/
Arachis hypogaea (peanut)	http://peanutgenetics.tamu.edu/cmap
Trifolium pretense (red clover)	http://clovergarden.jp
Tree of life: Fabaceae	http://www.tolweb.org/Fabaceae
International Legume Database	http://www.ildis.org/

being carried out by the International *Medicago* Genome Annotation Group (IMGAG), which involves participants from INRA-CNRS, JCVI/TIGR, NCGR, MIPS, MPIZ, UMN and VIB-Gent. A single uniform set of annotations (with periodic updates) of the gene-rich pseudomolecules, the unanchored BACs and the Illumina assemblies not captured by the BAC-based project are hosted at JCVI and MIPS (www.medicago.org/genome, see also Table 11.1).

Now the international *Medicago truncatuala* (*Mt*) genome sequencing consortium is in its final phase, with ~2,600 unique BACs has already been sequenced. Assembly of these BACs, guided by an optical map of the entire genome, resulted in 8 pseudomolecules spanning ~375 Mb and containing ~250 Mb of sequence, plus ~150 sequenced BACs that do not have chromosomal assignments. Annotation of the unmasked pseudomolecules and orphan BACs resulted in the prediction of around 58,000 gene models of which ~11,500 appear to be transposon-related. Overall, the about 35% of *Medicago euchromatin* appears to be transposon-related. Assembly of Illumina whole genome sequence generated by the Hap-Map project produced an additional ~55 Mb of unique sequence containing ~20,000 gene models (www.medicagohapmap.org/genome). Whole genome comparisons reveal a high degree of synteny with Lotus and soybean, but duplication blocks within the *Medicago* genome are relatively short (~500 kb). Analysis of duplicated genes and expression patterns suggests that a genome duplication early in the 'papilionoid' legume group (containing e.g. soybean, *Medicago*, and peanut) was important for the evolution of the symbiotic nitrogen-fixing nodule in this group of legumes; but also that an even earlier genome duplication may also have played a role in evolution of this capacity (Town 2006; Verdier et al. 2008).

Whole-genome association studies (GWAS) in the model legume *Medicago truncatula* will enable the discovery of genes playing a role in the evolution of symbiosis. To achieve this goal, The International *Medicago truncatula* HAPMAP Consortium, including participants at the University of Minnesota, the National Center for Genome Resources, Boyce Thompson Institute, Hamline University, the University of Southern California, the Noble Foundation, INRA-Montpellier, ENSAT-Toulouse, and INRST-Tunisia, is developing a hapmap GWAS resource for *Medicago*. More than 300 inbred lines spanning the range of *Medicago* diversity are being resequenced using Illumina next generation technology (http://www.jcvi.org/cgi-bin/medicago/overview.cgi).

The *Medicago* genome is highly syntenic with alfalfa and pea and moderately syntenic with soybean. A project funded by the Noble Foundation and the University of Oklahoma is now sequencing 1,000 BAC clones, providing 100 Mbp of sequence and seed points for a minimum tiling path (George et al. 2008; Hougaard et al. 2008; Umezawa et al. 2008). Currently huge efforts have been taken by genetists and plant molecular biologists to develop resources in order to decipher the functions of many *Medicago* genes. These resources include a gene expression atlas/database that can be used to obtain developmental and other types of expression data for most *Medicago* genes, and two large mutant populations, a fast-neutron-bombardment-deletion and a Tnt1-insertion population, which can be used for both forward and high-throughput reverse genetics (Benedito et al. 2008; He et al. 2009). About 15,000 Tnt1 insertion lines have been generated in the R108 genotype and phenotypic screens found 177 of these lines to be defective in SNF. Flanking sequence tag (FST) analysis and a PCR-based approach have identified insertion alleles of the following known symbiotic genes amongst the mutants: DMI1, DMI2, DMI3, NSP1, NSP2, ERN1, NIN, LYK3, SUNN, SST1, HMGR1, SST1, IRE, and EIN2. High-throughput 454 sequencing

using a 2D-pooling strategy is being used to accelerate current FST sequencing efforts, which will lead to a comprehensive FST database for all Tnt1 insertion lines. The mutant resources and gene expression atlas have been used to identify novel symbiotic genes, including VAPYRIN, which is required for intracellular accommodation of rhizobia in nodules (Murray et al. 2011).

The *Lotus japonicus* (*Lj*) genome sequencing project is being carried out by the Kazusa DNA Research Institute in Japan (http://www.kazusa.or.jp). This project is also primarily using a clone-by-clone approach, sequencing transformation competent artificial bacterial chromosomes (TACs), with average insert size of approximately 100 kb. The clone-by-clone sequence is also being augmented by a combination of whole genome shotgun (WGS) and low coverage TAC sequencing. The *Lj* sequence coverage has spanned approximately 177 Mbp, or roughly 60% of the euchromatic regions of the *Lj* genome (Young et al. 2005; Cannon et al. 2006).

The *Glycine max* (*Gm*) genome is being sequenced primarily with a Whole Genome Shotgun (WGS) approach, with sequence coming from a combination of random reads, paired fosmid ends, and paired BAC end sequences (http://www.plantgdb.org/GmGDB). This project was initiated by combined effort of the U.S. Department of Energy's Joint Genome Institute (DOE_JGI Community Sequence Program) and the NSF and USDA-ARS (managing mapping and physical mapping components of the project) (http://www.phytozome.net/soybean, Umezawa et al. 2008). The sequence consortium was led by Gary Stacey, Randy Shoemaker, Scott Jackson, Jeremy Schmutz, and Dan Rokhsar. Genome size is around 975 Mb and is captured in 20 chromosomes with a small additional amount of mostly repetitive sequence in unmapped scaffolds. Around 46,430 protein-coding genes have been predicated (Schmutz et al. 2010). The soybean genome is one of the largest high quality draft- whole-genome shotgun sequenced plant genome and will be having a remarkable impact on the genomic research of other economically important legume crops. After more than a decade-long effort, the genome sequences of these three legume species, *Medicago truncatula, Lotus japonicus*, and *Glycine max* (Soybean), seems to be complete. The genome sequences of these three species can be compared with each other and are likely to be predictive of other agriculturally important legume crops (Sato et al. 2008). These three model legumes are all part of the Papilionoideae subfamily of Leguminosae, and their genes are found in large syntenic regions, which should facilitate positional cloning of genes from other related legumes. The impact of these assembled, annotated genomes from all three model plants will be enormous in crop biotechnology. New technologies, for example high-throughput sequencing, high multiplexed mapping techniques and rapid development of new bioinformatics tools will glean new information about current model systems and emerging new models as well (Doyle and Luckow 2003).

The information obtained from genomic data of all three model legumes should be useful for the understanding of traits and the isolation of corresponding genes in target crop species.

3 Genome Resources in Model Legumes

The sequence information of *Medicago truncatula* (as a reference species for legume genomics) will revolutionize legume research, just as the *Arabidopsis* and rice genome sequences have transformed plant research. Groups interested in positional cloning will quickly begin to discover the genes responsible for fundamental biological processes like symbiosis and nitrogen fixation – and this capability will be available to people working both on *Medicago* and related legume species. The researchers working in proteomics will get high quality, full-length sequences as a basis for identifying peptide fragments. Studies in functional genomics will be based on a more solid foundation, where the results of microarray and gene-knockout experiments will be informed by complete sequences for members of relevant gene families. The molecular breeders will be able to discover candidate genes for the QTLs they have mapped in *Medicago* or other legumes and plant pathologists can explore the entire genetic complement of resistance genes and disease response pathways.

This genome sequence of model legume plants opens the door to the crop improvement for other important legumes used for human and animal food production and more recently for biofuel production. As genome sequencing begins in other crop legumes, constructing sequence assemblies will be far more efficient (as rice has aided in the sequencing of maize) (Sato et al. 2008; Young and Udvardi 2009). The latest information on the status of development of genomic resources and of related information in model legumes and some other economically important species are given in Table 11.1.

4 Transcriptomic Analysis

The combination of high-throughput transcript profiling and next-generation sequencing technologies is a prerequisite for genome-wide comprehensive transcriptome analysis. A growing array of genomic tools is being developed for *M. truncatula*. This great interest in transcriptome studies in *Medicago truncatula* is evidenced by the generation and sequencing of more than 70 cDNA libraries, in total yielding more than 250,000 expressed sequence tags (ESTs) deposited in Genbank (Quackenbush et al. 2001). Parallel to the generation of EST data, thousands of oligonucleotide microarrays were hybridized with targets from different biological conditions (Kuster et al. 2007), using layouts such as Mt16kOLI1 (Hohnjec et al. 2005) and Mt16kOLI1Plus (Thompson et al. 2005). The GeneChip® *Medicago* Genome Array was specifically designed to monitor gene expression in *Medicago truncatula, M. sativa*, and the symbiotic organism *Sinorhizobium meliloti*. The *Medicago* Genome Array is particularly useful for molecular biologists and agronomists studying legume genomics and symbiotic relationships between nitrogen fixing bacteria and plants. Affymetrix GeneChip array was designed from the sequence information collected

from data sources including the TIGR *M. truncatula* gene index (The Institute for Genomic Research), gene predictions from the International *Medicago* Genome Annotation Group (IMGAG), gene predictions from the *S. meliloti* genome, and *M. sativa* EST information made available by TIGR. This array contains over 61,200 probe sets: 32,167 *M. truncatula* EST/mRNA-based and chloroplast gene-based probe sets; 18,733 *M. truncatula* IMGAG and phase 2/3 BAC prediction-based probe sets; 1,896 *M. sativa* EST/mRNA-based probe sets; and 8,305 *S. meliloti* gene prediction-based probe sets (Frickey et al. 2008) (Affymetrix GeneChip@ *Medicago* Genome array).

This genechip forms the basis for a recently developed gene expression atlas (Benedito et al. 2008; He et al. 2009). For reverse genetics, *M. truncatula* has large and characterized Tnt1 (Tadege et al. 2008; Rogers et al. 2009), fast neutron bombardment (FNB) (http://bioinfo.noble.org/), and targeting-induced local lesions in genomes (TILLING) (Thompson et al. 2005) populations available, as well as RNA interference (RNAi)-based gene silencing (Limpens et al. 2004; Cannon et al. 2009). Recently Affymetrix *Medicago* GeneChips® are focusing on analysis of *Medicago* transcriptomics with precise accuracy. It is because of the availability of wide variety of genomic tools which can be used to do better comparison of gene expression data from a multitude of conditions leading to more accurate results (Benedito et al. 2008; Cannon et al. 2009; Young and Udvardi 2009).

The various sequence and expression datasets are being stored by different organizations which could be downloaded and can be used for further analysis. For example EST libraries around over 100 species have been clustered and assembled at J. Craig Ventor Institute which can be utilized for the identification of species specific gene expression (Quackenbush et al. 2001). *Medicago truncatula* GeneIndex 10.0 are now available at the Dana-Farber Cancer Institute (DFCI) (http://www.dana-faber.org) for further use. These Gene indices allow to relate EST data to the biological conditions which were used to generate cDNA libraries. In addition several statistical methods were developed to assess the differential expression of the gene under a given condition (Stekel et al. 2000; Journet et al. 2002). A range of other different databases such as GEO (Edgar et al. 2002; Barrett et al. 2007), Arrayexpress (Parkinson et al. 2007), PEPR (Chen et al. 2004) the Stanford MicroArray Database (Sherlock et al. 2001), and MedicagoPLEX Experiment Data (PLEXdb) (Wise et al. 2007) store microarray and GeneChip® expression data, offering biologists public access to results from transcriptomics experiments (Henckel and Arnaud 2010). PLEXdb is a MIAME/Plant-compliant database which serves as a public repository for raw and normalized expression data. It also provides annotation for the Affymetrix plant microarrays and for other important microarrays. A popular resource for expression profiles relying on *Medicago* GeneChips® has been developed (Benedito et al. 2008; He et al. 2009). This comprehensive gene expression atlas for *Medicago truncatula* is a rich of information for agronomists and biologists and enables a large scale comparison between the transcriptomes of different plant species. In case of the model legume *Medicago truncatula*, the TRUNCATULIX data warehouse currently integrates five different

sequence databases as well as oligonucleotide microarray and GeneChip® expression experiments from different source databases (Henckel et al. 2009). The user can quickly scan the complete database for the expression of genes of interest, but downstream analyses of expression data cannot be performed inside the warehouse. In order to eliminated this problem (lack of an integrated expression analysis), a new analysis tool MediPlEx (*MEDIcago truncatula* multiPLe EXpression analysis) was developed (Henckel and Arnaud 2010). It offers an integrated analysis pipeline for different sets of expression data generated for *Medicago truncatula* (http://www.cebitec.uni-bielefeld.de/mediplex). These resources are complemented by an extensive array of websites and bioinformatic tools (see Table 11.1).

5 Nodule Specific-Transcriptome

It has been shown that, during the nodulation process, there are major changes in plant gene expression (Hogslund et al. 2009). Several hundreds of genes (nodule-specific transcriptome) have been identified that are specifically and strongly up- or down-regulated during the nodulation process. The large majority of these genes are activated at later stages of organogenesis as deduced from the observation that mutants which are affected in the early signaling between the symbiotic partners fail to activate this "nodule-specific transcriptome" (El Yahyaoui et al. 2004). However, the link between the transcriptome activation and the key events of nodule formation – the establishment of a primordium, plant cell infection and cell differentiation of host and endosymbiont – remains unknown. The nodule-specific transcriptome involving several hundred of genes (El Yahyaoui et al. 2004; Benedito et al. 2008) exhibit three major characteristics. First, the repression of plant defense-related genes is probably necessary to avoid repulsion of the infecting rhizobia. Rhizobia possess "microorganism-associated molecular patterns" or MAMPs which are capable of eliciting the innate immune responses of the host plant whereas other rhizobial effectors, notably surface polysaccharides, are thought to attenuate or suppress this defense reaction during infection of root tissues for nodulation (Jones et al. 2007). Second, a transient activation of cell cycle and protein synthesis genes at the incipient stage of nodule development reflects an increase in cell division at this stage of nodule organogenesis. Third, the activation of the secretory pathway and a large number of secretory proteins and/or peptides throughout organogenesis can be understood in the context of a major activity of symbiotic cells in the hosting and maintenance of the symbiosomes. The secretory pathway ensures the correct trafficking of transmembrane or soluble proteins in different compartments of the root cells including in infection threads and symbiosomes of the symbiotic nodule cells. Recent studies with *Medicago truncatula* shows that during infection process secretory proteins are transported to the symbiosome, symbiosome lumen or the bacteroids (Maunoury et al. 2010).

6 Nodule Proteome

Transcriptome approach using microarray and gene expression analysis by real time PCR are useful tools in finding out the abundance of organ or nodule specific transcripts; however it is still a questionable to correlate mRNA abundance with protein levels (Gygi et al. 1999). In contrary, proteomics give relatively a direct assessment of biochemical processes by observing or identifying the actual proteins performing the regulatory and structural functions encoded by the genome and transcriptome. The proteomics studies does not rely on the availability of microarrays, or on extensive data regarding the genomes of interest in that tandem mass spectrometry (MS/MS) allows de novo sequencing and protein identification by homology search. By directly targeting proteins that are key effectors of biotrophic interactions, proteome analysis may thus unravel some actors of the symbiotic molecular dialog, including those of bacterial or fungal origin. Proteomic approaches have been successfully employed to investigate many different legume species including soybean (Komatsu and Ahsan 2009), *M. truncatula* (Bestel-Corre et al. 2002; Watson et al. 2003; Imin et al. 2004; Lei et al. 2005; Colditz and Braun 2010), alfalfa (Watson et al. 2003; Incamps et al. 2005), lupin (*Lupinus albus*) (Brambilla et al. 2009; Tian et al. 2009), *L. japonicus* (Dam et al. 2009), chickpea (*Cicer arietinum*) (Bhushan et al. 2007), pea (*Pisum sativum*) (Wienkoop and Saalbach 2003; Bourgeois et al. 2009), common bean (*Phaseolus vulgaris*) (Lei et al. 2011), and white clover (*Trifolium repens*) (Wilson et al. 2002).

Proteomic studies of the plant symbiosome membrane have shed light on nodule development. A novel 53-kDa late nodulin was isolated from the symbiosome membrane of soybean nodules and found to be regulated by *B. japonicum* inoculation (Winzer et al. 1999; Kistner et al. 2005). Two other known nodulins were identified from the most studied part of symbiosome membrane, the peribacteroid membrane (Panter et al. 2000). In a time-course analysis of protein expression in *M. truncatula* roots upon exposure to *Sinorhizobium meliloti*, two symbiosis related proteins were identified. One is leghemoglobin MtLb2, involved in maintaining oxygen levels, crucial for nitrogenase activity required for nitrogen fixation, and the other is enolase involved in carbon metabolism (Bestel-Corre et al. 2002). The symbiosome membrane protein profile from the model system *Medicago truncatula* and the corresponding bacterium *Sinorhizobium meliloti* was examined using two-dimensional electrophoresis and microcapillary high-performance liquid chromatography (HPLC) tandem mass spectrometry. The identities of 51 proteins were obtained and these proteins were categorized into functional classes to indicate biochemical roles (Catalano et al. 2004). Symbiosome membrane proteins include an H^+-ATPase, ENOD16, ENOD8, nodulin-25, BiP, HSP70, PDI, multifunctional aquaporin, a putative syntaxin, and other proteins of known and unknown identity and function.

Root proteome reference maps have been established for *Medicago truncatula* (Watson et al. 2003; Mathesius et al. 2008) identifying 179 and 40 proteins, respectively; 74 proteins were identified from white lupin; 532 proteins were identified

from several organs of soybean (Sakata et al. 2009), 1492 proteins were identified from soybean root hairs (Brechenmacher et al. 2010). In addition, more than 100 proteins excreted from the root tip and root border cells from pea (Wen et al. 2007). A study showed that around 33 proteins were differentially accumulated in meristematic and non-meristematic cells of *M. truncatula* root cells including proteins involved in carbon metabolism, defense and stress response and flavonoid metabolism (Holmes et al. 2008). Most of the previous legume proteomics studies have used the National Center for Biotechnology Information non-redundant (NCBI nr) protein database for protein queries and identifications. The NCBI nr protein database is a very comprehensive and large protein database, consisting of amino acid sequences translated from coding genome sequences from a vast number of different organisms and species including human, animals, plants and microorganisms.

In this data base (NCBI nr protein database), a large number of experimentally determined tentative consensus sequences and expressed sequence tags (i.e., TCs and ESTs) have not been included. Thus, it will be highly beneficial for the legume community to assemble a legume specific protein database that contained a comprehensive compilation of legume sequences. Due to that, a legume specific protein database (LegProt) has been created containing sequences from seven legume species, i.e., *Glycine max, Lotus japonicus, Medicago sativa, Medicago truncatula, Lupinus albus, Phaseolus vulgaris,* and *Pisum sativum* (Lei et al. 2011). The database consists of amino acid sequences translated from predicted gene models and 6-frame translations of tentative consensus (TC) sequences assembled from expressed sequence tags (ESTs) and singleton ESTs. This database (LegProt) significantly increases legume protein identification success rates and the confidence levels compared to the commonly used NCBI nr. These improvements are primarily due to the presence of a large number of legume specific TC sequences in the LegProt database that were not found in NCBI nr and will serve as a valuable resource for legume proteomics (http://bioinfo.noble.org/manuscript-support/legumedb).

6.1 The Rhizobium – Legume Interaction as a Model for Plant Cell Biology

Legumes are the key for sustainable agriculture because of their role in elemental nitrogen fixation through a symbiotic association with rhizobia in the roots and make renewable source of nitrogen available to crop plants. The symbiosis between legume plants and soil-living rhizobia provides the largest amount of organic nitrogen in the global nitrogen cycle. The infection of rhizobia to the plant root triggers several important events in the root cell, which finally ends up in a nodule formation – a nitrogen-fixing compartment. Some of the signal molecules like Nod-factor and genes related to the nod factor synthesis, thread and peribacteroid formation have been studied but little is known about the genes and proteins involved in membrane and protein trafficking and targeting in nodule which is one of the most important

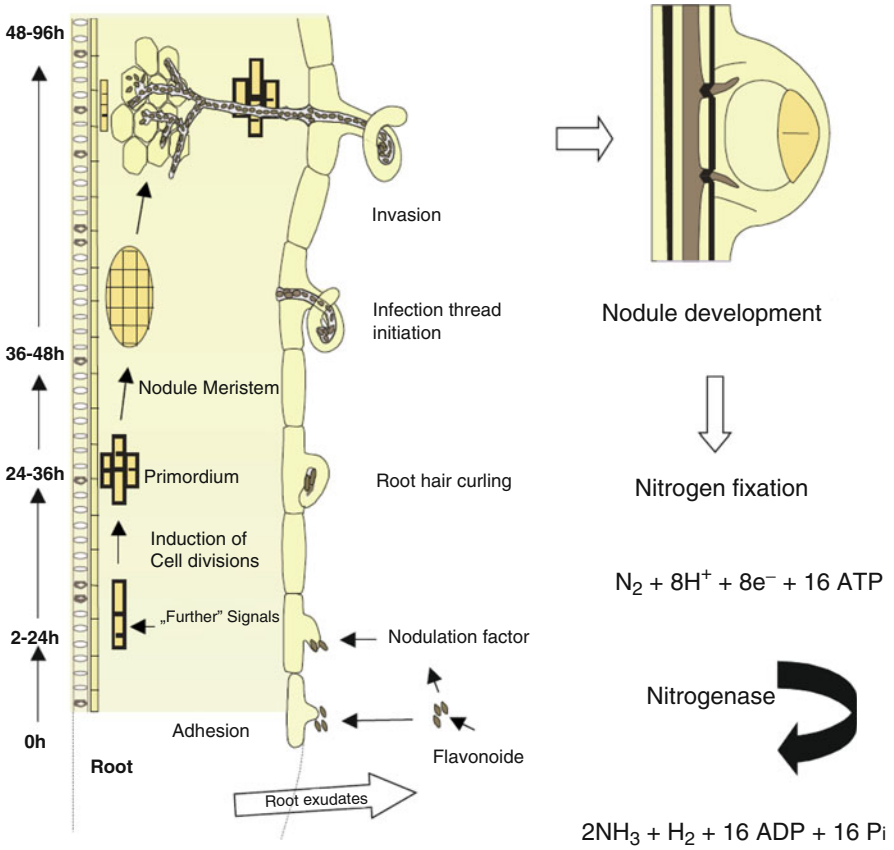

Fig. 11.1 Establishment of the *Rhizobium* – Legume symbiosis. A schematic diagram depict the infection process in early stages of the symbiosis (0–96 h) between *Medicago truncatula* roots and *Sinorhizobium meliloti* (FP7-PEAPOLE-2007-1-ITN; Niehaus, personal communication)

processes in nodule formation and development (Schultze and Kondorosi 1998). Here I will describe recent advances in understanding the molecular mechanisms involved in membrane and protein delivery to the symbiosomes and its effect on nodule development in two legume model systems (*Medicago truncatula* and *Lotus japonicus*).

The symbiosis between soil bacteria of the genus *Rhizobium* and leguminous plants is of outstanding interest to sustainable agriculture and builds one of the best-studied models to understand the molecular biology of plant-microbe interactions (Graham and Vance 2003). During the establishment of this symbiosis plant roots get infected by the microbe leading to cell division and the development of root nodules, specific organs build by the plant and colonized by the invading bacteria (Fig. 11.1). The rhizobia are released into the plant cells, surrounded by a membrane of plant origin, the so-called peribacteroid membrane. This membrane represents an "interface" between the engulfed bacteria and the plant. In a short time

these bacteria differentiate into bacteroids and fix atmospheric nitrogen, which is supplied as a nitrogen source to the plant and in return they are fed with assimilates by the plant (Hirsch and Oldroyd 2009).

The symbiotic interaction between legume plant for example *Medicago truncatula* (barrel medic) and their nodulating symbiont, S*hinorhizobium meliloti*, begins with the sensing of flavonoids and some other compounds including some specific proteins in root exudates by bacteria (Fig. 11.1) (Dela-Pena et al. 2008). The flavonoids released by host plants activates *S. meliloti* transcriptional regulators which then induce the expression of about several bacterial *nod* genes required for the biosynthesis of the lipooligosaccharide signaling molecule. Nod factor is essential for nodulation (Kondorosi et al. 1991; Geurts and Bisseling 2002) and its specificity related with each rhizobium strain and plant species is well documented. Rhizobia enters into root cortical cells via an infection thread which starts from infected root hair and then traverses several cell layers to deliver bacteria to root cells in the developing nodule(Gage 2004; Oldroyd and Downie 2004). Bacteria releases from infection thread and enter the cytoplasm of the nodule cells which differentiate into nitrogen-fixing bacteroids (Pessi et al. 2007). The infection thread is a bacteria filled invagination of the root cell wall and its underlying plasma membrane and is formed through fusion of vesicles containing cell wall and membrane material which move in an actin and microtubule dependent manner (Crespi and Frugier 2008).

6.2 Establishment of the Symbiosis

Rhizobia are chemotactically attracted by root exudates and colonize the host plants rhizosphere. Phenolic signal molecules (*nod*-inducers) present in the exudate activate the transcription of the rhizobial *nod* genes. As a result, host-specific lipochitooligosaccharide signal molecules, called Nod-factors, are synthesized by the bacterial symbiont (Schultze and Kondorosi 1998). Rhizobia attach to emerging root hairs – possibly with the help of lectins – forming microcolonies. As a consequence of Nod-factor activity the root tips coil entrapping the rhizobial colony within pockets formed by the walls of the curled root hairs. The hair walls adjacent to the microcolonies are degraded and the rhizobia invade the underlying periplasmic space of the root hair (Fig. 11.1). Plasma membrane derived infection threads, originating from curled root hairs, act as a trigger for rhizobia to enter the root tissues and eventually progress toward the root cortex where a nodule primordium has been initiated (Hirsch and Oldroyd 2009). The release of rhizobia from infection threads into the cytosol of a nodule primordial cells and subsequent cellular localization of bacteria lead to the formation of fully functional nitrogen fixing nodules. Large numbers of symbiosomes are formed and localized in each nodule, which means that enormous amounts of membrane material and proteins must be delivered to the symbiosome membrane for its development and efficient function (Ivanov et al. 2010).

Flavonoides are produced by host roots and Nod factor is synthesized by rhizobia. After nodule development N_2 is fixed in the nodule by the help of nitrogenase enzyme. The establishment of the *Rhizobium* – Legume symbiosis represents an example for a very intimate interaction of two organisms (Fig. 11.1). The cell biology and physiology of both partners is tightly interconnected. The ability of nodule cells to control protein trafficking and targeting to the symbiosome is essential for nodule development and function (Ivanov et al. 2010). The mechanisms by which proteins are targeted to this novel sub-cellular compartment to accommodate its demand for membrane proteins are largely unknown.

Protein targeting to the symbiosome must rely on the plant secretory system, however, very little is known about protein trafficking and targeting in symbiosomes and root nodules. In mammalian and yeast cells, proteins that are destined for the plasma membrane or endosomal organelles travel through the secretory system and are targeted to their final destination in cargo vesicles. Each target organelle in the secretory system must maintain unique biochemical properties for accurate targeting of the cargo vesicles from the *trans* Golgi (Bonifacino and Glick 2004; Gillingham and Munro 2007; Emr et al. 2009). Site-directed vesicle trafficking in plant cells is essential for plant growth and development (Memon 2004; Hanton et al. 2007). Small GTP binding proteins (Rop, Rab, Arf, Sar) and cytoskeletal reorganization are likely to fulfill a unique symbiotic role by contributing to the specialization of the plasma membrane material around the infection thread and specialization of infection droplets and the symbiosome membrane (Memon et al. 1993; Schiene et al. 2004; Yaneva and Niehaus 2005).

6.3 *Recognition of Symbiotic Microbes: Dissecting the Early Signal Transduction Network*

Most of the cultivated legumes are tetraploid and many have large genomes and are recalcitrant to transformation or difficult to regenerate. As a result, two main species, *Medicago truncatula* and *Lotus japonicus*, have been adopted as models for legume research by several research groups. Both legumes are among the best-established systems to study plant-microbe interactions on a molecular level. Mutants of these two model legumes helped to analyze molecular events during the early perception of the Nod-factor. Genes coding for membrane spanning receptors and downstream elements involved in calcium signalling have been identified first in these model plants; however, the chain of events from Nod-factor perception to initiation of plant cell division is not well understood. The model legumes *L. japonicus* and *M. truncatula*, are also able to establish symbiotic interactions with mycorrhizal fungi (Frenzel et al. 2005) with *Mesorhizobium loti* (Stougaard 2000) and *Sinorhizobium meliloti* (Oldroyd et al. 2009) resulting in nitrogen fixing root nodules.

6.4 Origin and Nature of the Symbiotic Interface

As shown in Fig. 11.1 rhizobia enters the root epidermis by intracellular infection thread and is first initiated in the root hair cells and concurrently the cells at the root cortex start to differentiate and divide to develop a nodule meristem. This coordinated process between the infection thread formation and elongation and nodule organogenesis is crucial for root nodule formation.

Now question arises that how rhizobial cells are released from the infection thread to the plant cells? And how plant membrane material is delivered to the developing bacteroid? This includes two other principal questions asking: How membrane identity is established? And how the vesicle transport is reprogrammed in infected cells? In this review, a strong focus is given on the role of the small GTP binding proteins in the "endocytotic" uptake of rhizobia by the plant. Bacterial surface polysaccharides are also important "virulence factors" that are needed for a successful infection but how these molecules contribute in the infection process is still not clear. Although isolated bacterial lipopolysaccharides (LPS) and flagellin in disease infected plant cells are taken up by a process that resembles a receptor-mediated endocytosis nothing is known about the uptake of rhizobia by root cells. After the uptake of rhizobia, the bacteria are surrounded by the peribacteroid membranes, which exhibit unique features. It is still not known that how the identity of these membranes is maintained.

Vesicle trafficking plays an important role in root tip growth, infection thread formation and the development of root nodules (Oldroyd and Downie 2008). Several studies have shown that membrane associated small GTPases belonging to the Arf/Sar, Rab, and Rop/Rac families, along with their interacting proteins, playing a vital role in the diverse aspects of root nodule formation and development (Yuksel and Memon 2008, 2009). For example, Rab1, Rab7 and Rab11f are implicated to be essential in the formation of the peribacteroid membrane in *Glycine max*, *Vigna aconitifolia* , *Medicago sativa* and *M. truncatula* (Niehaus et al. 1993; Cheon et al. 1993; Son et al. 2003; Schiene et al. 2004). RAB7 has been reported to play an essential role in vesicular fusion during rhizobial endocytosis and symbiosome formation in soybean (Son et al. 2003). In addition Borg et al. 1997 isolated a total of 33 small GTPbinding genes form a cDNA library of *Lotus japonicus*, the majority of these trascriptes were ubiquitously expressed in all plant tissues with the exception of RAB1, RAB2, RAB5, RAB7, and a RAC which were predominantly expressed in nodules. Similarly, (Schiene et al. 2004) and (Meschini et al. 2008) demonstrated that Rab11 f from *M. sativa* and *Phaseolus vulgaris* is predominantly expressed in the root nodules and possibly involved in targeting vesicles to a symbiotic structure in nodule. Rac-Rop subfamily of the Rho GTPases has been reported to be associated with the plasma membrane at the apex of elongating root hairs and pollen tubes in *Arabidopsis thaliana* and tobacco (Kost 2008). They control tip growth by coordinating actin organization and membrane trafficking.

Although the sorting and targeting of the many secretory proteins from TGN to vacuolar and plasma membrane compartment has been worked out in Arabidopsis, tobacco leaf and BY2 cells but the targeting to the peribacteroid membrane (PBM)

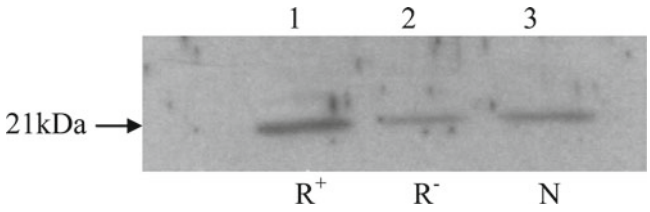

Fig. 11.2 Expression of SAR1 level in the membrane fraction of 3 month old *M. truncatula* infected with *Sinorhizobium meliloti* (R^+) and not infected (R^-) roots, N, nodules. Membrane fractions were isolated at 250,000 × g (Yuca and Memon unpublished data)

is still not clearly identified in legumes (Samaj et al. 2004; Richter et al. 2009). One pioneering work of the lab of D.P. Verma showed the involvement of the two GTP-binding proteins, Rab l and Rab7 in this process but there were several unresolved questions related to the role of these proteins in peribacteroid formation (Cheon et al. 1993).

The protein and gene expression studies could suggest a nodule-specific expression of small GTP binding proteins especially the expression of several Rabs and Rops (Son et al. 2003; Blanco et al. 2009). The targeting of peribacteroid membrane (PBM) proteins seems to vary and this could be due to the mosaic nature of PBM having properties common to both plasma membrane and vacuoles. Therefore involvement of several GTP binding proteins in PBM biogenesis may be more complex and could be worked out in detail. It is concluded that looking further into the small GTPases of legume plants, more of these genes could be found to be significant in the formation and maintenance of the symbiosome compartment through regulating functions such as shuttling of products between symbiont and host. To realize this we previously made an attempt to retrieve the EST databases of two model legume plants, *Medicago* and *Lotus* and searched the nodule specific expressed transcripts of small GTPases (Yuksel and Memon 2008). As a result of this bioinformatic analyses, two ARL like genes, a group of 10 Rab GTPases and one or two ROP like GTPases were found to be mainly expressed in nodules. The sequences determined as a result of this study, we decided to commence detail expression analysis of these GTP-binding proteins at RNA and protein levels in rhizobium inoculated and non inoculated roots and nodules of *Medicago truncatula*. Our results show three to five times more Sar1 and Arf1 protein content in rhizobium infected roots compared to non inoculated one (Figs 11.2 and 11.3).

Our qReal-time PCR experiments clearly showed about 5–10 times more expression of Sar1 and Arf1, in roots and nodules of inoculated plants compared to the roots of non inoculated ones (see Fig. 11.4a, b). Our results indicate that small GTPases playing a major role in the regulation of membrane and protein transport in root nodule development in legumes.

A schematic diagram (Fig. 11.5) shows the probable role of Small GTPases in various steps of nodule development in legume roots (Yuksel and Memon 2009).

Detailed studies should be carried out to elucidate the role of different GTPases at various steps depicting in the Fig. 11.5. My group and several other groups in

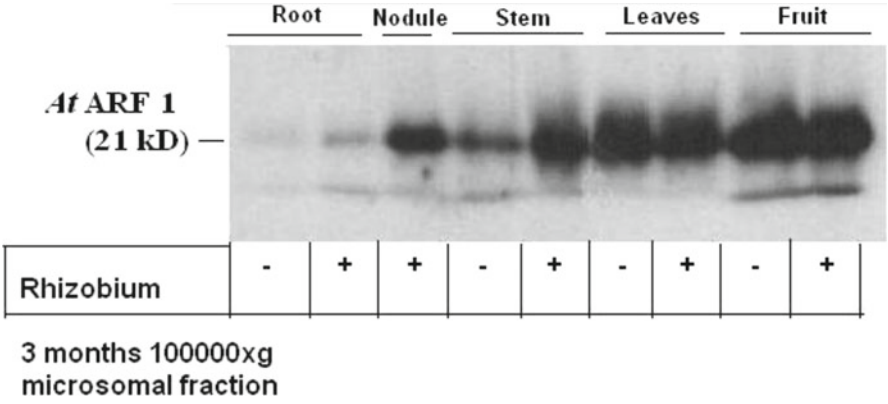

Fig. 11.3 ARF1 expression in the microsomal fraction isolated from different parts of the Medicago *truncatula* grown for 3 months (Yuzbasioglu and Memon unpublished data)

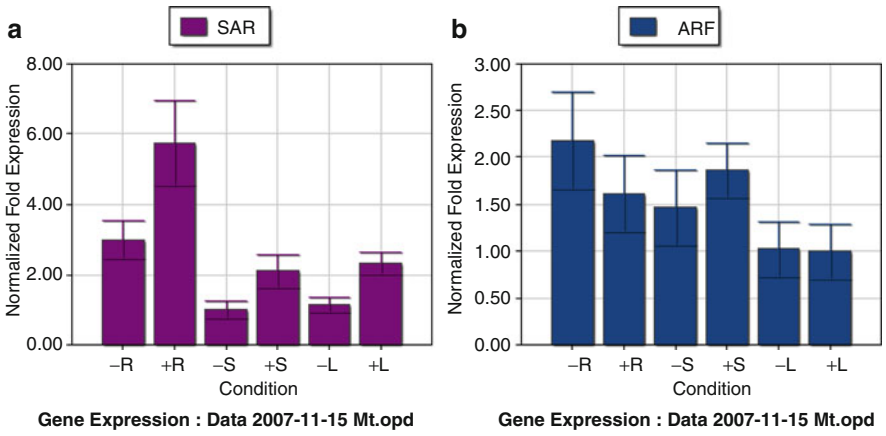

Fig. 11.4 (**a**, **b**): The effect of *S. meliloti* infection on SAR and ARF mRNA levels in *M .truncatula* roots after 30 days infection. Ubiqutine was used as an internal control. −R, −S and −L, roots, stems and leaves from uninoculated plants and +R, +S, +L, roots, stems and leaves from inoculated plants (Yuzbasioglu, Keskin, and Memon unpublished data)

Europe (for example Niehaus's group in Bielefeld, Germany, FP7-PEAPOLE-2007-1-ITN) are studying in detail the membrane trafficking from ER to Golgi and from the *trans* Golgi network (TGN) to peribacteroid membranes and/or plasma membranes. To identify the role of small GTP-binding proteins in these processes is one of the important goals of our research, since these proteins are (i) essential and (ii) specific regulators of all vesicle transport events (Samaj et al. 2006). We will take the advantage of the GFP technology and will use GFP/RFP-tagged GTPases as markers to locate the targeting sites and compartments of these proteins and manipulate their specific functions by the use of gain-of-function and loss-of-function mutants of these signal transduction components.

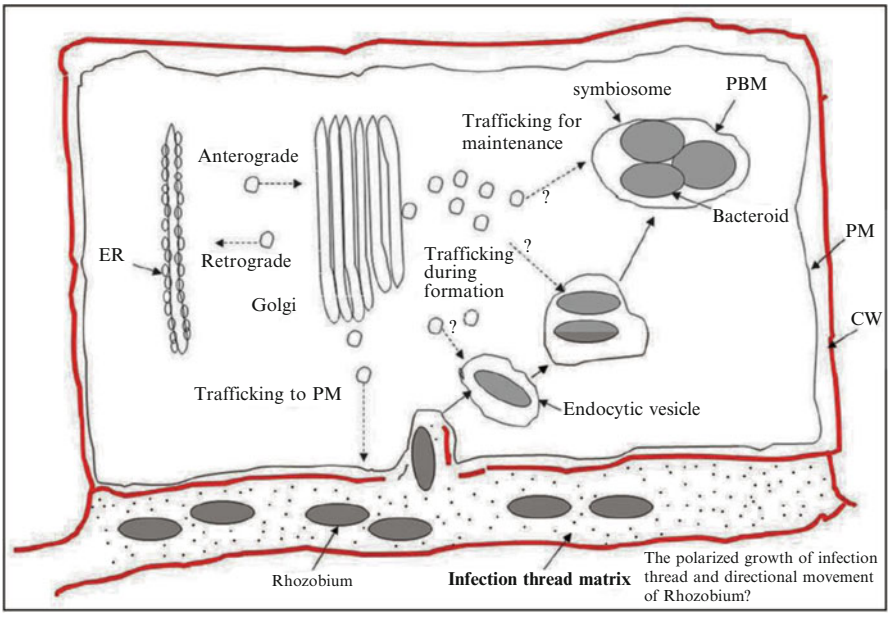

Fig. 11.5 A schematic diagram showing the possible role of various small GTP binding proteins at different steps of membrane trafficking in the formation of symbiosome and nodule development. The *dashed arrows* indicate the possible route of coated –vesicles carrying the cargo to the target compartment. Questions marks describes the involvement of some identified small GTPases in nodule specific trafficking. *PM* plasma membrane, *PBM* peribacteroid membrane, *CW* cell wall, *ER* endoplasmic reticulum

7 Conclusion

Legumes are one of the most economically important crop plants in the world. Because of their unique ability to fix natmospheric nitrogen through a symbiotic relationship with rhizobia, they are a linchpin of sustainable agriculture. This mutallistic interaction between host plant and bacteria provide a plentiful supply of nitrogen to the plants that in turn results in very high protein levels in legumes. They also provide a free and renewable source of usable nitrogen to the subsequent crops grown in rotation. Biological nitrogen fixation (BNF) and the understanding of beneficial plant microbe interactions are essential for a sustainable agriculture. The heavy use of chemical fertilizers especially nitrogen is a global concern for high economic cost and environmental pollution. For sustainable agriculture alternative nitrogen sources should be urgently sorted out. The unique nitrogen fixing features of legumes compare to non nitrogen fixing plants be exploited efficiently to maintain the sustainability in our cropping system. Nitrogen-fixing systems of legumes represent an economically attractive and ecologically sound means of reducing external inputs and improving internal resources. This notion has led to the development of three model legume species, *Lotus japonicus, Medicago truncatula* and *Glycine max* which are providing a rich source of genetic and genomic information.

Medicago truncatula is the first model leguminous plant with a completed genome sequence (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=9508) available. This allows utilization of molecular biology techniques to study the genomic, transcriptomic, and proteomic relationships of *M. truncatula*. A better knowledge of cellular and molecular processes involved in symbiosome formation may contribute in a long-term to improve the efficiency of BNF by leguminous plants and/or to extend it to other crops. The protein and gene expression studies could suggest a nodule-specific expression of small GTP binding proteins especially the expression of Arf1, Sar1, Rabs and Rops and their possible role in early infection process and nodule development. Recent availability of soybean genome sequence in public domain has not only accelerated the work in finding improved varieties of this economically important crop but will also be used as a key reference for more than 20,000 legume species. This genome sequence opens the door for legume researchers to develop new efficient nitrogen fixing and high quality protein and oil producing crops for a changing world.

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Chapter 12
Agrobacterium tumefaciens and its Use in Plant Biotechnology

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Contents

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Abstract For years, isolation of foreign genes from one plant and transferring them to another and then observing effects of new genes in transferred plants has only been a dream for a plant biologist. Today, many commercially important species are routinely transformed by different biotechnological methods. Methods available for plant transformation are arranged in three main groups: using biological vectors (virus- or bacteria-mediated transformation), direct DNA transfer techniques (chemical-, electrical-, or laser-induced permeability of protoplasts or cells) and non-biological vector systems (microprojectiles, microinjection or liposome fusion). Today in many countries a number of transgenic important crops such as soybean, maize, cotton, canola, sugarbeet, sugarcane and alfalfa are available and the mostly preferred method is *Agrobacterium*-mediated transformation. In this chapter, some information about this important bacterium and mechanisms of *Agrobacterium*-mediated gene transfer are presented.

Keywords Cotton • Crown-gall disease • Molecular marker techniques • Tissue culture • Transgenic • T-DNA • Vir proteins

1 Characteristics of *Agrobacterium tumefaciens*

The genus *Agrobacterium* is divided into a number of species mostly on the basis of disease symptomology and host range (Gelvin 2003). *Agrobacterium radiobacter* is an "avirulent" species, while *A. tumefaciens* causes crown gall disease; *A. rhizogenes* causes hairy root disease, and *A. rubi* cane gall disease. Recently, a new species *A. vitis* was proposed, which causes the growth of neoplastic tumors on the stem and crown of grapevines and induces necrotic lesions on grape roots and a few other plant species (Otten et al. 1984; Burr et al. 1998; Gelvin 2003; Escobar and Dandekar 2003).

Agrobacterium tumefaciens is a Gram negative, motile, rod shaped soil bacterium, which is non-sporing, and is closely related to the N-fixing rhizobium bacteria, which form root nodules on leguminous plants (Ream 2002). It is also a soil pathogen initiating tumors on plants (DeCleene and DeLay 1976; McCullen and Binns 2006). The bacterium is surrounded by a small number of flagella (Fig. 12.1) (Garrity 2005). The systematic classification of *A. tumefaciens* is given in Table 12.1.

Although today in nomenclature, the name *Agrobacterium tumefaciens* Conn 1942 (Smith and Townsend 1907) is mostly used, there are some synonyms of this bacterium as; *Bacterium tumefaciens* (Smith and Townsend 1907), *Pseudomonas tumefaciens* (Smith and Townsend 1907) Duggar 1909, *Phytomonas tumefaciens*

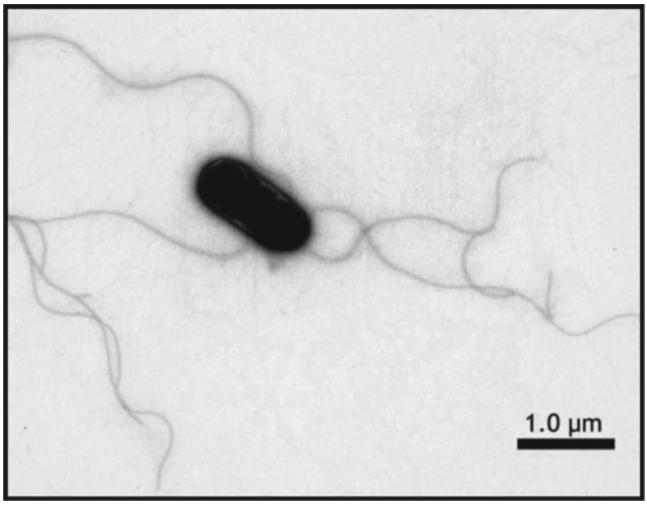

Fig. 12.1 Electron microscopic view of *Agrobacterium tumefaciens* with peritrichous flagella (Modified from Jeon et al. 2008)

Table 12.1 Systematics of *Agrobacterium tumefaciens* (Bergey's Manual of Systematic Bacteriology 2005)

Kingdom	Bacteria
Phylum	Proteobacteria
Class	Alphaproteobacteria
Order	Rhizobiales
Family	Rhizobiaceae
Genus	*Agrobacterium*
Species	*Agrobacterium tumefaciens*

(Smith and Townsend 1907) Bergey et al. 1923, *Polymonas tumefaciens* (Smith and Townsend 1907) Lieske 1928 (LPSN 2011).

Agrobacterium-mediated gene transfer has been of great help in modern plant molecular genetics and genetic engineering (Ream 2002). Nowadays, plant transformation by *A. tumefaciens* has become one of the most used methods for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants (de la Riva et al. 1998; Ziemienowicz 2001; Ream 2002; Özcan et al. 2004; McCullen and Binns 2006). In addition, the genus *Agrobacterium* can transfer a piece of its plasmid DNA into a remarkably broad group of organisms including numerous dicotyledonous and monocotyledonous species (Anderson and Moore 1979; de Frammond et al. 1986; van Wordragen and Dons 1992; Men et al. 2003; Li et al. 2007; Wanichananan et al. 2010) and gymnosperms (Loopstra et al. 1990; Stomp et al. 1990; McAfee et al. 1993; Yibrah et al. 1996; Tang et al. 2001; Grant et al. 2004). Furthermore, *Agrobacterium* can transform fungi (Chen et al. 2000),

including yeasts (Bundock et al. 1995; Bundock and Hooykaas 1996; Piers et al. 1996), ascomycetes (de Groot et al. 1998; Abuodeh et al. 2000; Zhiming et al. 2008) and basidiomycetes (Sharma et al. 2006; Okamoto et al. 2010). *Agrobacterium* has been reported to transfer DNA to human cells as well (Kunik et al. 2001; Gelvin 2003).

2 Crown Gall Disease

In nature, *A. tumefaciens* infects the wounded sites in dicotyledonous plants and some monocotyledonous as well, causing the formation of crown gall tumors (de la Riva et al. 1998). These tumors no longer require the continuous presence of the inciting bacterium for proliferation demonstrating that the plant cells have been transformed (White and Braun 1942; McCullen and Binns 2006). Crown gall disease is a common plant disease, affecting more than 600 types of plants. It affects nearly all dicotyledonous plants, woody and herbaceous plants and many commercially important and valuable crops such as brambles, rose, willow, grapes, rice and sugar beet (Burr et al. 1998; Jeon et al. 2008). Although *A. tumefaciens* has a wide host range, many plants are immune to the disease and can be planted in sites with a history of crown gall. Some of these well-known genera, which are resistant or immune to this disease are as follows; *Ailanthus*, *Berberis*, *Betula*, *Catalpa, Cedrus*, *Cercis*, *Fagus*, *Ginkgo*, *Larix*, *Magnolia, Picea*, *Pyracantha* and *Sambucus* (Lacy and Hansen 2002).

The disease can be identified by the appearance of tumors or galls of varying size and shape on the lower stem where it meets the soil (crown), main roots and sometimes branches of the plant (Fig. 12.2). Infected plant cells contain bacterial genes (plasmid) that replace some of the normal plant cell genes (Khawar and Özcan 2002; Jeon et al. 2008). When they are young, the galls can be white or cream colored and spongy or wart-like; as they age, they become dark and woody (Burr et al. 1998). The tumor usually either appears as a swelling of the plant tissue, or as a separate mass of tissue close to the plant surface, joined only by a narrow neck of tissue (Fig. 12.3). Some of them can reach up to 30 cm in diameter, though 5–10 cm is more common. When plants are infected with the bacterium they become stunted, produce small chlorotic leaves and become more susceptible to extreme environmental conditions such as winter cold and wind. Galls can interfere with the plant's ability to move water and nutrients through the stem, which may result in stunting or decline of the plant (Sule et al. 1995; Jeon et al. 2008).

Plant tumors normally do not kill the plant, but crown gall disease can be fatal if the tumors become too enlarged. Crown gall can also be eradicated using creosote based chemical compounds, copper-based solutions and strong oxidants such as sodium hypochlorite (Utkhede and Smith 1993; Polcaro et al. 2008). However, these are costly to apply in terms of both labor and buying the product. They are also very

Fig. 12.2 Tumors of Korean ginseng seedling roots collected from a seedbed at the harvest time of seedling roots (Modified from Jeon et al. 2008)

Fig. 12.3 Healthy (*right*) and diseased (*left*) carrot disc showing callus-like swellings (*arrows*) around vascular bundle 2 weeks after *Agrobacterium tumefaciens* inoculation (Modified from Jeon et al. 2008)

harmful to the surrounding environment and accumulation of large quantities of copper in the soil can have a disastrous impact on other plants in the area. Therefore, chemical controls are rarely used against *A. tumefaciens* (Deacon et al. 1988; Zauner et al. 2006).

3 Genome Structure of *Agrobacterium*

The first gene transfer into plants was not realized by scientists, it was realized by *A. tumefaciens*, which has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected plant cells (Fig. 12.4). Therefore, the first genetic engineers are from the genus *Agrobacterium*, not from the genus *Homo*. The molecular basis of genetic transformation of plant cells by *A. tumefaciens* is **(1)** transferring some genes from the bacterium and **(2)** their integration into plant nuclear genome located in a large tumor-inducing Ti plasmid of bacterium (Gelvin 2003; Özcan et al. 2004).

The virulent strain of *A. tumefaciens*, which induces crown gall disease, contains a large mega-plasmid (more than 200 kbp) called Ti plasmid (Fig. 12.5). In genus *Agrobacterium*, Ti plasmids are classified according to opines which are produced and excreted by the tumors they induce (Zhu et al. 2000; Ream 2002; Özcan et al. 2004). During infection, the T-DNA that is a mobile segment of Ti plasmid, is transferred into the plant cell nucleus and integrated into the plant genome (de la Riva et al. 1998; Gelvin 2003). T-DNA region generally represents less than 10% of the Ti plasmid. Some Ti plasmids contain one T-DNA whereas others contain multiple T-DNA regions (Zambryski et al. 1980; Barker et al. 1983; Gelvin 2003). The processing of T-DNA from Ti plasmid and its subsequent export from bacterium into plant cell result in large part from the activity of virulence (*vir*) genes carried by the Ti plasmid (Garfinkel and Nester 1980; Hooykaas et al. 1984; Knauf et al. 1984;

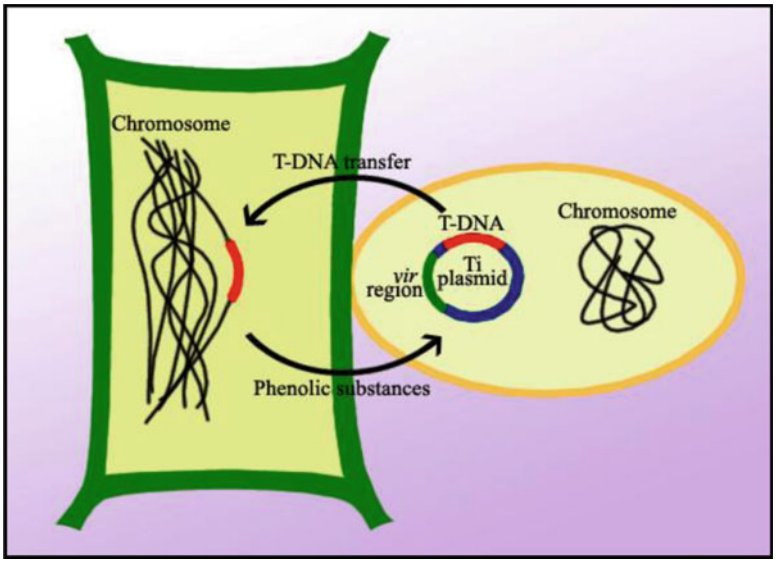

Fig. 12.4 Ti plasmid, T-DNA, *vir* region and T-DNA transfer into the plant cell (Modified from Özcan et al. 2004)

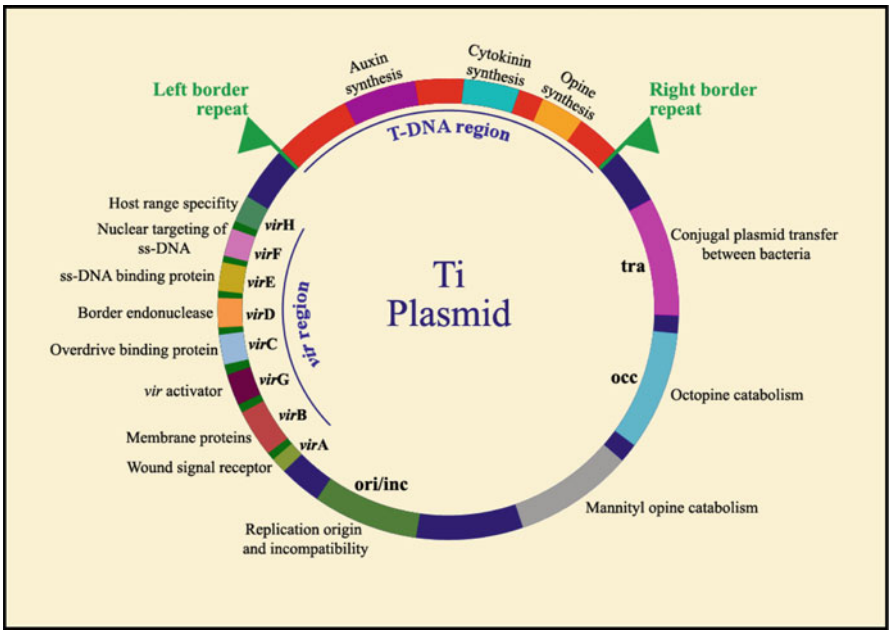

Fig. 12.5 Genetic map of octopine-type Ti plasmid (Modified from Ream 2002 and Özcan et al. 2004)

Lundquist et al. 1984; Horsch et al. 1986; Stachel and Zambryski 1986; Gelvin 2003). In addition, earlier studies have indicated that the Ti plasmid, rather than chromosomal genes, was the major genetic determinant of host range (Loper and Kado 1979; Thomashow et al. 1980).

In *A. tumefaciens*, the T-DNA contains two types of genes; (a) oncogenic genes which are encoding for enzymes involved in the synthesis of auxins and cytokinins that are responsible for tumor formation, and (b) the genes which are encoding synthesis of opines that are responsible for the formation of the novel amino acid-sugar conjugates (de la Riva et al. 1998; McCullen and Binns 2006). The amino acids and sugars are synthesized and excreted by the crown gall cells and consumed by *A. tumefaciens* as carbon and nitrogen sources (de la Riva et al. 1998).

The specific DNA region acting as a *cis* element signal for the transfer apparatus from the Ti plasmid is generated by 25-bp direct repeats. Through the cooperative action of proteins encoded by the genes defined in Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome, the process of T-DNA transfer is carried out (de la Riva et al. 1998; Ream 2002; Gelvin 2000). However, T-DNA transfer and integration into plant genome does not require tumorigenesis or T-DNA encoded proteins (Hoekema et al. 1983; Ream et al. 1983). This situation has allowed the plant scientists to use *A. tumefaciens* to transfer some desired genes into plants in place of T-DNA oncogenes (Ream 2002).

Table 12.2 The *vir* regions of Ti plasmids and their functions

vir Locus	Function
***vir*H**	Encodes VirH1 and VirH2 proteins (they could enhance the transfer efficiency, detoxifying certain plant compounds that can affect bacterial growth)
***vir*F**	Encodes 23 kDa protein (functions once the T-DNA complex is inside the plant cells via the conjugal channel or independently, as it was assumed for VirE2 export)
***vir*E**	Encodes ss-T-DNA binding protein (stabilizes T-DNA during or after transfer)
***vir*D**	Nicks Ti plasmid at T-DNA borders, covalently attaches to T-strand
***vir*C**	Binds to the 'overdrive' region to promote high efficiency T-strand synthesis
***vir*G**	Regulatory (transcriptional activator of other *vir* loci)
***vir*B**	Transfer apparatus (required for export of the T-complex and VirE2 into the plant cell)
***vir*A**	Regulatory (recognizes plant metabolites, activates *vir*G)

The Ti plasmid also contains the genes for opine catabolism produced by the crown gall cells and the regions for conjugative transfer and for its own integrity and stability (Fig. 12.5) (de la Riva et al. 1998; Özcan et al. 2004). Based on the kind of opines produced in the tumors, agrobacteria are classified as octopine, nopaline, succinamopine and leucinopine strains. The biosynthesis of opines is catalyzed by opine synthases, which are encoded by the T-DNA (Ziemienowicz 2001). There are more than 20 opines and each strain induces and catabolizes a specific set of opines. In general, each *A. tumefaciens* strain catabolizes only the opines synthesized by tumors that it will induce (Hooykaas and Shilperoort 1992; Zupan and Zambryski 1997; de la Riva et al. 1998). For example, octopine-type Ti plasmids direct their hosts to synthesize at least eight opines. The *ocs* gene encodes octopine synthase, which reductively condenses pyruvate with either arginine, lysine, histidine or ornithine to produce octopine, lysopine, histopine, or octopinic acid respectively, all of which can be detected in crown gall tumors (Dessaux et al. 1998; Ziemienowicz 2001). Furthermore, some opines induce conjugal transfer of self-transmissible Ti plasmids between strains of *A. tumefaciens* (Petit et al. 1978; Ellis et al. 1982), thereby conferring on other strains the ability to catabolize extant opines. Apparently, *A. tumefaciens* strains create a "niche" (a crown gall tumor-synthesizing particular opines) that offers a favorable environment for growth of the inducing strain (Ream 2002).

There are six operons organized in the *vir* region of Ti Plasmid (30 kb) essential (*vir*A, *vir*B, *vir*D, and *vir*G) or increasing of transfer efficiency (*vir*C and *vir*E) for the T-DNA transfer (Hooykaas and Shilperoort 1992; Zupan and Zambryski 1995; Jeon et al. 1998; de la Riva et al. 1998). In addition, there are also two more operons, which are not necessary for T-DNA transfer called *vir*F and *vir*H (Fig. 12.5) (Özcan et al. 2004). The protein products of these genes, respond to the specific compounds secreted by the wounded plant to generate a copy of the T-DNA and mediate its transfer into the host cell (Table 12.2, Sheng and Citovsky 1996). The *vir*A, *vir*G and *vir*F operons carry one gene, while *vir*E, *vir*C and *vir*H operons carry two genes.

However, *vir*D includes four and *vir*B includes 11 genes (de la Riva et al. 1998; Özcan et al. 2004). *vir* gene expression is induced by chemical signal molecules (phenolic substances and sugar) released from wounded plant cells (Winans et al. 1994). Recognition of these signals by the sensor protein VirA leads to phosphorylation of VirG protein (Stachel and Zambryski 1986; Jin et al. 1990a).

*vir*D gene products are needed for DNA processing which involves recognition and complexing of direct repeat borders of the T-DNA segment (Sheng and Citovsky 1996; Ziemienowicz 2001; Özcan et al. 2004). T-strand production is dependent on the cleavage of the border sequence which is performed by VirD2 in the presence of VirD1 protein. VirD2 cuts the border sequence in a site and strand specific manner and following cleavage it becomes covalently attached to the 5′ end of the nicked DNA (Dürrenberger et al. 1989; Howard et al. 1990; Howard et al. 1992). The nicked DNA is then removed from the plasmid producing single stranded T-DNA (ss-T-DNA). It is believed that by a pilus-like structure containing VirB and VirD4 proteins the T-DNA-VirD2 complex and the VirE2 protein are transferred to the plant (Citovsky et al. 1994; Rossi et al. 1996). In the plant cell, T-DNA becomes associated with the single stranded DNA-binding protein, VirE2 and then the T-DNA-protein complex is transported into the nucleus. Following the transportation, the integration of T-DNA into the nuclear genome occurs (Sheng and Citovsky 1996; Ziemienowicz 2001).

The third component is the cluster of chromosomal virulence (*chv*) genes found on the *Agrobacterium* chromosome. The *chv* genes play important roles in bacterial chemotaxis and attachment to the wounded plant cell (Citovsky et al. 1992; Zambryski 1992; Sheng and Citovsky 1996; de la Riva et al. 1998; Özcan et al. 2004). Different chromosomal-determined genetic element products have exhibited their functional role in the attachment of *A. tumefaciens* to the plant cell and bacterial colonization: the loci *chv*A and *chv*B code for inner membrane proteins essential for the transport of β-1,2 glucan from bacterial cytoplasm into periplasm and in the synthesis of β-1,2 glucan (Cangelosi et al. 1989), the *chv*E is required for the expression of *vir* genes and bacterial chemotaxis (Ankenbauer and Nester 1990; Cangelosi et al. 1990, 1991; de la Riva et al. 1998), the *cel* locus responsible for cellulose fiber synthesis to enable bacterial cells to be firmly adhere to plant cell wall (Matthysse 1983), the *psc*A (*exo*C) locus is involved in the synthesis of both cyclic glucan and acid succinoglycan (Cangelosi et al. 1987, 1991) and the *att* locus acts role in the cell surface proteins (Matthysse 1983; de la Riva et al. 1998).

As an important model system in understanding pathogen-host interactions including recognizing and delivering macromolecules into target cells resulting in disease, *A. tumefaciens* has been established by the intense mechanistic analysis of *Agrobacterium*-mediated transformation (McCullen and Binns 2006). From these studies, a basic model for the cellular transformation of plants by *A. tumefaciens* has been developed (Gelvin 2003; Palmer et al. 2004; Tzfira and Vaidya 2004; Brencic and Winans 2005; Christie et al. 2005; McCullen and Binns 2006). In this chapter, a hypothetical model depicting the most important stages of this process is presented, supported by the most recent experimental data and accepted hypothesis on T-DNA transfer.

4 Molecular Mechanism of Gene Transfer

The process of gene transfer from *A. tumefaciens* into plant cells implies several essential steps: **(1)** bacterial colonization, **(2)** induction of bacterial virulence system, **(3)** generation of T-DNA transfer complex, **(4)** T-DNA transfer and **(5)** integration of T-DNA into plant genome (Fig. 12.6).

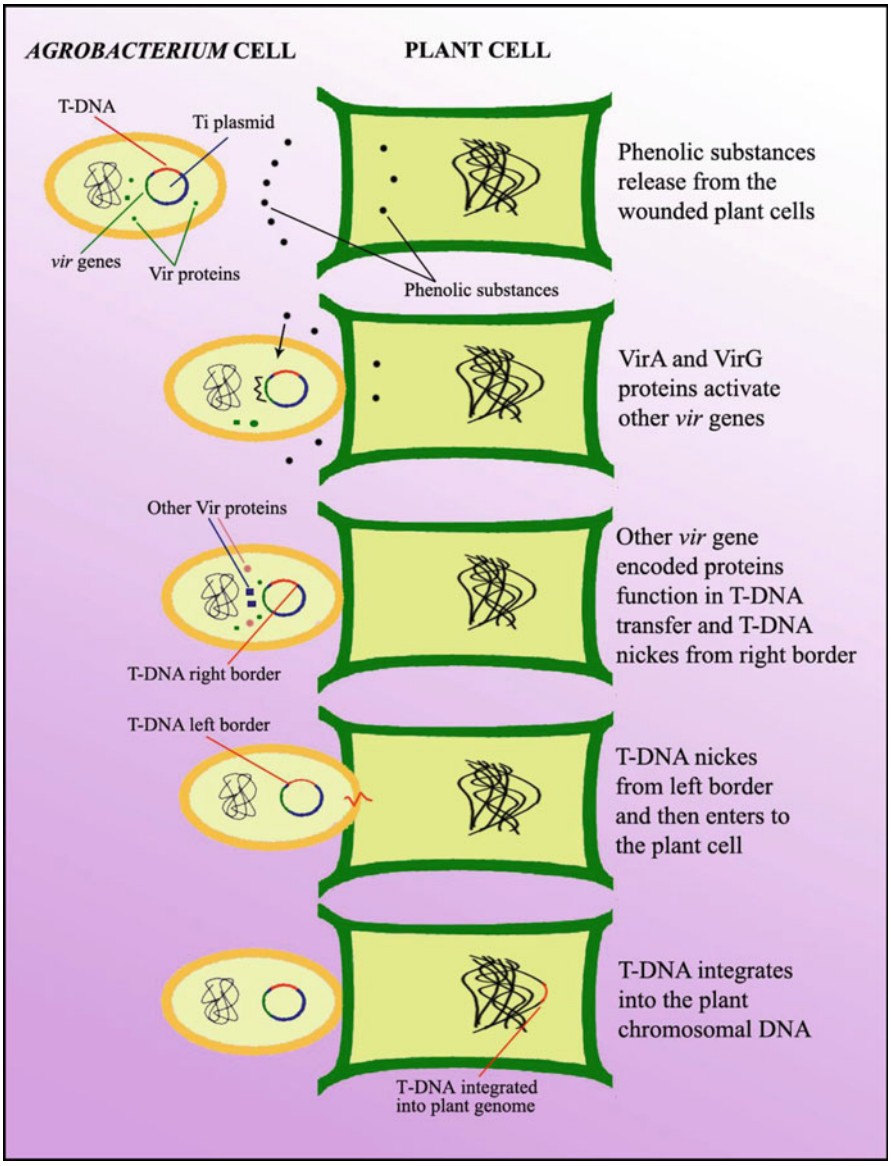

Fig. 12.6 Natural gene transfer from *Agrobacterium tumefaciens* to plant cell (Modified from Watson et al. 1992 and Özcan et al. 2004)

4.1 Bacterial Colonization

Phenolic compounds released to the surface of the host cells during injury act as signals that attract *Agrobacterium* cells to the wounded site in a plant before bacterial colonization (Parke et al. 1987; Ashby et al. 1988; Ziemienowicz 2001). Experiments have shown that some compounds like acetosyringone induce *vir* genes which attract a highly motile strain exhibiting marked pTi-dependent chemotaxis whereas a poorly motile strain does not show any movement. This pTi-dependent chemotaxis requires virulence genes, *vir*A and *vir*G (Shaw et al. 1988). Furthermore, sugars and amino acids also attract *Agrobacterium* (Hawes et al. 1988; Ziemienowicz 2001).

Bacterial colonization is the first step in tumor induction and it occurs when *A. tumefaciens* is attached to the plant cell surface (Özcan et al. 2004). Earlier studies showed that non-attaching *A. tumefaciens* mutants lose their tumor-inducing capacity (Douglas et al. 1982; Cangelosi et al. 1987; Thomashow et al. 1987; Bradley et al. 1997; de la Riva et al. 1998). The cell surface polysaccharides of *A. tumefaciens* are considered to be important in the colonizing process (Bradley et al. 1997). It was reported that the bacterial attachment could be inhibited when preparations of lipopolysaccharides (LPS) of virulent strains are applied to the plant tissue before bacteria (Whatley and Spress 1977; de la Riva et al. 1998).

The LPS are integral components of the outer membrane and contain a lipid moiety, which is embedded in the outer membrane, a conserved core oligosaccharide, and a polysaccharide side chain known as the O-antigen. In addition to LPS, *A. tumefaciens* also produces capsular polysaccharides. Some evidence indicates that capsular polysaccharides have specific functions in the interaction with the host plant (de la Riva et al. 1998). There is evidence that there was a correlation between the production of an acidic polysaccharide and attachment of wild-type bacterium to plant cells (Bradley et al. 1997; de la Riva et al. 1998). Genes involved in the attachment are located in the *Agrobacterium* chromosome, and include *chv*A, *chv*B, *psc*A (or *exo*C) and *att*. Mutations in these loci lead to a loss of virulence towards many plant species (Ziemienowicz 2001).

Experiments related with *chv*A and *chv*B genes showed that mutations in these genes produce non-specific alterations in the bacterial surface and result in absence of β-1,2-glucans in the bacterial membrane, increased production of extracellular polysaccharides, reduced motility and failure to bind to plant cells. The *chv*A gene codes for inner membrane protein essential for the transport of β-1,2-glucans from bacterial cytoplasm into periplasm (Cangelosi et al. 1989; Ziemienowicz 2001) while the *chv*B gene codes for an inner membrane protein likely to be involved in the synthesis of β-1,2-glucan (Zorreguieta et al. 1988). The genes at *att* left side are responsible for molecular signaling events, while the right side genes are involved in the synthesis of fundamental components. The *att* left side includes an operon composed by nine open reading frames (ORF). Four of these ORF exhibit homology to the genes involved in the periplasmic binding protein dependent (or ABC) transport system (Ames et al. 1990; Higgings et al. 1990). A failure observed in a mutant analysis showed that the production and accumulation of specific compounds

are necessary for the attachment of bacteria (de la Riva et al. 1998). The ABC transporter genes may be required for the secretion of these substances or some plant-originated activators are introduced into bacteria by the synthesis of compounds specific for the attachment (Matthysse et al. 1996; de la Riva et al. 1998).

After the attachment of bacteria to the plant cell wall, colonization of the plant cell wall begins through cellulose fibers produced by bacteria (Özcan et al. 2004). The *exo*C is required for the synthesis of glucose phosphate isomerase and mutants of the *exo*C are therefore unable to produce extracellular polysaccharides (Uttaro et al. 1990). Furthermore, mutants in the *Agrobacterium* chromosomal *cel* genes (*cel*ABCDE) show deficiency in cellulose synthesis and aggregate formation (Matthysse 1983), thus, these mutants exhibit weak binding capacity to plant wounded sites (Ziemienowicz 2001). In addition to bacterial adhesins and other adhesion factors, several plant factors (plant adhesins) are essential for attachment of *Agrobacterium* to plant cells. These include pectin acceptors, and vitronectin-like and germin-like proteins (Matthysse and Kijne 1998; Ziemienowicz 2001).

4.2 Induction of Bacterial Virulence System

The products of the genes encoded by the 30–40 kb *vir* region of the Ti plasmid mediate T-DNA transfer process. The activation of *vir* genes is carried out by two genes, *vir*A and *vir*G, during co-culture with plant cells (Stachel and Nester 1986). Although constitutive expression of *vir*A and *vir*G are basically at low levels but they are also highly stimulated in an autoregulatory fashion (Winans et al. 1988; McCullen and Binns 2006). There is substantial homology between *vir*A and *vir*G and the genes encoding two-component regulatory systems in which a response regulator is activated as a result of controlling the latter's phosphorylation status by a sensor kinase in response to signal input (de la Riva et al. 1998; Wolanin et al. 2002; McCullen and Binns 2006).

VirA is a transmembrane dimeric protein localized on the plasma membrane of the bacterial cell and it acts as a sensor in detection of signal molecules, mainly small phenolic substances, released from wounded plants (Pan et al. 1995; Gelvin 2003). Activation of VirA is achieved by the signals including acidic pH, phenolic substances such as acetosyringone (Winans 1992; Ziemienowicz 2001) and certain group of monosaccharides, which work synergistically with phenolic substances (Ankenbauer and Nester 1990; Cangelosi et al. 1990; Shimoda et al. 1990; Doty et al. 1996; de la Riva et al. 1998). VirA protein consists of three domains: the periplasmic or input domain and two transmembrane domains (TM1 and TM2). The transmembrane domains work as a transmitter (signaling) and receiver (sensor) (Parkinson 1993). The periplasmic domain is required for detection of monosaccharides (Chang and Winans 1992). This structure is a common structure for other transmembrane sensor proteins and the protein is anchored in the inner membrane by a simultaneous alignment with the inner membrane as a result of protein folding

(Seligman and Manoil 1994). The TM2 is the kinase domain and plays a crucial role in the activation of VirA (Huang et al. 1990; de la Riva et al. 1998).

VirA carries out amplification of the transformation system by detection of monosaccharides in the presence of low concentrations of phenolic compounds. This system could be induced only through the periplasmic sugar (glucose/galactose) binding VirE (Ankenbauer and Nester 1990; Cangelosi et al. 1990), which interacts with VirA (Shimoda et al. 1990; Chang and Winans 1992; Shimoda et al. 1993; Turk et al. 1994; de la Riva et al. 1998). In addition, for induction of *vir* gene expression, the phenolic substances are absolutely required whereas the other signals sensitize the bacteria to the phenols (McCullen and Binns 2006).

Activated VirA has the capacity to transfer its phosphate to a conserved aspartate residue of the cytoplasmic DNA binding protein VirG (Jin et al. 1990a, b; Pan et al. 1993). VirG functions as a transcriptional factor regulating the expression of *vir* genes when it is phosphorylated by VirA (Jin et al. 1990a, b; McCullen and Binns 2006). Transcription of *vir*BCDEFGH genes are activated by phosphorylated VirG protein with binding to "*vir* boxes". Except *vir*H gene, all other *vir* genes are involved in T-DNA processing, transport and integration and are essential for the infection (Ziemienowicz 2001).

External factors like temperature and pH also play important roles in activation of *vir* system. The expression of *vir* genes is not possible at temperature higher than 32°C due to a conformational change in the folding of VirA causing the inactivation of its properties (Jin et al. 1990a).

4.3 Generation of T-DNA Transfer Complex

Generation of single-stranded (ss) molecules is produced by activation of *vir* genes representing the copy of the bottom T-DNA strand. The copy number of T-DNA strands is approximately one copy per induced *Agrobacterium* cell and is derived from the coding strand of the T-DNA element (Stachel and Nester 1986; Sheng and Citovsky 1996). Two 25 bp long imperfect direct repeats flank the region known as border sequences. VirD1 and VirD2 proteins from the *vir*D operon recognize and cleave the T-DNA borders (Yanofsky et al. 1986; Stachel and Nester 1986; Filichkin and Gelvin 1993; Ziemienowicz 2001). The nick sites are considered to be the initiation and termination sites for T-strand recovery. After endonucleotidic cleavage by VirD2, the 5′ of the nicked T-DNA gets covalently bound to VirD2. Binding to the 5′ end it prevents 5′ exonuclease activity (Dürrenberger et al. 1989) and distinguishes the 5′-end as the leading end of the T-DNA transfer complex. VirD1 is considered as topoisomerase and it binds to the region where the ss-T-strand is originated. *In vitro* experiments proved that the presence of VirD1 is required for the cleavage of supercoiled stranded DNA by VirD2 (Zupan and Zambryski 1997; Christie 1997). The simultaneous establishment of the excised ss-T-strand is evolutionarily related to other bacterial conjugative DNA transfer processes (Lessl and Lanka 1994; Christie 1997; Zupan and Zambryski 1997; de la Riva et al. 1998; Zupan et al. 2000).

It seems that the right border acts as a starting site for T-strand synthesis, progressing leftwards and terminating at left border even when the left border is mutated or completely absent, although with lower efficiency. The left border can act as an initiation point for ss-T-strand synthesis but the efficiency is much lower (Filichkin and Gelvin 1993).

There is no difference between the right and left border sequences but the right border is more active than the left. The difference between two borders is the presence of an enhancer or "overdrive" sequence close to the right border (Peralta and Ream 1985). It has been found that VirC1 protein specifically recognizes this enhancer (Toro et al. 1989). VirC binds to overdrive region at the right end of the right border of T-DNA (Toro et al. 1989), and helps in unwinding the dsDNA and in activating cleavage of DNA at the right border (Zhu et al. 2000; Ziemienowicz 2001; Gelvin 2003). Deletion of *vir*C operon is followed by attenuation of virulence of the *Agrobacterium* strains (Rogowsky et al. 1987; van Haaren et al. 1988; Gelvin 2003).

4.4 The T-DNA Transfer

It is implied that the T-strand from the bacterium passes into the plant cell as a protein-nucleic acid (ss-T-DNA-protein) complex (Sheng and Citovsky 1996) and during translocation of ss-T-DNA-protein complex cross over three membranes, the plant cell wall and cellular spaces (de la Riva et al. 1998). This T-DNA transport is intermediately descripted as the T-complex (Howard and Citovsky 1990) and is a T-strand DNA molecule (carries the genetic information) associated with two proteins, VirD2 and VirE2 (protect the T-strand, help in transporting the DNA across shape and supply specific targeting signals) (Table 12.3) (Sheng and Citovsky 1996). VirE2 is a 69-kDa single stranded DNA binding protein and according to the most commonly accepted model, by binding of VirE2 to the ss-T-DNA-VirD2 complex, a cooperative association arises, preventing from any nuclease digestive activity, stabilizing the DNA into a rigid rod-like structure and making the translocation through membrane channels easier (de la Riva et al. 1998).

VirE2 contains two plant nuclear location signals (NLS) and VirD2 (Tinland et al. 1995). This fact indicates that both proteins play roles in importing proteins to the plant cell nuclei, and a plant import has been shown to mediate nuclear import of VirD2 (Ballas and Citovsky 1997; de la Riva et al. 1998) once the complex enters into the plant cell, the complex is transported to the nucleus (Herrera-Estrella et al. 1988; Shurvinton et al. 1992; Tinland et al. 1995; Rossi et al. 1996; Sheng and Citovsky 1996; Zupan et al. 1996). It is known that VirE1 is required for the export of VirE2 to the plant cell, but other specific functions still await characterization (Binns et al. 1995; Gelvin 2003).

An alternative model has been brought to light for ss-T-DNA complex transfer. This model proposes that covalent attachment of VirD2 to the single strand DNA 5′ end, forms the transfer complex but is uncoated by VirE2. Following the independent transfer of VirE2 into plant cell as the result of natural process, the naked

Table 12.3 The functions of Vir proteins both in *Agrobacterium* and plant cells

Vir protein	Function in *Agrobacterium*	Function in plant
VirA	Sensor for phenolic substances	
VirG	Phenolic response regulator	
VirB1-11	Synthesis of T-pilus, showing ATPase activity and provide the energy required for export of other protein subunits, for T-DNA transport. They thought to form pore for T-DNA export	
VirC1	Possible "overdrive" binding protein; increasing T-DNA transfer	
VirD1	*In vivo* T-DNA process and *in vitro* nicking in T-DNA borders	
VirD2	T-DNA border specific endonuclease activity (ss-T-DNA-VirD2 complex)	Nuclear targeting of the T-strand, protection of the T-strand from 5′exonucleolytic degradation, T-strand integration into the plant genome
VirD4	ATP-dependent linkage of protein complex and necessary for T-DNA translocation	
VirE1	Carrying VirE2 from *Agrobacterium* and its protection	
VirE2	Protecting the T-strand, shape it into a transferable (thin and unfolded) form and supplying specific targeting signals	Formation of a putative "T-complex" in the plant, protection of the T-strand from nucleolytic degradation, unclear targeting of the Ti-strand, passage of the Ti-strand through the nuclear pore complex
VirF	Nuclear targeting of the ss-T-DNA	Host range factor, possible interaction with Skpl proteins to regulate plant cell division cycle
VirH	Possible cytochrome p450 enzyme, plays a role in the host range specificity of bacterial strain	

ss-T-DNA-VirD2 complex is coated by VirE2 inside the plant cell (Lessl et al. 1992; Binns et al. 1995; de la Riva et al. 1998).

It is also possible that the process can be performed by one of the proposed alternatives according to the conditions of infection. Previous studies report that the *vir*B operon products organize suitable cell surface structures for the transfer of ssDNA across the membrane into host cell cytoplasm (Finberg et al. 1995; Stephens et al. 1995; Fernández et al. 1996; Beaupré et al. 1997; Dang and Christie 1997; Rashkova et al. 1997; Zhou and Christie 1997; de la Riva et al. 1998). The VirD4 protein is also required for the ss-T-DNA transport. VirD4 is necessary in the translocation of T-DNA by generating the ATP- dependent linkage of protein complex (Firth et al. 1996). VirB are proteins that present hydropathy characteristics similar to other membrane-associated proteins (Thompson et al. 1988; Ward et al. 1988;

Kuldau et al. 1990; Shirasu et al. 1990; Shirasu et al. 1994; de la Riva et al. 1998). VirD4 is a transmembrane protein but predominantly located at the cytoplasmatic side of the cytoplasmic membrane (Okamoto et al. 1991). Comparative studies show that the genetic organization of transfer regions of broad host range (BHR) plasmids reveals a high degree of homology to the *vir*B operon in genetic organization, nucleotide sequence and protein function (Lessl et al. 1992; Pohlman et al. 1994; de la Riva et al. 1998). Both systems deliver non-self transmissible DNA-protein complex to the recipient host cell. In addition, they are capable for DNA interkingdom delivery (Heinemann and Sprague 1989; Bundock et al. 1995; Piers et al. 1996) suggesting that the T-DNA transfer apparatus and conjugation systems are displaying relatedness and apparently share a common ancestral origin (Christie 1997; Oger et al. 1998). The majority of VirB proteins are organized in a membrane-associated pore-forming structure involving both membranes (Shirasu and Kado 1993a, b; Shirasu et al. 1994; Stephens et al. 1995; Das and Xie 1998). Two VirB proteins, VirB4 and VirB11, peripherally associate with others and located primarily in the cytoplasm, but a small part of VirB4 may span the inner membrane. These proteins serve as ATPases and are thought to provide energy for the physical movement of the DNA into the host cell across the cellular barrier and for export of other protein subunits or both. VirB proteins are thought to form pore for T-DNA export. Recent studies have demonstrated that VirB proteins form pili that show high degree similarity with the conjugative pili (Fullner et al. 1996; Ziemienowicz 2001) and VirB2 constitutes the major subunit of this pili. VirB7, an outer membrane lipoprotein, may facilitate to anchor pilus to the bacterial cell by forming disulfide bonds with the periplasmically localized VirB9 (Ziemienowicz 2001).

*vir*F and *vir*H operons are accessory in the octopine Ti plasmid. One of the *vir*F operon products is a 23 kDa protein that functions once the T-DNA complex is inside the plant cells via the conjugal channel or independently as it was considered for VirE2 export. VirF plays a role identified as assistance in the nuclear targeting of the ss-T-DNA complex (Hooykaas and Shilperoort 1992). The *vir*H operon consists of two genes that code for VirH1 and VirH2 proteins. These Vir proteins are functionally redundant but could enhance the transfer efficiency, detoxifying certain plant compounds that can affect bacterial growth (Kanemoto et al. 1989). If this is the function of VirH proteins, then they play a role in the bacterial attachment to the host plant species (de la Riva et al. 1998).

4.5 Integration of T-DNA into Plant Genome

The *Agrobacterium* T-complex is imported into the host cell nucleus and then the DNA becomes integrated into the host chromosomes (Sheng and Citovsky 1996). The ss-T-DNA complex is directed to the nucleus passing the nuclear membrane inside the plant cell. Both VirD2 and VirE2 proteins of the T-complex have been found to play roles in the integration process and VirF probably plays a minor role in this process (Hooykaas and Shilperoort 1992; Sheng and Citovsky 1996). In addition,

the nuclear location signals (NLS) of VirD2 and VirE2 are identified as important players in nuclear targeting of the delivered ss-T-DNA complex, as early described. VirD2 has one functional NLS (de la Riva et al. 1998).

A large nucleoprotein complex called ss-T-DNA complex is created when one molecule of VirD2 is covalently attached to the 5′-end of the T-strand. Nevertheless, the complex is coated with VirE2 molecules and each of them has two NLS. The two NLS of VirE2 are responsible for the continuous nuclear import of ss-T-DNA complex. They probably keep both sides of nuclear pores simultaneously open (de la Riva et al. 1998). The nuclear import process is initiated by certain NLS-binding proteins in the plant cytoplasm. The final step of T-DNA transfer is its integration into the plant genome. The mechanism of the T-DNA integration is largely uncharacterized. It is considered that the integration occurs by illegitimate recombination (Gheysen et al. 1991; Lehman et al. 1994; de la Riva et al. 1998; Puchta 1998).

A sequence at T-DNA 3′-end finds homology with plant DNA resulting in a first contact between the T-strand and plant DNA and generating a gap in 3′–5′ strand of plant DNA. Endonucleases digest the displaced plant DNA at the 3′-end position of the gap and VirD2 attached to the 5′-end accompanies with a nucleotide in the top (5′–3′) plant DNA strand. The 3′ overhanging part of T-DNA and displaced plant DNA are removed away by digesting either with endonucleases or 3′–5′ exonucleases (de la Riva et al. 1998). Then, the 5′ attached to VirD2 end and other 3′-end of T-strand joins the nicks in the bottom plant DNA strand (Tinland et al. 1995). Once T-strand is introduced into the 3′–5′ strand of the plant DNA, a torsional strain would then result in the introduction of a nick into the opposite plant DNA strand. The repair mechanism of the plant cell is activated because of this situation and using the early inserted T-DNA strand as a template, complementary strand is synthesized (Fig. 12.6) (Tinland et al. 1995; de la Riva et al. 1998).

The published data indicates that the function of VirD2 is the precise integration of T-strand into the plant chromosome. VirD2 may provide energy by releasing from its phosphodiester bond, at the Tyr29 residue, with the first nucleotide of T-strand providing the 5′-end of the T-strand for ligation to the plant DNA. This phosphodiester bond is electrophilic center and may act as substrate for nucleophilic 3′-OH from nicked plant DNA (Jayaram 1994). VirD2 is also implicated in other functions such as recruiting plant enzymes involved in DNA repair or recombination to the site of the integration and/or by interaction with some structural chromatin proteins (Ziemienowicz 2001).

5 Use of *Agrobacterium tumefaciens* in Plant Biotechnology

5.1 General View to Plant Biotechnology

Hungarian agricultural engineer Karl Ereky first recorded use of the term “biotechnology” in 1919 (Hopkins 2007). It is defined as “any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use”. In fact, the origin of biotechnology

could be traced back to prehistoric times. In those times microorganisms had already been used for the processes like fermentation, for production of yoghurt, cheese and vinegar (Chawla 2002). Nevertheless, biotechnology was applied throughout for these products without any real scientific understanding of what was occurring (Lindsay 2002). Biotechnology got boost in the 1970s with the discovery of restriction enzymes, which cut double stranded or single stranded DNA at specific recognition nucleotide sequences (Roberts 1976; Chawla 2002), gene technologies began developing rapidly after that. Today, biotechnology is synonymous with genetic engineering. The term genetic engineering, which was first coined by Jack Williamson, refers to a number of new techniques involving transfer of specific genetic information from one organism to another (Chawla 2002; Stableford 2004).

Plant biotechnology starts with tissue culture techniques (Chawla 2002). Plant cell and tissue cultures are sets of techniques that were designed for growing and cloning any plant parts by using some culture media in aseptic and controlled conditions (Vargas and Flota 2006). With the use of tissue culture techniques, elite plant varieties are clonally propagated, endangered plants are conserved, virus-free plants are produced, germplasm is conserved, and finally secondary metabolites are produced. Additionally, tissue culture serves as an indispensable tool for transgenic plant production (Cardoza 2008). These techniques were developed in the early 1960s and became standard procedures for modern biotechnology. Presently five major areas are recognised in this direction **(1)** large-scale propagation of elite materials, **(2)** generation of genetically modified fertile individuals, **(3)** as a model system for fundamental plant cell physiology aspects, **(4)** preservation of endangered species and **(5)** metabolic engineering of fine chemicals (Vargas and Flota 2006).

Tissue culture techniques have made it possible to transfer foreign genes from one plant to another. Recognition of the biology of *A. tumefaciens* and application of its T-DNA system has been a great step in plant biotechnology. In general, because of these modern gene technological methods, plant biotechnology has grown into a new dimension with putative future possibilities that can hardly be overestimated (Twyman et al. 2002). Today, related with the improved tissue culture and gene transfer methods, plant biotechnology has become one of the most important branches in science. The history of plant biotechnology, especially related to *Agrobacterium* sp. is given below chronologically (Table 12.4).

5.2 Agrobacterium and Plant Genetic Engineering

One of the most significant developments in plant biotechnology is stably introducing foreign genes into plant genome. Attempts have been made by using transgenic techniques for manipulating commercially important plants in conjunction with their secondary metabolic pathways for production of a variety of important metabolites (Merkli et al. 1997). The advantages of transformation over conventional cell culture systems are numerous including fast growth and stable high-level production of secondary metabolites making them ideal for biotechnological utilization (Chawla 2002; Twyman et al. 2002).

Table 12.4 The brief history of plant biotechnology related to *Agrobacterium* sp

1853	The first written report of crown gall disease (Fabre and Dunal 1853)
1897	*Agrobacterium vitis* identified as causal agent of crown gall in grape (Cavara 1897)
1902	First attempt of plant tissue culture (Haberlandt 1902)
1907	*A. tumefaciens* identified as causal agent of crown gall in Paris daisy (*Argyranthemum frutescens*) (Smith and Townsend 1907)
1941	*In vitro* culture of crown gall tissues (Braun 1941)
1947	Sterile plant tumor tissue can proliferate indefinitely on hormone-free medium in culture. Tumor cells are proposed to be 'transformed' by an *Agrobacterium*-derived tumor-inducing principle (TIP) (Braun 1947)
1952	First application of micrografting (Morel and Martin 1952)
1954	First plant from single cell (Muir et al. 1954)
1956	Unusual low-molecular weight nitrogenous compounds (opines) are identified exclusively in tumor tissue (Lioret 1956)
1959	Publication of first handbook on plant tissue culture (Gautheret 1959)
1962	Development of Murashige and Skoog nutrient medium (Murashige and Skoog 1962)
1970	Discovery of first restriction endonuclease from *Haemophilus influenzae* Rd. It was later purified and named *Hind*I (Smith and Wilcox 1970)
1971	*A. tumefaciens* loses virulence when grown at 37°C. The TIP can be transferred between virulent and avirulent *A. tumefaciens* strains (Hamilton and Fall 1971; Kerr 1971)
	Preparation of first restriction map using *Hind*I enzyme to cut circular DNA of SV 40 into 11 specific fragments was prepared (Danna and Nathans 1971)
1974	*A. tumefaciens* virulence depends on the presence of a large 'tumor-inducing' (Ti) plasmid. The TIP is probably a component of the Ti plasmid (Zaenen 1974)
	Biotransformation in plant tissue cultures (Reinhard 1974)
1976	Octopine and nopaline synthesis and breakdown found to be genetically controlled by the Ti plasmid of *A. tumefaciens* (Bomhoff et al. 1976)
1977	The T-DNA region of the Ti plasmid is present in the genome of crown gall tumor cells: the T-DNA is the TIP (Chilton et al. 1977)
1980	The opine concept: the synthesis of opines by transformed cells creates an ecological niche for the infecting strain of *Agrobacterium* (Guyon et al. 1980)
1983	The first plant transformed with a recombinant gene using *A. tumefaciens* as a vector (Zambryski et al. 1983)
1984	T-DNA oncogenes are identified that mediate overproduction of auxin and cytokinin (Klee et al. 1984; Lichtenstein et al. 1984)
	Development of the genetic fingerprinting technique for identifying individuals by analyzing polymorphism at DNA sequence level (Jeffreys et al. 1984)
1985	The *virA*/*vir*G two-component regulatory system is identified as a central component of signal perception and transduction in *Agrobacterium* transformation (Stachel and Zambryski 1986)
1987	Isolation of *Bt* gene from bacterium (*Bacillus thuringiensis*) (Barton et al. 1987)
1990	Development of the random amplified polymorphic DNA (RAPD) technique (Williams et al. 1990; Welsh and McClelland 1990)
2001	Publication of the complete genome sequence of two *A. tumefaciens* strains (Goodner et al. 2001; Wood et al. 2001)
2005	220 million acres of biotechnologic crops with herbicide tolerance and/or insect resistance traits were cultivated in 21 countries worldwide (James 2010)
2007	The global area of biotech crops reached 114.3 million hectares (282.4 million acres) worldwide (James 2010)
2010	The global area of biotech crops continued to soar for the fifteenth consecutive year at a sustained growth rate of 10% or 14 million hectares (35 million acres), reaching 148 million hectares or 365 million acres (James 2010)

One of the earliest biotechnology success stories in plant research is the *Agrobacterium* Ti plasmid, which is a classic example of how a useful research tool is created by years of effort combined with chance (Table 12.4) (Mugnier et al. 1986). Elucidation of molecular mechanism of plant genetic transformation via *A. tumefaciens* was made by scientists years ago and Armin Braun proposed the concept of a "tumor-inducing principle" that was stably transferred to and propagated in the plant genome (Braun 1947; Gelvin 2003). As a result of intense research in the 1970s on virulent *Agrobacterium* strains resulted in the identification of large plasmids (Zaenen et al. 1974), although we now know that many strains harboring plasmids are unrelated to virulence. Genetic experiments indicated that the Ti plasmids were involved in tumorigenesis (van Larebeke et al. 1974) and transmission to plant cells and integration into the plant genome is achieved by a specific segment of these plasmids called the T-DNA (Chilton et al. 1977; Zhu et al. 2000; Ream 2002; Özcan et al. 2004). It was thus obvious to suggest that Ti plasmids can be used as a vehicle to transfer and integrate foreign genes into plant cells. This knowledge, "the ability of *A. tumefaciens* to transfer a fragment of its T-DNA" provided the use of *A. tumefaciens* as highly efficient versatile vehicle for introduction of foreign genes into the desired plant genome and provided this bacterium to be used as a powerful tool for plant biotechnology applications (Ziemienowicz 2001; Chawla 2002; Twyman et al. 2002). However, the following properties of Ti plasmids prevent their direct use: **(1)** large size, **(2)** tumor inducing property and **(3)** absence of unique restriction enzyme sites (Chawla 2002).

Some manipulations are required for Ti plasmid before using *Agrobacterium* in genetic engineering. Hormones produced by oncogenes encoded by T-DNA are involved in tumor formation and establishment of a suitable regeneration to the tissue cultured explants can not be accomplished because of these tumors. The hormone imbalance in plant tissues causes disorganized callus growth (Hernalsteens et al. 1980; Twyman et al. 2002). Therefore, an important step in the development of T-DNA vectors is the realization that the transfer of T-DNA to the host plant was dependent on the *vir* genes and the 24-bp direct repeat structures marking the left and right borders of the T-DNA. Within the T-DNA, genes are not essential for transformation, and any sequence could be incorporated therein. This allowed creating disarmed Ti plasmids where all the oncogenes were removed, facilitating T-DNA transfer to plant cells without causing neoplastic growth. Systematically modified a nopaline Ti plasmid, pTiC58, could be used as a vector for the transfer of T-DNA into plant genomes but no longer cause tumors (Zambryski et al. 1983; Wood et al. 2001). This was achieved by deleting all of the T-DNA except for the left and right border sequences, and *nos* coding sequence and replacing this with a region of pBR322. The resulting plasmid pGV3850 was non-oncogenic and disarmed. *Agrobacterium*-carrying pGV3850 were used to infect discs of carrot or potato, or wounded petunia and tobacco plants. Crown gall-like tumors were not produced and callus tissue was only observed when the explants of wounded tissue or discs were cultured in the presence of phytohormones. These results showed that the deletion of internal sequences was required for hormone independent growth (Zambryski et al. 1983). These types of studies offered new promising approaches

for the production of transgenic plants where foreign genes could be transmitted through seeds (Barz et al. 2002).

Disarmed Ti plasmids are not preferred for use as primary cloning vectors because of their large size and lack of unique restriction enzyme sites. Therefore, two different types of vector systems called co-integrated and binary vectors have been constructed to be used in transformation carrying the foreign genes inserted into these vectors by plant scientists lately (Özcan et al. 2004).

An incorporation of suitable selectable markers into the T-DNA, Ti plasmids turn into very powerful gene delivery vectors. However, wild-type Ti plasmids are inappropriate for this situation as a result of their large size making them difficult to manipulate *in vitro*. An early approach to cope with this problem required subcloning of the T-DNA into a standard *Escherichia coli* plasmid vector which allowed *in vitro* manipulation by normal procedures, and then integrated into a resident disarmed Ti plasmid in *A. tumefaciens* via homologous recombination (Matzke and Chilton 1981). Although it was an easy system to use, and only required three bacterial strains (triparental matings), the system relied on a complex series of conjugative interactions between *E. coli* and *A. tumefaciens*. However, because the *vir* genes act in *trans* for mobilization and transfer of the T-DNA, it was soon realized that using natural large size of Ti plasmids was unnecessary. Intermediate vectors have been discarded and largely replaced by binary vectors (Bevan 1984), in which the *vir* genes and the T-DNA are cloned on separate plasmids. These can be inserted into *A. tumefaciens* by conjugation with an *E. coli* donor or by freeze-thaw cycles or electroporation. Binary vectors are employed in most contemporary *Agrobacterium*-mediated transformation systems (Barz et al. 2002).

5.2.1 Cointegrate Vectors

Vectors that combine via DNA homology into a resident Ti plasmid are often referred as integrative or co-integrate vectors, or hybrid Ti plasmids (Chawla 2002). For production of an intermediate vector, a conventional *E. coli* plasmid vector was used in subcloning of T-DNA isolated from a parent Ti plasmid for easy manipulation (Matzke and Chilton 1981). These vectors were not capable of replicating themselves in *A. tumefaciens*, and also had no conjugation functions (Fig. 12.7) (Primrose and Twyman 2006).

Transfer capacity for plasmids between different species of Gram-negative bacteria relies on several properties of plasmids. Both *cis*-acting region on the plasmid and *trans*-acting plasmid-encoded molecules are necessary for conjugal transfer. Additionally, many plasmids have a narrow host range for replication and/or they are incompatible with another plasmid in the same cell (Paszkowski et al. 1988; Chawla 2002). Three structures necessary for establishing this system easily are; **(1)** an *E. coli* strain carrying a helper plasmid, which is able to mobilize the intermediate vector in *trans*, **(2)** the *E. coli* strain carrying the recombinant intermediate vector and **(3)** disarmed *Agrobacterium* Ti plasmid (Primrose and Twyman 2006).

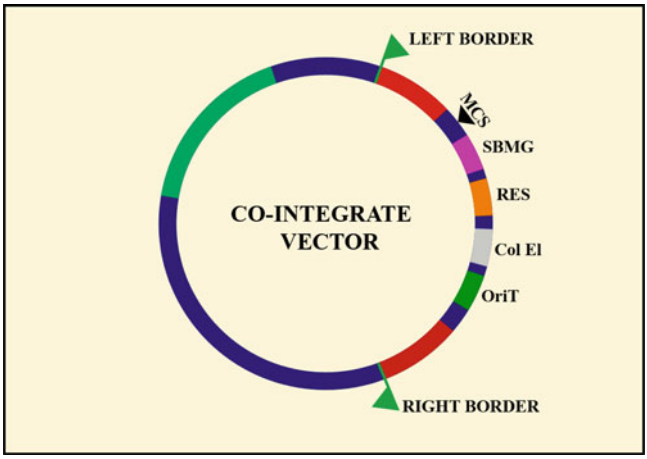

Fig. 12.7 Cointegrate plasmid and its components. *MCS* Multiple cloning site, *SBMG* Selective plant marker gene, *RES* Antibiotic resistance gene, *OriT* Origin of transfer, *Col E1* Replication origin, which belongs to pCol E1 plasmid (Modified from Armitage et al. 1988; Walkerpeach and Velten 1994 and Özcan et al. 2004)

For recombinant T-DNA, maintenance appears to be dependent on recombination. Efficiency of recombination is enhanced if there is a large homologous region shared by the two plasmids. For example, Ti plasmid pGV3850 carries a segment of the pBR322 backbone in its T-DNA (Chawla 2002; Primrose and Twyman 2006). However, a co-integrate plasmid constructed *in vitro* is assembled from parts of different plasmids. Normally these parts are; **(1)** *vir* genes, **(2)** left and right T-DNA borders, **(3)** an exogenous DNA sequence between the two T-DNA borders, and **(4)** plant and bacterial selectable markers (Chawla 2002).

5.2.2 Binary Vectors

The generation of transgenic plants is greatly simplified by T-DNA binary systems. Sophisticated and complex microbial genetic approaches are no longer required to integrate T-DNA regions located on large Ti or Ri-plasmids (Lee and Gelvin 2008). The binary vector systems consist of two autonomously replicating plasmids within *Agrobacterium*; **(1)** a shuttle (binary) vector containing gene of interest between the T-DNA borders and **(2)** a helper Ti plasmid which provides the *vir* genes necessary for facilitating transfer into plant cells (Chawla 2002). A T-DNA is considered disarmed if its tumor-inducing (oncogenic) genes are removed by genetic engineering methods (Chawla 2002; Lee and Gelvin 2008).

A standard binary vector consists of **(1)** multiple cloning site, **(2)** a broad host range origin of replication functional in both *E. coli* and *A. tumefaciens,* **(3)** a selectable marker for both plant and bacteria and **(4)** the left and right T-DNA borders (or at least the right T-border) (Fig. 12.8) (Hoekema et al. 1983; Bevan 1984).

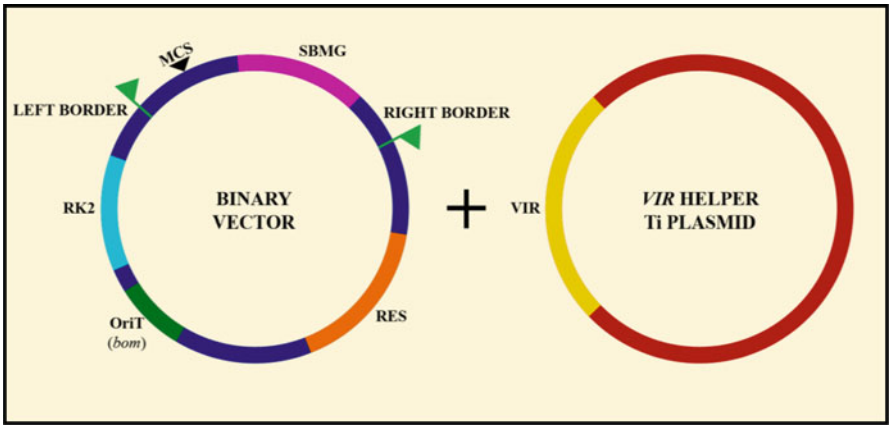

Fig. 12.8 Binary plasmid, its components and *vir* helper Ti plasmid. *MCS* Multiple cloning site, *SBMG* Selective plant marker gene, *RES* Antibiotic resistance gene, *OriT* Origin of transfer and ***bom*** region, *RK2* Replication origin, which belongs to pRK2 plasmid (Modified from Armitage et al. 1988; Walkerpeach and Velten 1994 and Özcan et al. 2004)

In general, the transformation procedure is as follows; **(1)** direct transfer of the recombinant small replicon to *A. tumefaciens* harboring a helper Ti plasmid via bacterial conjugation or direct transfer, **(2)** co-cultivating the plant cells with the *Agrobacterium* for allowing transfer of recombinant T-DNA into the plant genome and **(3)** selecting transformed plant cells under appropriate conditions. The stability of wide host range replicons in *E. coli* and *Agrobacterium* varies considerably, some having possible disadvantages in this type of plasmids. Two different origins of replication known to be active *E. coli* may be unstable depending on the orientation (Lee and Gelvin 2008). There are many advantages of these types of vectors when compared with co-integrated vectors; **(1)** no recombination process takes place between the molecules involved in binary vectors **(2)** high transformation efficiency from *E. coli* to *Agrobacterium* is achieved by using small vectors instead of using large, recombinant, disarmed Ti plasmid (2–3 days versus 4–7 days) **(3)** *Agrobacterium* carrying plant ready genes on a binary vector is easily and efficiently obtained **(4)** the only requirement for binary vectors is an intact plasmid to be inserted into the target bacterium, making the bacterial transformation process more efficient and faster (2–3 days versus 4–7 days) (Chawla 2002; Lee and Gelvin 2008).

5.3 Marker and Reporter Genes

The success rate generally appears to be at a very-low frequency for integration of introduced foreign genes during the genetic transformation of plants (Hopkins 2007). Therefore, the availability of monitoring techniques for analyzing the expression of

Table 12.5 The mostly used marker and reporter genes, their encoded enzymes and conferred resistances

Marker Gene	Enzyme Encoded	Resistance Conferred
Antibiotics		
npt II	Neomycin phosphotransferase	Kanamycin, neomycin, G418, paromycine
hpt or *aph* IV	Hygromycin -phosphotransferase	Hygromycin
dhfr bacterial or mouse	Dihydrofolate reductase	Methotrexate
bla	TEM-1 β-lactamase	Ampicillin
aadA	Association with several transposons (Tn7, Tn21, …)	Streptomycin and spectinomycin
Herbicides		
bar	Phosphinothricin acetyltransferase	Phosphinothricin
aro A	5-enolpyruvylshikimate-3-phosphate synthase	Glyphosate
Modified *als* genes	Acetohydroxyacid synthase (or acetolactate synthase)	Chlorsulfuron, imidazolanones
Reporter Genes		
CAT	Chloramphenicol acetyltransferase	
GUS	β-glucuronidase	
npt II	Neomycin phosphotransferase	
Luc	Luciferase	
bar	Phosphinothricin acetyltransferase	
β-gal	β-galactosidase	

introduced foreign genes in cells and tissues is necessary in gene transfer studies (Galbraith et al. 1995). This can be accomplished by using a marker gene for selection and regenerating plants under the appropriate culture conditions in the genetic transformation (Twyman et al. 2002). For optimization of co-cultivation parameters, culture media, explants and *in vitro* growth conditions for developing efficient transformation protocols in morphogenic tissues and protoplasts, marker genes have been utilized in most of the transformation experiments (Rugini et al. 2000). To achieve the above target, cloning vectors are constructed containing marker genes along with the 'gene of interest'. The marker genes are broadly of two types: **(1)** Selectable markers and **(2)** Reporter genes (Stirn and Lörz 2006; Miki 2008). Selectable marker genes encode easily detectable traits enabling to distinguish marked cells from non-marked cells. The two most widely used selectable marker genes encode proteins that provide herbicide and antibiotic tolerance (Table 12.5) (Galbraith et al. 1995).

A reporter gene is a gene, which encodes a product that can readily be assayed. Reporter genes have been attached to another gene of interest in cell culture, animals or plants (Koo et al. 2007). While the selectable marker genes help the researcher select transgenic tissue, reporter genes usually report which cells are transgenic (Miki 2008). In contrast with selectable marker genes, reporter genes do not confer resistance to selective agents inhibitory to plant development (Stirn and Lörz 2006).

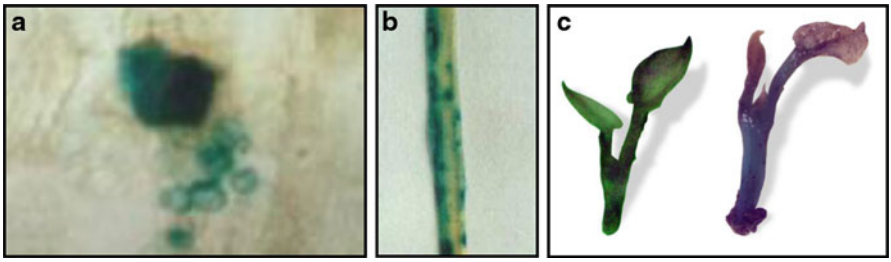

Fig. 12.9 A-*GUS* gene expression in a transversal section of a mature embryo of barley (*Hordeum vulgare* L.) (*Magnification*: 100×) and B-*GUS*-expressing cell clusters in a mature leaf segment from a 3-month-old plantlets of barley (From Gürel and Gözükirmizi 2003) C-Control (*left*) and *GUS* gene expression showing (*right*) young cotton (*Gossypium hirsutum* L.) plantlets (From Özyiğit et al. 2006)

Reporter genes code for products which can be detected directly or catalyze reactions whose products are detectable and as a result, a scientist can detect when and where the gene is active in the genome (Table 12.5) (Schrott 1995; Stirn and Lörz 2006).

The *CAT* (Chloramphenicol acetyl transferase) gene has been employed as a reporter gene in assessing the conditions for polyethylene glycol (PEG)-mediated transfection of kiwifruit protoplasts (Oliveira et al. 1991). A transient expression of *CAT* gene was observed in protoplasts of cv. Harward by using 30% polyethylene glycol 4,000 and by having protoplasts at 45°C for 5 min before transfection. Further research on DNA uptake clarified that transient expression rates of either the *CAT* or the β-glucuronidase (*GUS*) genes were influenced negatively when electroporation was tried without reproducing plasmid uptake (Oliveira et al. 1994; Rugini et al. 2000). The transient *GUS* gene expression was studied in two protoplast populations obtained from *in vitro*-grown leaf material of the same cultivar. One population originating from epidermis and leaf veins was composed of hyaline or light green protoplasts and the other originating from mesophyll was composed of green protoplasts very dense in chloroplasts (Raquel and Oliveira 1996; Rugini et al. 2000). One of the most important systems used to study gene regulatory elements and mechanisms in plants has been the *GUS* gene system (Fig. 12.9) (Miki 2008).

5.4 PCR and Blotting Techniques

Polymerase chain reaction (PCR) and Southern blot analysis can be used in confirmation of the transgenic nature of regenerated plants (Narula et al. 2004). However, screening can be carried out by using Southern blotting or PCR for the confirmation of whether regenerants have integrated the antibiotic resistance gene (and also a gene of interest if this was linked to it within the T-DNA) (Maniatis

et al. 1982; van der Meer 2006). Lately, for DNA analysis, northern and western blotting methods have been used (Vargas and Flota 2006). The basic principles of these methods are given here.

5.4.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a highly sensitive scientific technique that is widely used to amplify specific DNA sequences requiring appropriate primers (Wu 1995a; Wu et al. 1993, 2004). The PCR technique developed by Kary Mullis in 1983 is now a common and often indispensable technique used in biological research labs for a variety of applications (Bartlett and Stirling 2003). PCR provides the ability to amplify and clone the genomic DNA, cDNA or RNA strands thousands of times, in a matter of minutes, by using the natural ability of an enzyme called polymerase. It is also a relatively rapid and inexpensive procedure (Edwards et al. 1991; Ausubel et al. 1995; Wu 1995a, b; Mohan et al. 1998; Wu et al. 2004). PCR makes studying a certain piece of DNA easier for scientists DNA and RNA expression, genetic diagnosis, detection of mutations and transgene analysis became routine applications by using of this technology (Mohan et al. 1998). Components required to carry out a PCR reaction are; **(1)** the DNA or RNA fragment to be copied, **(2)** two primer fragments, **(3)** the polymerase enzyme and **(4)** a special machine that carefully controls temperature.

With respect to the general principles of PCR procedures, oligonucleotide primers complementary to opposite strands of DNA region are prepared and separated by up to a few hundred base pairs. Denaturing is the word used for separation process (Mohan et al. 1998). *Taq* DNA polymerase purified from thermophile bacterium called *Thermus aquaticus* is a temperature-tolerant enzyme and catalyzes the synthesis of a new DNA strand complementary to a template DNA from 5′ to 3′ direction by using special short sequences called primers during the PCR (Nair 2008). The *Taq* DNA polymerase is stable in high-temperatures (90–96°C) and therefore amplification of target sequences is possible for many cycles using excess primers in a commercial thermocycler. In recent times, high- *Taq* polymerases, such as recombinant polymerase *Tth*, long-span polymerase and high-fidelity PCR polymerase, have been developed (Wu et al. 2004; Nair 2008). The next step involves adding the primers and lowering the temperature to facilitate bonding formation. Since forward and reverse primers are complimentary to the up- and downstream regions of the target DNA, they will bind to those areas. They are employed in the copying process as building blocks to up- and down regions. At this point, the polymerase and deoxyribonucleoside triphosphates are added and temperature is raised to match the optimal working temperature for polymerase. It recognizes the primers and starts to copy. After 20–30 cycles of PCR are performed, hundreds or thousands of copies of the original target sequence are produced (Mohan et al. 1998; Wu et al. 2004; Nair 2008).

The PCR technique has become a valuable tool in the field of molecular genetics because it offers rapid analysis, ease of automation, its relative economy and extraordinary specificity (Mohan et al. 1998). By contrast, the fact that false-negative

results may present a problem if positive and negative controls are omitted or do not work in PCR process. Additionally, extra precautions should be taken when assaying putative transformants in PCR screens for eliminating the problem of false positives produced by the plasmid having a source of nonintegrated DNA in the plant cell in the process of *Agrobacterium*-mediated gene transfer. (Cubero and López 2005; Zale 2008). Young plantlets or seedlings regenerated directly after an *Agrobacterium* treatment without an intervening antibiotic treatment should not be used in PCR proceedings, and even then, there might be alive *Agrobacterium* cells existing in plant tissues. An antibiotic such as carbenicillin (Cheng et al. 1997) or cefotaxime (Broothaerts et al. 2005) could be used to control bacterial contamination in regenerated plantlets and whole seedlings (Zale 2008).

5.4.2 Southern, Northern and Western Blotting

There are three different blotting techniques, which are called Southern, northern and western blot. The Southern blot detects DNA (DNA-DNA hybridization), the northern blot detects mRNA (messenger ribonucleic acid, DNA-RNA hybridization) and the western blot detects proteins (protein-protein hybridization such as antigen-antibody binding or protein-ligand binding) (Nair 2008). The blotting techniques are often used to detect genetic abnormalities, detection of mutations and transgene analysis (Mohan et al. 1998). A mixture of DNA, RNA or protein fragments can be separated by gel electrophoresis and the obtained bands can be stained and visualized directly in the gel. However, confirmation and identification of these bands or finding the similarity of one or more of these bands with a known and available molecular probe, it is possible to hybridize these bands with a labeled probe (Wu et al. 2004).

To facilitate this hybridization, the bands are often transferred to a nitrocellulose membrane through a technique described as blotting. When DNA bands are blotted, it is called Southern blotting; when RNA bands are transferred and described as northern blotting and similarly when protein bands are transferred, the technique is described as western blotting.

The Southern Blot

The Southern blot technique was developed by E. M. Southern, begins with the isolation of genomic DNA from cells like peripheral leukocytes or fetal cells (Southern 1975). The technique is used to detect and identify specific DNA fragments on an agarose gel after the separation by electrophoresis (Mohan et al. 1998; Einspanier 2006). This technique consists of six main steps. They are; **(1)** preparation of DNA for analysis **(2)** separation of DNAs by an agarose gel **(3)** denaturation of dsDNAs into ssDNAs **(4)** transfer of DNAs onto a membrane **(5)** hybridization with a non-radioactive or a radioactive probe **(6)** detection of hybridized signals (Wu et al. 2004).

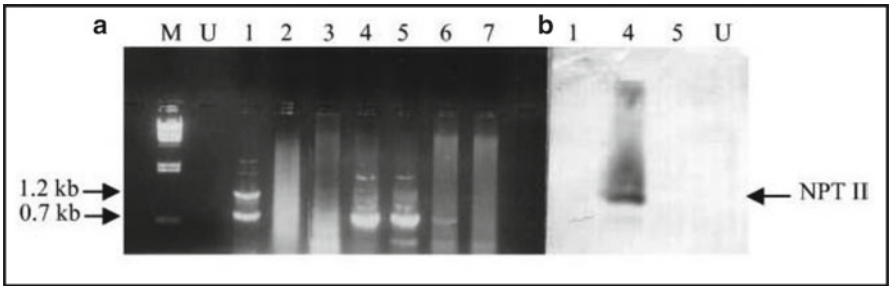

Fig. 12.10 A-PCR and B-Southern blot analysis of barley plants developed from transgenic embryos (From Gürel and Gözükirmizi 2003)

A series of DNA fragments which are reproducible can be obtained by digesting high molecular weight genomic DNA with restriction enzymes. These fragments are separated by electrophoresis in agarose gels based on their molecular weights (Mohan et al. 1998). The resulting dsDNA species are denatured into ssDNAs and then blotted or immobilized onto nylon or a nitrocellulose membrane. The transfer is exact and does not disturb the location and grouping of the DNAs from gel to paper. This process is called "blotting" (Beaudet 1991; Wu et al. 2004). Since there are usually many fragments on a blot, DNA probes are used to localise specific pieces of nucleotide sequences (Fig. 12.10). If the specific labeled probe finds a fit (because of complementarity) on the paper blot, it will bind to the DNA. The membrane is washed to remove unbound probe and then labeled probe is detected by using x-ray film (Beaudet 1991; Mohan et al. 1998).

The Northern Blot

Northern blot hybridization, which was developed by James Alwine, David Kemp and George Stark in 1977, is a procedure in which at different sizes of RNA molecules are separated by agarose gel, fixed onto a membrane following electrophoretic separation, and then incubated with a labeled DNA or RNA probe for hybridization process (Alwine et al. 1977; Wu et al. 2004). The northern blot is similar to the Southern blot except that the northern blot uses mRNA from samples instead of DNA (Wu 1995b; Mohan et al. 1998). One of the most fundamental and powerful tools used in analysis of gene expression is the northern blot technique (Wu 1995b). This technique is a sensitive, reliable and quantitative method for characterization of the steady-state level of RNA transcripts. It consists of five main steps; **(1)** preparation of RNAs for analysis, **(2)** separation of RNAs on an agarose gel, **(3)** transfer of RNAs onto a membrane, **(4)** detection of hybridized signals and **(5)** hybridization with a non-radioactive or a radioactive probe (Wu et al. 2004). RNAs migration through the gel is relative to their size during electrophoresis. Small RNA species move faster than large RNA molecules (Wu 1995b). Northern blot analysis provide

information regarding of the species, sizes and expression levels of diversity of RNAs that cannot be revealed by alternative techniques such as the dot/slot blot and RNA protection assays (Wu et al. 2004).

The Western Blot

Proteins translated from mRNA species are regarded as the end products of gene expression. The protein facilitates the specific function of a gene as result of a meaningful gene expression (Wu and Welsh 1996a, b). Therefore, protein expression is one of the most important parts of modern molecular biology. Electrophoresis in which a net charged molecule moves in an electric field is one key approach used for the analysis of proteins. For this aim, the most widely utilized system for separating proteins on the basis of their net charges, sizes and shapes is the SDS-PAGE (sodium dodecyl sulfate poly-acrylamide gel electrophoresis) which was developed in the mid-1960s (Knudsen 1985; Wu 1995b; Wu and Welsh 1996a). Protein antigens can be detected by using another technique called "western blotting" (Burnette 1981). In western blotting, cellular total protein is isolated and separated in a polyacrylamide gel, electroblotted onto a solid PVDF (polyvinylidene difluoride) or nitrocellulose membrane, and then specified with primary and secondary antibodies (Reece 2004; Zale 2008).

The antibodies that are bound to the protein fixed to membrane are detected and analysis of the membrane is carried out following the procedure (Mohan et al. 1998; Wu et al. 2004). Western blot technique is a sensitive, reliable and quantitative method widely employed in the analysis of proteins. The information about the species, sizes and expression levels of diverse proteins can be tracked simultaneously by using this technique. Other alternative techniques cannot provide such information. (Kyhse-Anderson 1984; Wu and Welsh 1996a, b; Wu et al. 2004). This technique consists of five main steps; **(1)** preparation of proteins, **(2)** separation of proteins by SDS-PAGE, **(3)** incubation of the membrane with primary antibodies, **(4)** incubation of the membrane with secondary antibodies, **(5)** detection of specific protein band and **(6)** transfer of proteins onto a membrane (Wu et al. 2004).

The mobilities of proteins on the gel will be influenced by concentration of polyacrylamide gel. Depending on the particular size of the protein of interest, a general chart is displayed below. Normally, lower percentage gels are better for resolving very high molecular weight proteins (Wu et al. 2004). The electrophoretic blot method can be employed for the transfer of proteins from a polyacrylamide gel onto a solid nitrocellulose or nylon membrane. The membrane is incubated with an antibody raised against the transgenic protein, and the antibody binds primarily through hydrogen bonds (Memelink et al. 1994; Sambrook and Russell 2001). During the detection process the protein of interest with an antibody which is linked to a reporter enzyme is detected by using a secondary antibody that drives a colourimetric reaction and produces a colour on film (Memelink et al. 1994). Size approximations are taken by comparing the stained bands to that of the marker loaded during electrophoresis. The amount of target protein can be semiquantified by band intensity (Zale 2008).

5.5 *A Model Study*, *Agrobacterium-Mediated Gene Transfer to Meristematic Tissues of Cotton (Gossypium hirsutum L.)*

Agrobacterium-mediated transformation of plants is now applicable to many dicotyledonous and several monocotyledonous plant species (DeCleene and DeLay 1976; McCullen and Binns 2006). It can be used to transform many different species based on various factors; **(1)** the broad host range of *Agrobacterium*, **(2)** the regeneration responsiveness of many different explant tissues and **(3)** the utility of a wide range of selectable marker genes (van der Meer 2006). In this chapter, *Agrobacterium*-mediated gene transfer to meristematic tissues of cotton is given as a sample gene transfer protocol (Fig. 12.11).

5.5.1 Surface Sterilization

Before sterilization, cottonseeds were kept under flowing tap water for 1 h. They were surface sterilized by immersion in 70% ethanol for 3 min, followed by stirring in 20% commercial bleach (ACE Lever Co.) for 20 min. The surface sterilized seeds were rinsed 3 times with sterile distilled water for 5 min and they were dried onto sterile filter papers. Seed coats were removed with sterile scalpel and pliers prior to germination (Ozyigit et al. 2007; Ozyigit 2008)

5.5.2 Germination

The seeds were germinated on hormone free Murashige Skoog (MS) medium (Murashige and Skoog 1962) containing macro and micro nutrients supplemented with 100 mg L^{-1} myo-inositol, 0.5 mg L^{-1} thiamine-HCl, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine-HCl and 3% sucrose. 2.2 g phytagel was added into medium and then pH was adjusted to 5.7 with 1 M NaOH before autoclaving. 20 mL MS medium was poured into Magenta vessels and five seeds were germinated in each Magenta (Özyiğit et al. 2006; Özyiğit and Gözükirmizi, 2008).

5.5.3 Culture Conditions

Seeds were left in growth chamber under a photoperiod of 16 h light (7,500 lux) and 8 h dark, at 25°C and 70% humidity (Ozyigit 2009; Ozyigit and Gozukirmizi 2009).

5.5.4 *Agrobacterium*-Mediated Transformation

A. tumefaciens strain LBA 4404 was used as a transformation vehicle to mediate gene transfer into cotton, cv. Çukurova 1518. Plant expression vectors of pBI 121

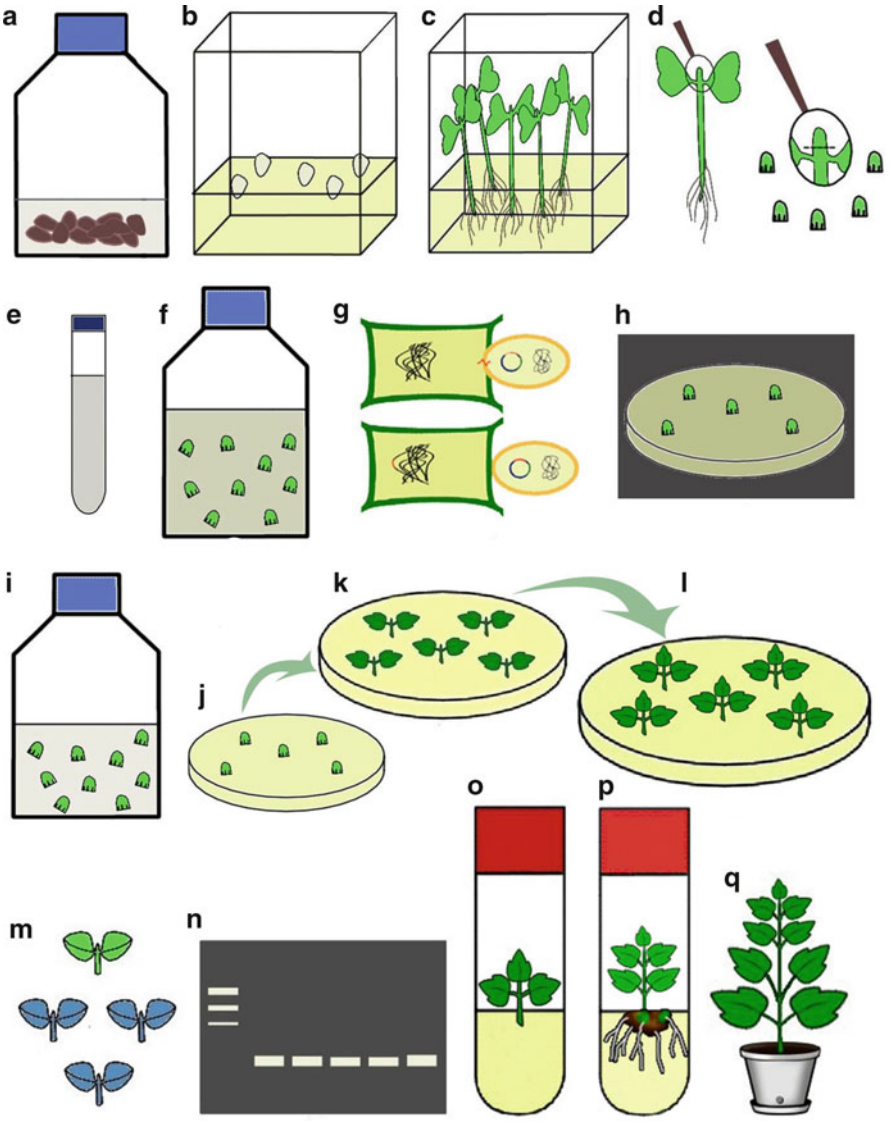

Fig. 12.11 *Agrobacterium*-mediated gene transfer protocol for cotton, (**a**) Surface sterilization, (**b**) Planting seeds, (**c**) 1-week-old young plantlets, (**d**) Isolation of meristematic shoots, (**e**) Breeding of *Agrobacterium*, (**f–g**) Gene transfer to meristematic cells with *Agrobacterium*, (**h**) Culturing explants in darkness, (**i**) Removing *Agrobacterium* by washing with kanamycin, (**j–l**) Culturing explants on selective media, (**m–n**) Histochemical and molecular analysis, (**o–p**) Rooting procedure, (**r**) Adaptation to the soil (Modified from Özyiğit and Gözükirmizi 2008)

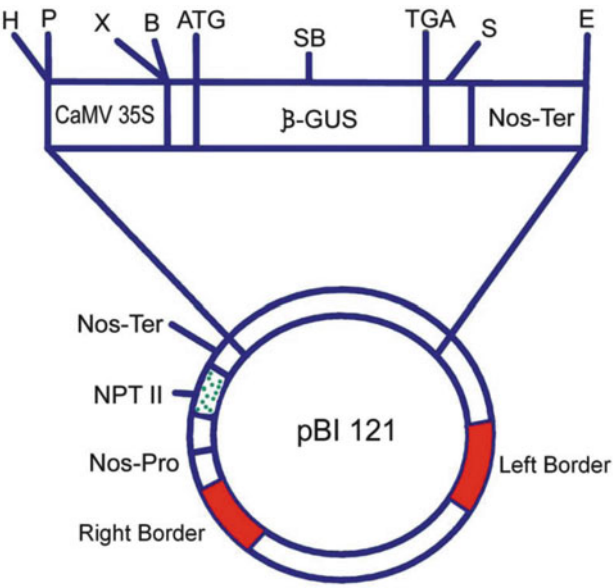

Fig. 12.12 The gene map of pBI-121 plasmid. *Nos-Pro NOS* promoter gene region, *Nos-Ter NOS* gene's terminator region, *CaMV 35S* Cauliflower mosaic virus 35S RNA promoter region, *npt* II Neomycin phosphotransferase II gene, *GUS* β-Glucuronidase gene, Enzyme restriction sites, *H* Hind III, *P* Pst I, *X* Xba I, *B* Bam HI, *SB* Sna BI, *S* Sac I, *E* Eco RI (Modified from Chen et al. 2003)

and pGUS-Int were mobilized into *A. tumefaciens* via triparental mating with pRK 2013 as helper plasmid (Fig. 12.12). The vectors have a selectable marker neomycin phosphotransferase (*npt* II), which confers resistance to kanamycin. The *GUS* and intron-containing *GUS* genes were under the control of CaMV 35S promoter and meristematic tissues carried out the transformation. Meristematic shoot apexes of cotton 1–2 mm long, infected with plasmid-harboring *A. tumefaciens* having final optical density as OD 600 = 0.4. Primary transgenic tissues (TR) were selected on 100 μg/ml kanamycin and 500 μg/ml cefotaxime-containing MS supplemented with 0.1 mg/L KIN + 2 mg/L NAA plates (Uğraş and Gözükirmizi 1999; Özyiğit et al. 2006).

5.5.5 Histochemical GUS Assays

The histochemical GUS assay was also performed with the leaves of 2-weeks-old plantlets. They were washed for 30 min with 50 mM phosphate buffer (pH 7.0) and immersed for 10 min in fixation solution (0.3% formaldehyde, 10 mM MES, 0.3 M mannitol). The samples were put in 1 mM X-gluc (5-bromo-4-choloro-3-indolyl-b-glucuronic acid) solution and incubated at 37°C overnight for blue

color development. *GUS* activity was also detected with overnight cultures of *Agrobacterium* harboring pBI 121 and pGUS-Int by histochemical assays (Özyiğit et al. 2006).

5.5.6 PCR Analyses

Genomic DNA was isolated from 2-month-old plant leaves. PCR was run with specific forward and reverse primers ("*GUS* 1" 5′ GGT GGG AAA GCG CGT TAC AAG 3′ and "*GUS* 2" 5′ GTT TAC GCG TTG CTT CCG CCA 3′) of the *GUS* gene and 35 cycles were driven by thermocycler for 60 s at 95°C, 60 s at 36°C and 90 s at 72°C. The PCR was completed for 10 min at 72°C (Uğraş and Gözükirmizi 1999).

5.5.7 Southern Hybridization

It was performed with XbaI digested genomic DNAs (8 μg) to determine the integration of the *GUS* gene into plant genomes and to estimate the number of insertion in the transformants. The overnight digested DNA was fractionated on a 1% agarose gel and transferred to nylon membrane with fixation by UV crosslinking. The non-radioactive digoxigenin (DIG) hybridization system was used for hybridization analysis. The PCR-amplified product of the *GUS* gene was labeled with DIG-dUTP and used as a probe for Southern hybridization. Hybridization was carried at 68°C and immunological detection steps were performed according to manufacturer's instructions (Uğraş and Gözükirmizi 1999).

6 Conclusion

Plant biotechnology has developed into a separate scientific discipline in a very short span of time and *Agrobacterium*-mediated transformation has emerged recently as an efficient method for genetic manipulation of plants, while it was only a dream 25 years ago. Although other direct DNA transfer methods are also being used, *Agrobacterium*-mediated transformation has major advantages over these systems. Many transgenic economically important plant species, or elite varieties have been developed by the *Agrobacterium*-mediated methods. This situation is especially relevant, because plants are the major and most important source of our nutrition. As a result of developments in plant biotechnology and transgene technologies, during the period 1996–2010, biotech crops have been grown on more than 1 billion hectares. As mentioned by James, in 2010 the global area of biotech crops continues to soar for the fifteenth consecutive year at a sustained growth rate of 10% or 14 million hectares, reaching 148 million hectares. United States, Brazil, Argentina, India, Canada, China, Paraguay, Pakistan and South Africa are the principal

adopters of biotech crops globally. Although there are many discussions about the transgenic crops and transgene technologies, the amount and variety of transgenic crops will increase in the future parallel to increasing world population and demands.

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Chapter 13
Progress and Prospects for Efficient Micropropagation of Woody Plants

Faheem Aftab

Contents

Abstract Despite the progress made so far, tissue culture studies involving woody plants have always remained quite challenging. Amongst several reasons, lack of a suitable explant source still remains a major bottleneck to limit success. The present review describes the progress in micropropagation of difficult to manipulate woody plant species. It discusses the possibility of forcing epicormic or latent buds in a wide range of adult woody plant species of both temperate as well as tropical origin

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where procurement of clean, juvenile explant material is extremely scanty. Forcing may be accomplished under different environmental conditions such as fog, mist or lab and media including sand, sawdust, perlite, vermiculite and their combinations. The explants derived from such forced shoots have shown good potential for either rooting directly under greenhouse conditions or further manipulation under *in vitro* conditions for axillary bud activation, shoot apex proliferation, multiple shoot formation and somatic embryogenesis. This paper intends to review the progress made so far for an efficient micropropagation of several woody species of economic significance making use of the above technology. Several factors limiting the scope of forced epicormic shoots and possible solutions have also been detailed and discussed herein.

Keywords Adventitious shoots • Bud break • Chilling • Epicormic buds • Micropropagation • Shoot forcing • Somatic embryogenesis

1 Introduction

Over the past few decades, plant tissue culture studies have made considerable progress. Amongst plants belonging to diverse groups and families, reproducible protocols exist for callus induction, its maintenance and regeneration. Owing to the relative ease with which these plants may be maintained under *in vitro* conditions (Aftab et al. 2008a, b), much focus, however, has been on herbaceous and crop plants. It is the possibility of maintaining totipotent plant material *in vitro* that has also paved way for several breakthroughs in the genetic engineering of various crop plants (Aftab et al. 1996; Aftab and Iqbal 1999).

Compared with the above-mentioned plants, woody plants including many tree species have posed special problems and thus considered to be quite recalcitrant under *in vitro* conditions (Aftab and Preece 2007). Lack of suitable explant source and reproducible regeneration protocols, release of polyphenols, slow growth and high rate of contamination have limited success with micropropagation of these plants of economic significance.

Several nursery or greenhouse techniques may now be combined with tissue culture techniques in order to get meaningful results in the propagation of several woody plant species (Preece and Read 2003, 2007). The purpose of this paper is to highlight both the problems as well as the prospects of woody plants' propagation under such an experimental setup. Softwood shoot forcing and forcing epicormic buds has emerged as a great tool resulting in the production of plant material that has shown potential as propagules for further development under greenhouse conditions or as a suitable explant source for the establishment of *in vitro* cultures. Since these techniques have mainly been investigated for the plants of temperate origin, this review also outlines several associated problems and forecasts the potential use of such techniques in the micropropagation of a wide range of woody plants.

2 Forcing Epicormic or Latent Buds

Epicormic or the latent buds may prove to be an ideal source material in plants where clean and juvenile tissue is usually not readily available for either greenhouse propagation or *in vitro* manipulations including micropropagation by tissue culture means. Over the past several years, its use has proven to be of great significance and the resulting forced softwood shoots have either been used directly for rooting purposes or as a source material to obtain nodal or apical explants for *in vitro* culture establishment (Preece and Read 2003). Softwood shoots are usually visible within a week's time on the surface of logs in most species that have earlier been derived or cut from adult trees and further cut to a length of some 40 cm and placed some one-fourth embedded in several possible media (details to follow in the next section) in flats. Several studies have shown the significance of the caliper size on the number of forced shoots (Henry and Preece 1997; Ledbetter and Preece 2003; Fishel et al. 2003), but the log length seems to be relatively less critical a factor influencing softwood shoot forcing (Henry and Preece 1997). Experience with some tropical trees like teak (*Tectona grandis* L.) in this lab have shown that the areas on logs surrounding cut branches have several hidden buds that are usually easier to force and may result in greater number of softwood shoots. This is also true for pecan (*Carya illinoensis* (Wangenh.) C. Koch), a temperate nut-producing tree species of the family juglandaceae (Haroon 2011).

Although the logs have been taken from plants and forced in lab conditions on year-round basis but seasonal influence seems to hold good for the production of larger number of softwood shoots. Epicormic buds on pecan logs thus forced easily and at a greater frequency during the spring season rather than autumn where the logs mostly remained quite dormant and produced either a few shoots or none at all (Aftab and Preece 2007).

Perhaps the most important aspect to consider for softwood shoot forcing is to obtain the branches from within the cone of juvenility (Preece and Read 2003). This allows obtaining softwood shoots with juvenile characteristics that may be rooted easily and thus may be used for large scale propagation. Retaining juvenile characteristics is also important if the aim of the studies is to use softwood shoots as an explant source for *in vitro* culture establishment. Detailed account on juvenility in woody plants can be found in a review article by Preece and Read (2003).

2.1 Media and Culture Conditions

Forcing softwood epicormic buds involves placing the logs either in empty flats of suitable size or containing forcing medium. In case of empty flats, it is important that the logs be drenched with water daily in order to retain moisture and keep the buds alive. Drenching is also important in case the medium is dispensed in the flats and

logs are placed embedded in the medium in such a manner that some three-fourth of the logs is exposed to the external environment. Some of the media that in particular have been used for a wide variety of plants are perlite and vermiculite (Aftab et al. 2005). Vermiculite adsorbs and retains more water as compared to perlite and hence provides a better opportunity for the logs that have been forced under the lab conditions (Aftab et al. 2005). The choice of medium thus seems to be interacting with the environment. Combinations of perlite and vermiculite have also been used and it is not surprising that epicormic buds have also been forced in empty flats provided the environmental conditions are humid enough (Aftab et al. 2005). From the past experience, it is also known that even sand may prove to be a good forcing medium and may allow forcing of epicormic buds at a reasonable frequency (Aftab and Preece 2007; Akram and Aftab 2007). The use of some other forcing media such as peat moss, sawdust, cocopeat and even cotton have also been evaluated in the author's lab for such forcing experimentation under different environmental conditions, but so far none of these has been found superior to the ones mentioned above.

As pointed out earlier, the media seem to interact with the culture environment. Epicormic buds have been forced in different environmental conditions such as lab, under mist or fog in a greenhouse (Aftab et al. 2005). In all the environmental conditions, temperature and light conditions may be adjusted to suit the plant in question. Greenhouses in the western countries are usually well-equipped with the facilities to regulate temperature, light quality and intensity and produce mist or fog as desired. The intensity of fog or the duration of mist spray and interval in between is also electronically regulated. Both fog as well as mist have shown good results for the epicormic bud forcing in silver maple (*Acer saccharinum* L.) and white ash (*Fraxinus pennsylvanica* Marsh) though the lab conditions, supported relatively lesser number of softwood shoots (Aftab et al. 2005). Use of ambient temperature and light conditions has also been evaluated in teak during the spring season in order to force a large number of epicormic buds (Akram and Aftab 2007). This way of producing softwood shoots may also help in those conditions where a lot of softwood shoot production is desired but greenhouse facilities are non-existent. This may, however, result in lack of reproducibility of results thus limiting its experimental scope.

3 Shoot Forcing

'Shoot forcing' is essentially different from 'forcing epicormic buds' on a medium in flat trays as mentioned above. In the words of Read and Preece (2007), "This technology involves immersing the bases of cut woody stems into solutions containing 2% sucrose and 8-hydroxyquinoline citrate at the rate of 200 mg L^{-1}" for bud break in a number of woody plant species (Yang and Read 1992, 1993; Hamooh and Read 2000). Preece's group at Southern Illinois University at Carbondale, Illinois has conducted research on this aspect for many years. A useful information on several aspects of shoot forcing can be found in the earlier reports by Preece and Read (2003), Read and Preece (2007) and Preece and Read (2007). These review papers

and the literature cited therein strongly suggest the possibility of extending this research to woody plant species of tropical regions where work of this nature is almost non-existent till this point in time.

4 Chilling and Bud Break

Ashby et al. (1991) explained that "plants that set buds, become leafless, and do not respond readily to favorable growing conditions are considered dormant". Such plants were said to have a 'chilling requirement' usually of several 100 h before the bud break occurs (Swartz and Powell 1981; Ashby et al. 1991). Vast literature on various aspects of chilling and bud break in woody plant species is available (Weinberger 1950; Kriebel and Wang 1962; Spiers and Draper 1974; Hunter and Lechowicz 1992). The literature hence includes (but not limited to) studies on chilling exposures (Swartz and Powell 1981; Couvillon and Erez 1985), oxidative processes (Shulman et al. 1986), use of bioregulators for bud break (Shulman et al. 1986; Steffens and Stutte 1989) and the associated biochemical changes (Wang and Faust 1988; Wang et al. 1991). This alone necessitates a separate review in order to throw some light on this important physiological aspect of woody plants' propagation. In the present paper, this information only aims at indicating the potential use and importance of such studies to get maximum efficiency in propagation (or micropropagation) through the epicormic buds. The dormant stem sections of woody plants in particular limit the scope of a year-round forcing of epicormic buds and hence the main focus is on plant material that had undergone chilling under natural environment. This is for the same reason that stem sections that are collected during the late spring or early autumn in the temperate region have shown good potential for a greater number of epicormic bud forcing (Aftab et al. 2005). Dormant stem sections collected during other seasons may become an appropriate plant material only if their chilling requirements have been met. This aspect needs further investigation especially in woody plants of tropical regions of the world where a comprehensive information about the chilling requirements of a large number of woody plant species is still lacking.

5 Rooting and Acclimatization

Softwood shoots may be rooted directly under the greenhouse conditions in flats or beds of different sizes containing a suitable medium. This is accomplished usually by cutting softwood shoots of various sizes (usually 4–6 cm but may be longer) from the logs and treating the basal 2 cm cut ends with either indole-3-acetic acid (IAA) or 0.1% indole-3-butyric acid; IBA (Henry and Preece 1997). Softwood shoots after this treatment are transplanted to flat trays of appropriate sizes containing a variety of greenhouse media as just mentioned above. Peat moss is an important

medium at this stage. The purpose is to produce roots and acclimatize the plants in the greenhouse environment. Mist or fog conditions also play a vital role during the earlier establishment since the plants cannot afford water loss due to the absence of root system. Even after the establishment of a well developed root system, most plants need humid conditions for quite some time before being transferred to the field conditions. High humidity tents provide an opportunity to maintain the desired humidity level till that time or else may be maintained under the fog or the mist system. The hardening-off or acclimatization of plants derived either from the propagation bench or through micropropagation has been referred to as "the most tricky part of micropropagation" (Preece 2001). This is so because the plants are grown, regenerated and maintained under almost 100% relative humidity. This does not allow a propagator to transfer the plants directly to the field conditions. A stepwise hardening-off or acclimatization process thus needs to be followed and it has been reviewed by Preece (2001) in detail. A successful acclimatization will ensure survival of most plants in the normal greenhouse environment (without mist, fog or frequent watering) but 100% survival is seldom achieved.

6 *In Vitro* Establishment

An important application of softwood shoot forcing is subsequent establishment under *in vitro* conditions. This makes sense in that the plant material is generally cleaner as compared to the one derived from field-grown plants. Though the possibility exists for *in vitro* culture initiation in a large number of plant species, contamination is still a major issue at least for those explants that have been obtained from the shoots grown under mist, fog or high humidity tents. This is because the high humidity condition not only facilitates an optimum growth of plants but also favors the establishment of microbial contamination on the plants' surface. Such contamination is usually so severe that it is difficult to disinfest and culture-contamination during *in vitro* manipulations is usually high. In their experiments, Aftab et al. (2005) have recommended the use of 0.18% H_2O_2 (obtained in the form of a commercial preparation under a trade name of Zerotol; Biosafesystems, USA) on the stems of silver maple and green ash placed under mist or fog in greenhouse. Contamination rate was reduced to 68 and 92.2% in silver maple from the explants derived from stems placed under fog and mist conditions, respectively. The explants derived from shoots forced under the lab condition, however, were readily adapted *in vitro* with only 4% microbial contamination. Consequently, to get the true advantage of softwood shoots in raising *in vitro* cultures, more work is required to find out measures to minimize the microbial contamination of the plant material raised under mist or fog conditions. As a result, if the ultimate objective of forcing softwood shoots is to raise plant material for micropropagation or *in vitro* establishment for somatic embryogenesis, a tissue culturist is usually left with only one option, i.e., forcing softwood shoots under the lab conditions.

6.1 Micropropagation

For micropropagation, the best explant seems to be the nodal segments. Shoot apices may also be used but nodal explants offer potentially more chances of multiple shoot formation that is desired in micropropagation. Additionally, the softwood shoots (usually 2–4 cm) offer a unique opportunity of direct root formation under *in vitro* conditions and may then be hardened-off for further propagation.

6.1.1 Through Nodal Explants

Each nodal explant may provide one, two or more axillary buds that may be developed further under the influence of growth regulators. Multiple shoot formation is of great significance and is obtained using nodal explants subjected to a wide range of hormonal treatments (George and Sherrington 1984; Akram and Aftab 2008). It is important to give attention to details in order to establish nodal explants *in vitro* successfully. Disinfestation of explants must follow trimming at both the cut ends in order to avoid subsequent browning and necrosis of the entire culture. As stated above, the nodal explants, if obtained from the softwood shoots should preferably be forced under the lab conditions. Of the literature available on micropropagation involving a wide variety of plants, nodal explant seems to be the best choice (Quraishi and Mishra 1998; Fraga et al. 2004) amongst others such as hypocotyls (Daysuis and Chagvardieff 1990) and inflorescence (Eizenga and Dahleen 1990), but the choice of an explant may vary from species to species. It is also important that factors governing stock plant growth and physiology be known (Read 1990; Read and Preece 2007).

6.1.2 Through Shoot Apices

Although micropropagation has been reported using shoot apex (Nand et al. 2004; Akram and Aftab 2008), it poses certain disadvantages over the use of nodal explants. One of the most important being a relatively slower growth rate followed by a lesser number of micropropagated plants at the end of an experiment (Akram and Aftab 2008). However, variation in results in different plant species does not allow us to 'label' shoot apices as a second choice and may in fact be of great significance in certain plant groups (Birmeta and Welander 2004; Kadota and Niimi 2004). If enough literature is not available on a certain plant species of interest, its micropropagation should be attempted using all possible explant sources.

6.1.3 Direct Rooting

Direct rooting of softwood shoots under greenhouse conditions is discussed under Sect. 5. For micropropagation, direct rooting of softwood shoots has also been

attempted in teak (Akram and Aftab 2009) and Araucaria (Shafiq and Aftab 2008). There are no reports for such an attempt in other plant species. In these studies, softwood shoots (2–4 cm) were surface disinfested and transferred directly to half or full strength MS (Murashige and Skoog 1962), DKW (Driver and Kuniyuki 1984) or WPM (Woody Plant Medium; McCown and Llyod 1981) media containing several doses of IBA. The purpose of such attempts for *in vitro* rooting was simply academic and did not work well at least in teak and Araucaria, but the results with other plant species may be different and the author expects to see some progress in direct rooting of softwood shoots under *in vitro* conditions in the near future.

6.2 Problems Associated with Micropropagated Plants

Phenotypic inconsistency due to somaclonal variation has been attributed to be one of the main problems associated with micropropagated plants (Preece and Trigiano 2001). They have thus suggested the need to avoid adventitious shoots "especially from callus, during micropropagation of woody species". They have further described lack of chlorophyll in "portions of the leaves of some plants" as a result of this variation in some micropropagated plants. "Another fairly common variation in plants *in vitro* is the formation of fasciated stems (flat, wide stems that appear as if two or more stems are fused together lengthwise). Fasciation can have a genetic cause, as the result of a mutation, but it can also be a physiological response of shoots to conditions *in vitro*, such as the cytokinin used" (Preece and Trigiano 2001). Interplay of the growth regulators may be of use to overcome the above-mentioned problems.

It is a well-known fact that the micropropagated plants are usually quite fragile. Since these plants are raised *in vitro* with an ample supply of nutrients and growth regulators and the fact that relative humidity is almost 100%, growth rate is usually optimum. The plants are at the same time quite fragile and cannot cope with the external environment. A successful acclimatization or hardening-off phase is thus a prerequisite to obtain the benefits of micropropagation.

Micropropagation through multiple shoot formation is reported by many workers in a wide variety of plant species (Biroscikova et al. 2004; Ozden-Tokatli et al. 2005; Akram and Aftab 2008). Multiple shoot formation offers production of many shoots within the confines of a limited lab space and thus becomes quite economical. Micropropagation even without a greater number of multiple shoots still is economically favorable in those plants where it is the only means of propagation and conventional methods fail to yield the desirable results. Micropropagation through multiple shoot formation requires standardization of medium for each species and many reports in the available literature may be found focusing on standardization of culture conditions for this purpose.

6.3 Somatic Embryogenesis or Adventitious Shoot Formation – Must Have Option for Certain Recalcitrant Woody Plants

Focus on somatic embryogenesis and adventitious shoot formation in the literature seems to be for two main reasons. Firstly, in those cases where conventional propagation methods fail to yield desirable results and hence need supplemental *in vitro* approaches. Secondly, and perhaps more importantly, development of a reliable plant regeneration system either through somatic embryogenesis or organogenesis including adventitious shoot regeneration is extremely important in those studies where cells, protoplasts or tissues are used as target material to obtain transgenic plants (Aftab and Iqbal 1999). In view of the significance of the later point, a vast literature exists on callus induction, regeneration and somatic embryogenesis in woody plants of significance (Rakesh et al. 1995; Ipekci and Gozukirmizi 2004; Kvaalen et al. 2005; Aftab and Preece 2007). Adventitious shoot formation in particular for micropropagation is an approach that has got somewhat lesser interest of the workers until recently but the continuous advances in the understanding of the role of plant hormones and growth regulators *in vitro* have opened up further possibilities in the development of fast and reliable *in vitro* approaches for the propagation of various recalcitrant woody plant species (Wilhelm 1999; Akram and Aftab 2008).

6.4 Role of Growth Regulators

Generally speaking, exploitation of *in vitro* approaches for the potential development of woody plant species rests mainly on an appropriate use and interplay of various plant hormones and growth regulators; the main focus being on auxins and cytokinins (Vandenbussche and Straeten 2004; Gahan 2007). 'Plant hormone' is a strict term used for only naturally-synthesized signaling molecules that must be synthesized and translocated within the plant body for its physiological action at much lower concentrations of μM or even nM range. Since both naturally-synthesized as well as synthetically-produced signaling molecules are used in tissue culture studies, hence a broader term of 'plant growth regulators' is preferred in this text. The general philosophy for the use of plant growth regulators in woody plant species *in vitro* seems simple. Auxins, e.g., Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 2,4-D (2,4-dichlorophenoxyacetic acid) are generally known to stimulate somatic embryogenesis (Raghavan 2004), rhizogenesis in callus cultures (Wadegaonkar et al. 2006) and root formation of regenerated shoots (Akram and Aftab 2008) and cytokinins promote cell division (Sánchez-Moreiras et al. 2006) or shoot regeneration via organogenesis (Bhagwat and Lane 2004). In most studies, it

is the combination of the two or more growth regulators from each class that is reported to influence various growth behaviors *in vitro*. There is an optimum concentration for each growth regulator for a certain growth response or cellular behavior and this optimum may differ slightly or considerably when used in combination with another growth regulator. In general, higher cytokinin-to-auxin ratios have favored shoot initiation in callus cultures. On the other hand, higher auxin-to-cytokinin ratios usually promote rhizogenesis and equal or nearly equal 'moderate levels' of auxins and cytokinins support callus induction. It is essentially the choice of the worker to select a particular concentration or combination of growth regulators on the basis of experimental objectives.

In the language of Huetteman and Preece (1993) "Few innovations in woody plant micropropagation have been as dramatic as the introduction of thidiazuron as a plant growth regulator in culture media". For this reason, thidiazuron (N-phenyl-N′-1, 2, 3-thiadiazol-5-yl urea; TDZ) has been reported as an effective signaling molecule having cytokinin-like activity in most of the literature dealing with many *in vitro* aspects of woody plants during the 1990s and onwards (Huetteman and Preece 1993; Ledbetter and Preece 2004 and Akram and Aftab 2008). In the above-cited review paper of Huetteman and Preece (1993), TDZ's properties have been discussed with reference to chemical activity, axillary shoot formation, callus, adventitious regeneration and rooting besides certain special concerns such as elongation, tolerance, vitrification and shoot fasciation. In fact, in most of the recent literature on temperate woody plants, TDZ's usefulness has been proven in somatic embryogenesis (Bates et al. 1992), axillary shoot formation (Wilhelm 1999; Akram and Aftab 2008), adventitious shoot regeneration (Ledbetter and Preece 2004) and more reports about its 'strong effect' on various growth parameters of woody plants are continuously pouring in. From the personal experience of the author, the use of TDZ has proven to be of great significance in overcoming certain growth and developmental barriers in difficult-to-manipulate woody plants such as *Acer saccharinum* L. and *Fraxinus pennsylvanica* Marsh (Aftab et al. 2005), *Tectona grandis* (Akram and Aftab 2007, 2008), *Carya illinoensis* (Aftab and Preece 2007) and *Araucaria* (Shafiq and Aftab 2008; Batool and Aftab 2008) thus opening doors to the development of essentially rapid and reproducible micropropagation protocols for many related woody plant species. It is, however, surprising that most reports are on plants of temperate origin and its usefulness has not been encashed in the studies pertaining to micropropagation of woody plants of tropical origin (Aftab and Preece 2007; Akram and Aftab 2008). One obvious reason in the developing countries seems its price but effectiveness at very low concentrations may evade its price consideration.

Apart from the use of auxins and cytokinins, gibberellins have also been used in woody plant tissue culture albeit less frequently but its influence in regulating plants' height is well documented (Glocke et al. 2006). A new era on the studies pertaining to brassinosteroids has already started (Brosa 1999; Khripach et al. 2000; Ferrie et al. 2005; Haubrick and Assmann 2006) though its use in woody plants' tissue culture is yet to be determined. Similarly, jasmonic acid and salicylic acid and several other signaling molecules may also be strong candidates for the determination of their morphological effects on various growth and developmental aspects of woody plant species. Further work on these lines will hopefully throw light on these aspects.

7 In Search of Other Novel Candidate Explant Sources

If we see the history of plant tissue culture and the concept of totipotency, every living cell of a plant is considered to have an inherent capability to divide and regenerate into a plant. Based on this, explants have been collected from almost every tissue and organ of a plant. Leaves, nodes, internodes, shoot apices, roots and cambium have been the most common explant sources in wide groups of plant species. However, choice of explants is relatively less in case of recalcitrant woody plant species. The above-mentioned explants in case of adult woody trees are generally hard, lignified and mature enough and pose serious difficulties *in vitro* to revert back or dedifferentiate to meristematic nature. One possible solution is the one mentioned in Sect. 2 and involves the development of epicormic shoots that may be used as an explant source quite effectively. The other approaches have been to use softer tissues such as nucellus obtained from immature fruits in *Mangifera indica* (Rivera-Domínguez et al. 2004), immature seed tissues in *Juglans nigra* (Long et al. 1995) and immature zygotic embryos in *Carya illinoensis* (Mathews and Wetzstein 1993). This approach, however, considerably reduces the period of availability of suitable explants since immature fruits can be collected over a very brief developmental phase of a particular woody plant.

7.1 Immature Bark Explants and Pith Tissues

The author in view of the above limitations associated with woody plants suggests trying several uncommon explants as well. In *Carya illinoensis* (Wangenh.) C. Koch (Haroon and Aftab 2008) and *Araucaria heterophylla* (Batool and Aftab 2008), immature bark explants collected directly from adult trees during active growth phase have yielded quite satisfactory results for dedifferentiation leading to an ample callus formation and further morphogenesis. In addition, embryogenic callus from nucellar tissue of *Mangifera indica* spp. has also been obtained on several TDZ levels (Malabadi et al. 2011). Although the availability of epicormic shoots seems to mask the importance of such unusual explants, they may still have a potential role especially in the light of the established usefulness of growth regulators like TDZ in woody plants' micropropagation.

8 Conclusion

Combining relatively newer propagation techniques including shoot forcing and forcing epicormic buds with plant tissue culture holds a promise for the development of rapid and reproducible propagation and micropropagation protocols of many recalcitrant woody plant species. Studies on these lines may potentially ensure propagation of those woody tree species where non-viable seeds or associated

problems such as low germination rate and dormancy are frequently encountered or the aim of propagation is to retain genetic fidelity. Since most work cited in this paper is on woody plants of temperate regions of the world, its usefulness in woody plant species including ornamentals and forest trees of tropical origin is yet to be determined and the prospects are wide open. One needs a little caution though since the limitations are many (and many more may be reported in the future) once the work on similar lines is carried out in labs dealing with research on tropical woody plant species.

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Chapter 14
Novel Methods in Micropropagation of Pistachio

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Contents

Abstract The focus of this paper is to describe the novel methods developed for the different stages of micropropagation: installation of mature apical shoot tips and elimination of browning exudates; forcing hardwood shoots from the lignified stem sections; forcing axenic leaves; initiation of embryogenic masses (EMS); encapsulation of somatic embryos and cryopreservation of axillary buds for storage, and the facilitation of rooting. These developed methods are not being used by commercial micropropagation laboratories yet. Exudation of phenolics is inhibited

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when the disinfested and rinsed explants are cut at the base of the shoot tips and washed twice for 1 h by shaking them in sterile distilled water on a shaker at 100 rpm. For shoot initiation, 15–20 cm long terminal stem sections are cut into 3–4 cm sections and placed in pots filled with peat, perlite and soil. Two or three weeks later, the developed softwood shoots are excised; or freshly harvested three to nine apical tips of 1–2 cm are disinfested and used as explants. Leaves excised from axenic shoot cultures were also used to induce organogenesis on a Murashige and Skoog (MS) medium with Gamborg vitamins supplemented with combinations of different concentrations of 6-benzylaminopurine (BA) and indole-3-asetic acid (IAA). Calcium alginate gel was used to encapsulate somatic embryos to produce synthetic seeds. The encapsulated somatic embryos can be stored under refrigerated conditions for short-term conservation. For long-term conservation, some axillary buds can also be stored by using vitrification and one-step freezing technique; however, other cryogenic techniques should also be tested. With these improved stages, application of *Pistachio vera* L. micropropagation in commercial clonal orchards will be feasible as an alternative to traditional propagation in the near future.

Keywords *Pistacia vera* L. • Leaf forcing • Shoot forcing • Synthetic seeds • Somatic embryo • *In vitro* germplasm conservation

1 Introduction

The pistachio is usually propagated outside of Turkey by budding selected scions onto seedling rootstock of the same species or other pistachio species such as *P. atlantica, P. terebinthus*, *P. mutica*, *P. khinjuk*, *P. integerrima* or hybrids such as *P. atlantica*, *P. integerrima* (PGI), *P. atlantica* x *P. integerrima* (PGII and UCB-1) (Ferguson et al. 2002). Many of the institutions that currently produce plants for production are successful and have developed their protocols based on past research. Usage of pistachio as a rootstock results in a great variability in the performance of the grafted cultivars affecting diverse traits such as fungal resistance, shell splitting, blank nut production or yields (Hormaza and Wünsch 2007). Consequently, a greater uniformity is desirable and could be obtained by using vegetative propagated rootstock (Crane 1984). There are some techniques that are currently being developed that have the potential to impact commercial micropropagation in a positive manner. Breakthroughs are largely due to organogenesis and embryogenesis of the commercial cultivars, but may also be utilized in the micropropagation of stock cultivars.

The focus of this paper is concerned with the newer techniques of *in vitro* micropropagation of pistachio, such as the installation of mature apical shoot tips and the elimination of browning exudates; the forcing of the lignified stem sections and axenic leaves; the initiation of embryogenic masses; the encapsulation of somatic embryos; cryopreservation of axillary buds for storage; and the facilitation of rooting. None of these techniques is in widespread use in commercial micropropagation in an effective manner, but all appear to have a great potential and may become adopted by labs in the near future.

2 Revitalization of Pistachio

Clonal propagation is not always successful with older material due to poor rooting. Plants in the juvenile phase of growth generally are easier to propagate than plants that have reached the adult phase. The change from the juvenile to the adult phase is considered to be the most serious constraint to root cuttings (Howard 1990). As far as the rooting of adult *P. vera* material is concerned, rooting was attempted by several researchers (Joley 1960; Joley and Opitz 1971; Sakoury 1976; Bustamante-Garcia 1984) *in vitro* using a mist system. Unfortunately, no more than 5% rooting was achieved. Perhaps the major dilemma with rooting of cuttings of pistachios is the rapid loss in rooting capacity with the increasing age of the parent tree. Seedling explants are usually most readily established *in vitro* and proliferate more rapidly with tree species where micropropagation of adult material is often difficult. The majority of juvenile growth occur when the plant is young and still exhibiting juvenile characteristics. As plant ages, the older and lower parts retain their juvenile traits and an adult phenotype appears on the newest growth (Preece and Read 2003). In the case of pistachio *in vitro* culture, the origins of explants, in particular their positions on the mother plants, have been reported to influence their establishment and growth (Barghchi and Alderson 1985; Abousalim 1990; Onay 1996). We have been conducting research for the revitalisation of adult Turkish pistachio cultivars for years. At first, studies were carried out to investigate whether the degree of juvenility or maturity of an apical meristem depends on its distance (along the trunk or along the branches) from the root-shoot junction (Onay 1996). A diagram of the sampled gradients of the apical meristems is shown in Fig. 14.1, and the data reported are given in Table 14.1.

Depending on the time the explants were harvested, the position of the explants did not significantly influence the survival rate obtained by the explant positions A, B and C with 90%, 90%, and 100%, respectively (Table 14.1). However, the greatest length of shoots was obtained using the explants harvested from position C. There were significant differences between the positions tested. Explants from position C (terminal tips) produced most buds, and the mean was also significantly greater than the explants harvested from the other positions. This older, but more juvenile portion of the plant, is called “the cone of juvenility” (Preece and Read 2003). The challenge is to collect freshly sprouted shoot tips from the different positions of a mature tree, or if such juvenile shoot tips are not available, one may even go to the extreme measure of cutting down a tree to encourage juvenile shoots to grow from the stump, and then forcing them, especially during the dormant season, in a forcing solution indoors.

3 Shoot Forcing

When outdoor plants were used, it is difficult to remove microflora growing on them with using surface disinfestion techniques (Preece and Read 2003). The microbes are often in tiny cracks and crevasses and the disinfestant may not penetrate these areas. Many woody species are either too large or growing in the ground and cannot

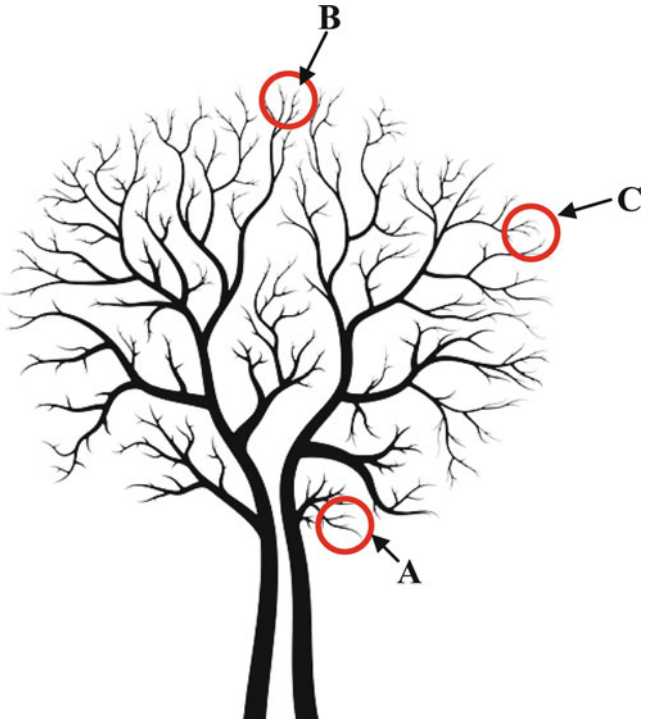

Fig. 14.1 Diagram of the sampled shoot tip gradients: (**a**) fresh sprouts from the trunk, (**b**) apical tips from the orthotropic far end of the root-shoot junctions, and (**c**) plagiotropic apical tips from far part of the root-shoot junction

Table 14.1 The effects of explant position harvested from different parts of a mature tree on viable culture and shoot growth

Position of the explant	Viable culture rate (%)	Mean shoot length (mm)	Mean shoot number
A	90	6.5 ± 0.61 a	4.2 ± 0.36 b
B	90	4.3 ± 0.50 b	3.8 ± 0.35 b
C	100	6.8 ± 0.82 a	5.3 ± 0.33 a
χ^2 (2 df)	P > 0.05		

be removed easily to greenhouse or growth room. Therefore, it is often necessary to remove parts of the plant such as branch tips or segments, usually during the dormant season, and force new shoot growth indoors or in a greenhouse environment. This new growth can then be excised, successfully surface disinfected and used for explant material. We have been conducting research on forcing shoots for years.

Much of the focus has been done on shoot tips harvested from mature trees during the dormant season. Terminal lignified stem sections, 3–4 cm long were collected from 30-year-old trees and then the cut ends were immersed in a 10 mg L^{-1} BA solution for 24 h before placing them in a greenhouse medium. Subsequently,

the cuttings were potted in a sterile 1:1 mixture of sand and soil and placed in a greenhouse (65 ± 5% R.H.) at 25°C/24 h. The photoperiod was supplied by mercury fluorescent lamps (25 mmol photons m^{-2} s^{-1}) in order to force the axillary buds to sprout. After 3 weeks, the newly formed leafy shoots from the forced buds were excised and surface sterilized by a 2-min dip in absolute ethanol followed by a 10-min soak in a 20% (v/v) NaOCl solution. Similarly, 20–25 cm long stems (Read and Yang 1991) were harvested from deciduous trees and then soaked for 15 min in a 0.78% NaOCl solution with Tween 20 (Yang and Read 1993). Following the bleach treatment, a fresh cut was made at the base. The lower cut ends of the stems were placed in containers with water, 200 mg L^{-1} 1,2,8-hydroxy-quinoline citrate (8-HOC), sucrose and, sometimes plant growth regulators. Read and Yang (1991) reported that faster bud break and greater bud and shoot elongation were promoted if the stems received the 15 min soak in the bleach solution prior to the forcing when compared to those that did not. Because these shoots were excised from the tips of branches of the most adult portion of a plant, they can be prone to be somewhat difficult to propagate. The effects of plant growth regulators in the forcing solution are, at least somewhat, species and plant growth regulator specific.

4 *In Vitro* Installation of Mature Pistachio Shoot Tips and Exudation of Phenolics

Juvenile explants are usually more readily established *in vitro*, grow and proliferate at a more rapid rate, and are more rootable than adult material (Preece and Read 2003). This is especially true with pistachio species where micropropagation of adult material is often difficult. Eradication of fungal and bacterial contaminants and browning were encountered as major problems in the establishment of pistachio tissue culture reported by many pistachio researchers (Bustamante-Garcia 1984; Barghchi and Alderson 1985; Abousalim 1990; Onay 1996; Mederos-Molina and Trujillo 1999; Ozden Tokatli et al. 2005). Data on the effectiveness of a sterilization method has not been published by these researches, although Abousalim (1990) and Onay (1996) gave optimum conditions for certain materials. Especially in the case of explants from mature pistachio trees, disinfestation was best achieved when the explants were obtained from actively growing meristem tip cultures of 50-year-old pistachio. (*P. vera* L.) (Onay 1996). However, when nodal bud segments and meristem tips were used for the initiation of a culture (Tilkat et al. 2008), a very high level of media browning was frequently observed. Browning, especially during explant establishment, appears to be the main limiting factor in the establishment of tissue cultures of pistachio species. There are a few interesting recent reports on the use of anti-oxidants, glutation, ascorbic acid, citric acid, PVP, L-cysHCL, Na-DEDC, and Rasmonal, but they did not make a visible difference on the absorption of phenolic compounds. To obtain satisfactory results, we have developed a new method that allows a faster and safer initiation and establishment of adult material. In our protocol, apical shoot tips were excised from 25-year-old mature trees. The branches were cut into 10–15 cm shoot tips. The softwood shoot

tips were excised into 1–2-cm long tips. The explants were surface disinfested with 10% sodium hypochlorite (NaOCl) for 30 min and rinsed three times with sterile distilled water, then the base of the shoot tips was cut. After this, these explants were washed twice for 1 h by shaking them in sterile distilled water on a shaker at 150 rpm. The exudation of phenolics was not observed when the explants were kept in twice-sterile distilled water for 1 h immediately after the excision of the basal ends, which were then blotted with sterile filter paper. During preparation, polyphenolic compounds are oxidized by polyphenol oxidates (PPO), inducing a strong browning on the cut surface and media and leading to the subsequent death of the explant (Tilkat 2006). Applying the new method described above, actively growing shoot tips of pistachio were cleaned and disinfested, dissected to 4–6 mm and placed on a modified MS medium of 1 mg L^{-1} BA. A yield of 100% sterile and non-browned explants was obtained in comparison to the formerly nearly 100% losses of non-washed shoot tips. The reason for obtaining a high survival rate from the treatment described above is that the cultured shoot tips could accumulate Na^+ ion from NaOCl which was used for surface sterilization. Therefore, phenolics were released from the basal-cut-ends of the apical shoot tips; they could react with Na^+ ion and form salt. This salt possibly dissolved in the water and the phenolic compounds lost their specific properties (Onay et al. 2007a; Tilkat et al. 2008).

5 Forcing Axenic Leaves

This technique is very different from forcing stem sections onto shoot tips, because already regenerated shoots can be used in a BA and IAA forcing solution. The details of the method are as follows: Healthy mature tips of 30-year-old male *P. vera* L. "Siirt" were collected from the pistachio research institute in Gaziantep province, a province of southeastern Turkey, and were used as the source of material (Tilkat and Onay 2009). To establish aseptic shoot cultures, apical shoot tips were disinfested by the protocol described above, and cultured on a modified Murashige and Skoog (MS) (Murashige and Skoog 1962) medium (initiation medium) with Gamborg vitamins (Gamborg et al. 1968) supplemented with 1.0 mg L^{-1} BA, 30 g L^{-1} sucrose and 5.5 g L^{-1} agar. Adventitious shoot buds from the initiation medium were subcultured on the fresh initiation medium every 3–4 weeks. The regenerated adventitious shoot buds from the *in vitro* cultures were maintained, and proliferated on the initiation medium for more than 1 year before the forcing studies. Semi-mature leaves along with a portion of the petiole were excised and cultured on an agarified MS medium with their petioles and abaxial sides in contact with the medium. The MS basal medium was supplemented with 3% (w/v) sucrose, 6-benzylaminopurine (BA) thidiazuron (TDZ) and kinetin (Kin) (each at 1 mg L^{-1}) and different concentrations of BA or TDZ (0,0, 1,0, 2,0, 3,0 and 4,0 mg L^{-1}) and IAA (0,5, 1,0 and 2,0 mg L^{-1}) were tested on supplements on the MS basal medium for shoot regeneration experiments. The formation of direct shoot buds from different woody species requires more exacting culture conditions of light and temperature, and complex medium

composition, especially with regard to plant growth regulators (Tomar and Gupta 1988; Gharyal and Maheshwari 1990). Gharyal and Maheshwari (1990) reported 36% direct shoot regeneration from petioles on a B5 medium supplemented with 0.5 mg L^{-1} IAA and 1 mg L^{-1} BA. However, 31.9% of direct shoot regeneration was obtained in female cultivar ("Siirt") from culturing leaflet explants on a MS medium supplemented with 1 mg L^{-1} IAA and 2 mg L^{-1} BA (Tilkat and Onay 2009). Shoots subcultured on the 1:2 IAA:BA media also proliferated rapidly, but the medium regenerated hyperhydric shoots. Once shoot buds were obtained from axenic leaves, the use of only BA on the proliferation medium proved to be more beneficial than the combination of IAA and BA. The results reported by Tilkat and Onay (2009) and Tilkat and co-workers (2009b) showed consistency with other studies where BA and IAA promoted the proliferation and elongation of shoots in buds from *Ladebouria hyacinthiana* (Turakhia and Kulkarni 1988) leaves. The protocol for forcing the regenerated leaves for organogenesis summarized above on the female cultivar "Siirt" showed consistency with the pistachio male cultivar "Atli" (Tilkat and Onay 2009; Tilkat et al. 2009b). These results show that a combination of BA and IAA was crucial for direct shoot regeneration.

6 Initiation of Embryogenic Mass

Somatic embryogenesis in *P. vera* L. has been reported in a number of studies (Onay et al. 1995, 1996, 1997; Onay and Jeffree 2000; Onay 2000a, b; Onay et al. 2005). In these reports initiation of embryogenic cultures from immature kernels ranged from 0.0% to 10%. More recently, Onay et al. (2007b) obtained somatic embryogenic lines with five open pollinated cultivars at frequencies of between 7.0% and 19%. Previous studies have suggested that somatic embryo formation was also influenced by the type of media and explant and by the type of cytokinin used in the culture medium (Onay et al. 1995; Onay and Jeffree 2000). In pistachio we have standardized the efficient protocols for direct somatic embryogenesis from immature fruits (Onay et al. 1995, 1996; Onay and Jeffree 2000), mature zygotic embryos (Onay 2000a; Onay et al. 2007b), and juvenile leaf explants (Onay and Namli 1998). The protocol involves multiple stages, including embryogenic mass formation, maturation, germination, and plantlet development.

Another factor pertinent to somatic embryogenesis (SE) in pistachio is the composition of the initiation medium. Various media have been used to culture immature fruits of pistachio, the most common of which is MS (Murashige and Skoog 1962). The only plant growth regulator for inducing direct embryogenic tissue from mother tissue has been BA. We have also demonstrated that TDZ was also another effective agent for the induction of callus from leaf explants of pistachio, but BA was again essential for the expression of somatic embryogenesis (Onay and Namli 1998).

The color of EMS varied depending on the genotype from yellowish green to deep green with some red pigmentation. Proliferative ability was influenced by all genotypes. Once an EMS was initiated in a liquid medium containing 3% sucrose,

with or without (after a few subcultures) 0.5 mg L^{-1} BA, it could be maintained successfully in liquid or the agarified medium (if the agarified medium was used for maintenance with or without BA, the proliferation of EMS takes a longer time). Therefore, subculture on the initiation medium with or without BA is a prerequisite for the maintenance of embryogenic tissues.

7 Cryopreservation and Encapsulation of *In Vitro* Grown Pistachio Regenerants

7.1 In Vitro Conservation and Cryopreservation of Pistachio Germplasm

As the diversity of wild pistachio types is destroyed by severe anthropogenic pressures, such as land clearance, charcoal burning, and over-grazing (Barghchi and Alderson 1989; Padulosi and Hadj-Hassan 2001), both wild and cultivated pistachio germplasm are today under threat of genetic erosion and a comprehensive gene bank for the pistachio has not been established yet. Thus, it is important to develop suitable conservation strategies for the medium and long-term preservation of pistachio germplasm in which slow-growth storage strategies can be carried out at low temperatures (above-freezing) in the former, whereas the material can be cryopreserved in liquid nitrogen (LN) at ultra-low temperatures (−196°C) in the latter. However, interestingly, up to now only a few reports can be found in the literature about both slow-growth storage and the cryopreservation of pistachio germplasm (Ozden Tokatli et al. 2008, 2010; Akdemir et al. 2012).

7.2 Slow-Growth Storage

Slow-growth storage of plant materials aims to reduce growth with increasing intervals between subcultures and, therefore, it is used as a strategy for germplasm conservation and exchange. In slow growing species standard culture conditions can also be used for medium-term conservation; however, in most cases, modified environmental conditions and/or a culture medium are preferred to be used in association with a decrease in light intensity or even its complete suppression for the induction of growth reduction (Engelmann 1997).

The potential of slow-growth storage of *P. vera* microshoots has also been investigated. An attempt to reduce growth by incorporating different concentrations of growth reducing substance like abscisic acid (ABA, 0.25–4 mg L^{-1}) or an osmotic inhibitor of growth like mannitol (2.5–40 mg L^{-1}) to the media in combination with a decreasing of the incubation temperature from 26ºC to 4°C and/or by using different photoperiods and light intensities was tried (Barghchi 1986). Using this approach some cultures have been stored for nearly 18 months, and shoots taken from these

stored cultures were able to resume growth after their transfer to normal proliferation conditions.

Apart from the usage of microshoots for the conservation of germplasm, propagules can also be preserved in synthetic seeds (synseeds) which can mimic the formation and germination of natural seeds. The concept of encapsulation was first applied to somatic embryos (Murashige 1977), and then, extended to other vegetative propagation units (Bapat et al. 1987) by using buds and nodal segments as encapsulated explants. In time, several methods of encapsulation have been tested for the production of synseeds, including gelation, complex coacervation, and interfacial polymerization (Redenbaugh et al. 1987). Since the gelation technique proved to be the most efficient, different gels were compared to form sufficiently hard capsules and to guarantee the maintenance of somatic embryo viability (Redenbaugh et al. 1991). Among them, Na-alginate is today by far the most commonly used compound, due to its ability to form hydrogels in the presence of divalent cations (Ca^{+2}), its nontoxicity and its low cost. These features have led to the technology being applied to a wider range of species and explants (Piccioni and Standardi 1995; Lambardi et al. 2006).

When both axillary/apical buds or somatic embryos are used for encapsulation, important advantages of this technology rely on its potential to improve not only the mass propagation of plants, but that they are also suitable for short-term conservation studies at cold (above-freezing) conditions with the ability of germplasm storage, and reducing the need for transferring and subculturing during off-season periods (West and Preece 2006), a condition which diminishes the labor costs of commercial micropropagation laboratories. Last but not least, the synseeds can also be used for the long-term preservation of germplasm with recently developed "encapsulation-dehydration" and "encapsulation-vitrification" techniques (Panis and Lambardi 2006).

In the case of pistachio, synseeds were prepared by using both axillary buds (Ozden Tokatli et al. 2008) and somatic embryos (Onay et al. 1996) with using Na-alginate as the gelling and $CaCl_2.2H_2O$ as the complexing agent. In the case of encapsulated axillary buds, more than 75% of the synseeds were able to convert to shoots after 4 weeks of culture in 2 mg L^{-1} BA containing MS media, whereas 14% of the somatic embryos showed germination in 4% (w/v) sucrose and 1 mg L^{-1} BA enriched MS media. Moreover, with encapsulation of somatic embryos, it was possible to conserve the propagules up to 2 months at 4°C with a 14% recovery rate which may confirm that encapsulation is a practical procedure for short-term storage of embryogenic pistachio tissue and may be applicable to the preservation of desirable elite genotypes (Onay et al. 1996).

7.3 Cryopreservation

Cryopreservation refers to the storage of biological specimens at ultra-low temperatures (196°C) in liquid nitrogen (LN) as a cryogenic medium (Withers and Engelmann 1997).

As at this ultra-low temperature, all cellular divisions and metabolic processes are stopped, it is the only sound technique that allows the conservation of specimens for a theoretically unlimited period of time (Engelmann 2004). For pistachio cryopreservation, protocols were developed initially for seeds by using dehydration and one-step freezing techniques (Ozden Tokatli et al. 2007). Using this technique, a maximum of 90% germination was obtained following 8 h of drying in silica gel (corresponding to 11.7% moisture content on a FW basis) and direct immersion in LN. Then, more recently, a different cryogenic approach, vitrification and one-step freezing, was applied for the cryopreservation of pistachio axillary buds (Ozden Tokatli et al. 2008) in which excision of buds from *in vitro* grown and cold-acclimated shoots were followed by their pre-treatment with a sucrose enriched medium for 2 days at 4°C. This freezing procedure, referred to as vitrification, comprises a loading treatment with a cryoprotectant solution (2 M glycerol and 0.4 M sucrose, Matsumoto et al. 1994) at room temperature (RT), followed by the exposure of explants to a plant vitrification solution (PVS2; 30% glycerol (w/v) + 15% ethylene glycol (w/v) + 15% DMSO (w/v) in a culture medium with 0.4 M sucrose, Sakai et al. 1990) at 25°C or 0°C for various incubation periods (15, 30, 60, 90 min), rapid cooling and warming, and final removal of the vitrification solution by washing samples with an unloading solution consisting of a liquid culture medium supplemented with 1.2 M sucrose. After 4 weeks of culture in 2 mg L^{-1} BA containing MS medium, both vitrified (control) and vitrified/ cryopreserved (LN+) axillary buds were analyzed. The results showed that prolonged exposure of buds to the PVS2 solution up to 90 min resulted in reduction in the survival and the proliferation percentages of the control samples in both applied temperatures. The initial survival percentage of axillary buds (100%) were decreased to 75.8% with exposure in 0°C and to 56.2% in 25°C; whereas, the proliferation rates were also decreased from 90% (initial) to 72.7% and 66.7%, respectively. These results clearly indicate that prolonged exposure of samples to a vitrification solution leads to detrimental effects due to its toxicity. However, it was also possible to recover some axillary buds after cryopreservation when PVS2 was applied for 60 min in 0°C and 90 min both in 0°C and 25°C prior to their direct immersion in LN. It should be noted that other cryogenic procedures, including encapsulation-dehydration, encapsulation-vitrification and droplet-vitrification techniques, should be carried out on pistachio in order to develop more efficient long-term conservation techniques.

7.4 Facilitation of Rooting

The *in vitro* initiation of roots for the regenerated pistachio shoots was found to be especially dependent upon the auxin type and the concentration (Barghchi and Alderson 1989; Parfitt and Almehdi 1994; Onay 1996; Onay 2000b; Ozden Tokatli et al. 2005; Tilkat 2006). Successful reports on the tissue culture of adult *P. vera* L. produced somatic plantlets, but obstacles pertaining to the low incidence of the rooting

of plantlets reproducibility during the rooting regime and the poor establishment of *in vitro* propagated plants after acclimatization as a result of fungal infections have been reported (Onay 2000b; Tilkat et al. 2008). Abnormality *in vitro* morphologies related to the callus production of cultured plant tissues is also a hindrance to commercial *in vitro* plant propagation as losses of up to 50% can be observed (Tilkat 2006). Although a high rate of rooting (84%) of *P. vera* cv. Atli was obtained on a medium supplemented with 2.0 mg L^{-1} IBA (Tilkat et al. 2008), the plantlets were at times prone to severe callusing and hyperhydricity.

Very recently, a new protocol was described by Tilkat and Onay (2009a). The washing of the cut ends of the microshoots before the rooting treatment had an effect on the rooting. There was evidence of a significant difference in the frequencies of rooted shoots between the results obtained from shoots excised from the washed and unwashed, which is not a fact that has been reported by other researchers concerned with the pistachio. This method provides a clean environment for microcutting by solving the released phenolics and other metabolites which hinder rhizogenesis. Improvements obtained in rooting percentages (from 67.5% to 87.5%) were produced when cloned shoots were used (Tilkat and Onay 2009a); this finding has been previously reported for seedling material of the pistachio (Onay 1996). This indicates that not only growth substances, but also some other factors may limit root induction, and this merits attention before any practicable application. The rooting responses are significantly influenced by the applied dipping IBA concentration and its duration, a maximum of 90.2% shoot-forming roots was obtained with 2 mg L^{-1} IBA after a 5-mi dipping in 10 mg L^{-1} IBA (Tilkat and Onay 2009a). Even though high levels of rooting were obtained, a dipping protocol was not suitable for future routine use as roots associated with callus are often vulnerable to pathogen attack once plantlets are transferred to soil. A quick dip approach for the rooting of pistachio under *in vitro* conditions has also been reported by another pistachio researcher (Abousalim 1990). The rooting of the regenerated 4-year-old *P. vera* L. material was tested using the quick auxin-dip approach. Up to 50% of the plantlets were rooted with a mean of three roots. A quick dip approach for in vivo rooting was also tested. After 5 weeks of culture, an 85% rooting was obtained on mature softwood cuttings of *P. vera* under mist, but only after treatment with an extremely high level of IBA (35,000 mg L^{-1}) (Al Barazi and Schwabe 1982).

The use of longer shoots (≥4 cm long) for rooting also showed a strong effect on root regeneration. The enhancement of root formation in mature male pistachio on 2 mg L^{-1} IBA support is new and of interest. Similar to these results, 80% of the shoots forming roots have also been reported by Tilkat and Onay (2009) using the regenerated shoots from mature female trees. Although a high percentage (90%) of shoots forming roots has been reported by Parfitt and Almehdi (1994) using juvenile material (*P. vera* "Kerman" seedlings), our percentage of shoots forming roots was the highest (92%) of these obtained by others working with *P. vera* (Barghchi and Alderson 1989; Parfitt and Almehdi 1994; Onay 2000b; Ozden Tokatli et al. 2005; Tilkat 2006). Once healthy and strongly rooted plantlets were obtained, acclimatization of the pistachio was easy (Onay et al. 2007a; Tilkat and Onay 2009).

8 Conclusions

In this chapter, the novel methods developed for the different stages of micropropagation of *Pistacia vera* L. have been reviewed, with a special attention paid to recent reports. Pistachio is one of the most important nut crops and its world production has increased significantly over the past 30 years mainly due to the growing demand in the world markets and the future appears to be more optimistic for the pistachio nut. However, traditional methods used for pistachio propagation and improvement are apparently not adequate to meet these demands. Application of biotechnological methods, such as *in vitro* micropropagation and conservation, to pistachio can create new opportunities in breeding, cloning, disease control and germplasm conservation. As regards micropropagation, mature pistachio cultivars have been extremely difficult to establish due to the high incidence of contamination and the oxidation of phenolic compounds. However, the successful protocols concerned with the different stages of micropropagation were described: installation of mature apical shoot tips and elimination of browning exudates; forcing hardwood shoots from the lignified stem sections; forcing axenic leaves; initiation of embryogenic masses (EMS) and the facilitation of rooting. The studies carried on both mature pistachio female "Siirt" and male "Atli" cultivars showed that a combination of BA and IAA (2 mg L^{-1} and 1 mg L^{-1}, respectively) should be incorporated to MS medium in order to force direct organogenesis from leaves, whereas BA and/or TDZ should be added to the media for induction of somatic embryogenesis from immature fruits and juvenile leaf explants. At present, somatic embryogenesis is restricted only to juvenile tissues as explants from mature trees, when cultured, have not demonstrated any embryogenic potential. To extend the expression of embryogenic potential to the explants from mature trees, it is necessary to identify cell or tissue types which are physiologically identical to these juvenile tissues. In the case of conservation, both slow-growth storage and cryopreservation methods were established for short and long-term preservation, respectively. It was shown that pistachio microshoots and encapsulated somatic embryos could be stored for a period in above freezing temperatures in former, while it was possible to cryopreserve both pistachio seeds and axillary buds with using appropriate cryogenic methods in ultra low temperatures, in latter. Cryobanks for seeds and synthetic seeds for somatic embryos of pistachio are a reality and investigations based upon cryopreservation of plumules will have a great impact on storage and management of genetic resources. Continued researches into the use of biotechnological methods will contribute to improve the vegetative propagation of the pistachio and ultimately should play an important role in future breeding programs for pistachio and other important woody species.

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Chapter 15
Crop Productivity and Water Use Efficiency: The Role of Carbon Isotope Discrimination Technique

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Abstract At a time when global population continues to increase, global agriculture now accounts for 70% of the amount of water used on earth and crop losses due to water loss are much higher than crop losses due to any other causes. Reliable selection of crop varieties or genotypes with increased grain yield is essential both under irrigated and rain-fed conditions, due to water shortage commonly experienced in many environments. This can be obtained by increasing crop water use efficiency (WUE) either by breeding, or by soil, water and crop management. WUE can be enhanced by reducing water losses (runoff, drainage, evaporation), thus increasing the proportion of the water used by the crop. There is, indeed, a clear relationship between the amount of water transpired and yield across a diverse range of crop species. The carbon isotope discrimination technique has been proposed by several authors as a mean of selecting cultivars with improved WUE. Carbon isotope discrimination (Δ) of plant tissues (leaf or grain) has been shown to correlate negatively with transpiration efficiency and positively with grain yield in a wide range of crops. In the present chapter, recent progress in improving crops for yield and water-use efficiency using the carbon isotope discrimination technique are reviewed and some possible avenues for making further advances are discussed. The review describes the basis of carbon discrimination in C_3 and C_4 crops with a special emphasis on cereals, dominant food crops grown in irrigated and rain-fed areas.

Keywords Carbon isotope discrimination • Isotopic fractionation • Water scarcity • Water-use efficiency • Transpiration efficiency • Drought tolerance • Crop productivity

1 Introduction

Humanity is dependent on freshwater for life, and water is increasingly becoming a strategic commodity. In several countries, including Pakistan, water resources current availability and future water supplies are at risk as a result of population increase and urbanization. Increasing water scarcity and high population growth have already increased the food imports in arid and semi-arid countries (UK meteorological Report 2006), the local agricultural sector becoming less and less able to produce staple crops in sufficient quantity. As the world population continues to increase, more people will require more water for the cultivation of food, fibers and industrial crops. The Comprehensive Assessment of Water Management in Agriculture (2007) reveals that around 1.2 billion people, or almost

one-fifth of the world's population, live in areas of physical scarcity, while 500 million people are approaching this situation.

In many regions, rainfall, a major source of freshwater, is expected to be also severely affected by changes in global climate, caused by higher concentration of greenhouse gases. The International Panel for Climate Change (IPCC 2007), in its third Assessment Report on climate change, emphasized that global warming is happening at a much faster rate than expected. Global temperatures are rising nearly twice as fast as previously thought. The same report predicts that temperatures could rise by as much as 5.8°C by the end of the century and that rainfall will increase in regions that already have sufficient rains, while dry regions will become even drier. Climate change is expected to account for about 20% of the global increase in water scarcity. Countries that already suffer from water shortages will be hit hardest. Significantly, there will be major increases in water scarcity even if the water impacts of climate change prove to be neutral or enhance of the world's hydrological budget. With neither being reasonably expected to happen, the impact of a changing climate will affect not only bulk water availability but will also worsen the extremes of drought and floods. A 2006 study by the UK Meteorological Office concluded that, with no mitigation of climate change, the severe droughts that now occur only once every 50 years will occur every other year by 2100. Globally, it is estimated that food and feed crop demand will nearly double in the coming 50 years (UN Water 2007).

2 Agriculture and Water Scarcity

Today, agriculture accounts for 70% of all water use globally, up to 95% in several developing countries. Increasing the efficiency of water use and enhancing agricultural water productivity at all levels of the production chains are becoming priorities in a growing number of countries. Water use efficiency in this sector is very poor, not exceeding 45%. Thereby, enormous quantities of water could be saved in the agricultural sector, and increasing water-use efficiency of both irrigated and rain-fed crops is an urgent imperative (Hamdy et al. 2003). Several strategies are required to improve water use in irrigated and rain-fed agriculture (Wang et al. 2002). Breeding crop varieties that are more efficient in water use is one strategy (Condon et al. 2004; Condon and Hall 1997; Rebetzke et al. 2006). Others include better management of the water resource and changes in crop management (Gregory 2004). None of these strategies should be seen as operating in isolation. Rather, it is likely that the greatest gains will be obtained through complementary approaches involving each of them. A systematic approach to agricultural water productivity requires actions at all levels, from crops to irrigation schemes, in order to ameliorate inter-sectoral competition for water resources and optimize social and economic outcomes (Bacon 2004). To keep pace with the growing demand for food and taking into consideration an increase in water productivity, it is estimated that 14% more freshwater will need to be withdrawn for agricultural purposes by 2030 so as to obtain the 55% increase in food production needed.

3 Water Use Efficiency in Agriculture

Water use efficiency is a measure of the amount of water used by plants per unit of plant material produced. The term can be applied at the leaf, whole-plant, and ecosystem levels (Jones 2004). At the leaf level, it is more precisely referred to as "instantaneous water use efficiency" or "transpiration efficiency", TE (Farquhar et al. 1982) and represents the moles of CO_2 taken up by photosynthesis (A) divided by the moles of water lost through transpiration in a unit of time and per unit leaf area (T). At the whole-plant level, water use efficiency referred to as the units of dry matter synthesized divided by the units of water lost (Gregory et al. 1997). At the ecosystem level, WUE (often referred to as "agronomic WUE") can be calculated as the grams of dry weight gained by plants during the growing season per unit land area divided by the millimeters of water lost (including evaporation directly from the soil). Agronomic water use efficiency, or yield of harvested product achieved from the water used by the crop through all means (i.e., precipitation and/or irrigation) may be important for farmers and agronomists. The prospect of improving agronomic water-use efficiency by manipulating leaf-level water-use efficiency (i.e., TE) has long been an attractive one (Condon et al. 2004).

According to Gregory et al. (1997), the ratio of total biomass produced against total available water may be expressed as:

$$\mathrm{WUEB} = \mathrm{M}/\left(\mathrm{Es}+\mathrm{T}+\mathrm{R}+\mathrm{D}\right) \tag{15.1}$$

where M, Es, T, R and D are the biomass produced, the evaporation from the soil surface, the transpiration during the growing season, the runoff, and the drainage below the root zone, respectively.

Equation 15.1 can be rearranged to give:

$$\mathrm{WUEB} = \left(\mathrm{M}/\mathrm{T}\right)/\left[1+\left[\left(\mathrm{Es}+\mathrm{R}+\mathrm{D}\right)/\mathrm{T}\right]\right] \tag{15.2}$$

Agronomic water use efficiency on a grain basis, the ratio of production of total biomass against total available water can be expressed as:

$$\mathrm{WUEG} = \mathrm{HI}*\left(\mathrm{M}/\mathrm{T}\right)/\left[1+\left[\left(\mathrm{Es}+\mathrm{R}+\mathrm{D}\right)/\mathrm{T}\right)\right] \tag{15.3}$$

where HI is the harvest index, i.e., the ratio of grain yield to above-ground biomass.

The potential role of carbon isotope discrimination (Δ) for improving water use efficiency.

Equation 15.3 makes clear that improved WUEG can be obtained by either an increase of M/T, a reduction of Es/T, or an increase of HI. It has been postulated by several authors (Jones 1997; Impa et al. 2005) that Δ, the carbon isotope discrimination by the crop, can be used to estimate or predict WUEG. This assumption is mainly based on the significant association repeatedly observed between transpiration efficiency and Δ (Farquhar et al. 1982; Condon and Richards 1992) and on the fact that Δ was found largely determined by stomata opening and transpiration (Morgan et al. 1993).

3.1 Biochemical Basis and Variability of Δ in Plants

Isotopes are atoms of the same element that differ in mass. Carbon is made up of isotopes with masses 12, 13 and 14 (written ^{12}C, ^{13}C, ^{14}C, respectively). ^{14}C is radioactive and decay to produce isotopes of a different element over time. ^{12}C and ^{13}C are stable isotopes and do not experience radioactive decay.

During physical, chemical and biological processes, the heavy and light isotopes partition differently. This phenomenon is called isotope fractionation. In almost all reactions the light isotopes react faster than the heavy ones. Heavier isotopes have lower mobility, lower diffusion velocity and collision frequency with other molecules, and generally higher binding energies, compared to lighter isotopes. The differences in binding energy and reaction rates are proportional to the mass difference between isotopes. Thus, light elements are more likely to exhibit isotopic fractionation than heavy ones. For example, the isotopes ^{12}C and ^{13}C of the relatively light C atom have an 8% mass difference and undergo stable isotope fractionation. In contrast, the isotopes ^{87}Sr and ^{86}Sr of the heavy Sr atom have a 1.1% mass difference and do not exhibit detectable mass fractionation. Almost 98.9% of atmospheric CO_2 contains the ^{12}C and only a small part (1.1%) contains ^{13}C. Carbon, the major building block of carbohydrate and proteins in plant tissues contains both light (C^{12}) and heavy (C^{13}) stable isotopes.

Organic matter in plants is depleted in ^{13}C and consequently have lower $^{13}C/^{12}C$ ratio compared to atmospheric CO_2 (Craig 1954; Bender 1968), because plants fractionate carbon isotopes during photosynthesis. The magnitude of fractionation depends upon photosynthesis type, environment, genotypes and many other factors. The biochemical and physical phenomena underlying isotope fractionation are well recognized now and have been used to address a large range of problems in plant physiology and ecology (Rundel et al. 1979; Troughton 1979; Vogel 1980; O'Leary 1988; Farquhar et al. 1989).

3.2 Measurement of Carbon Isotope Discrimination

Measurements of ^{13}C contents of CO_2 are carried out with an isotope ratio mass spectrometer specially designed for high precision measurement of ratio R defined as:

$$R = {}^{13}CO_2 / {}^{12}CO_2 \tag{15.4}$$

R values are converted to carbon isotope composition (δ) as:

$$\delta^{13}C\ (‰) = \left[\left(\text{Rsample} / \text{Rstandard} - 1\right) \times 1000\right] \tag{15.5}$$

The standard is the CO_2 obtained from a limestone from Pee Dee Belmenite "PDB" formation in South Carolina, USA with absolute $^{13}C/^{12}C$ (Rstandard) of 0.0112372. The units are 'per mil' or ‰ and $\delta^{13}C$ has negative values. In the

Table 15.1 Carbon isotope fractionations associated with photosynthesis

Process	Fractionation (‰)[a]	Reference
Solubility of CO_2 in water	1.1	O'Leary (1984)
Hydration of CO_2	−9.0	Mook et al. (1974)
CO_2 diffusion in air	4.4	Hersterberg and Siegenthaler (1991)
CO_2 diffusion in aqueous solution	0.7	O'Leary (1984)
Spontaneous hydration of CO_2	6.9	Marlier and O'Leary (1984)
Carbonic anhydrase – catalyzed hydration of CO_2	1.1	Paneth and O'Leary (1985)
Carboxylation PEP	2.0	O'Leary (1984)
Carboxylation of RuBP	29.0	Roeske and O'Leary (1984)

[a]Positive values in this table indicate that the product is depleted in ^{13}C compared to the starting state; negative values indicate enrichment

absence of industrial activity the CO_2 in air has $\delta^{13}C$ value of −8‰ and become more negative after each year as result of fossil fuel combustion and deforestation (Keeling et al. 1979). The values are converted to carbon isotope discrimination (Δ) as the isotopic difference between source and product using formula (Farquhar et al. 1989):

$$\Delta(\text{‰}) = \left[\left(\delta_a - \delta_p\right)/\left(1+\delta p\right)\right] \times 1000 \tag{15.6}$$

where δp is $\delta^{13}C$ of samples and δa, −8.00‰ the $\delta^{13}C$ of atmospheric CO_2 (Keeling et al. 1979).

3.3 Components of Isotope Fractionation

Isotope fractionations values of different physical and chemical processes involved in photosynthesis are listed in Table 15.1.

3.3.1 Thermodynamic and Kinetic Fractionations

Two main types of isotope fractionations have been recognized, thermodynamic and kinetic. Thermodynamic fractionation reflects differences in equilibrium constants and will cause one isotope to concentrate more in one component of a reversible system that is in equilibrium. If the heavier isotope concentrates in a component, then that component is commonly referred to as enriched or heavy. If it is the light isotope that concentrates, then the component is referred to as depleted or light. In most circumstances the heavy isotope concentrates in the component in which the element is bound more strongly and thus equilibrium isotope effects usually reflect relative differences in the bond strengths of the isotopes in the various components of the system.

Kinetic fractionation reflects differences in rate constants for isotopic species and occurs when one isotope reacts more rapidly than the other in an irreversible system or a system in which the products are swept away from the reactants before they have an opportunity to come to equilibrium. Normally, the lighter isotope will react more rapidly than the heavy isotope and thus the product will be lighter than the reactant. The isotope fractionation will only occur in systems in which there is both an isotope effect and a reaction that does not proceed to completion. Thus, even in the presence of an isotope effect, there will be no isotope fractionation if all the reactant goes to a single product because all the atoms have reacted. The ratio of the heavy to the light isotope must be the same in the product as it was in the reactant. The magnitude of an isotope effect is expressed as a fractionation factor, represented as f and defined as the ratio of the heavy to light isotope in the product divided by the ratio of the heavy to light isotope in the reactant (f=[(heavy/light) product]/[(heavy/light) reactant]). When f is greater than 1, the product is heavy or enriched. When it is less than 1, the product is light or depleted. Most fractionation factors lie between 0.9 and 1.1. A fractionation factor of 1.050 is often referred to as a 5% isotope effect (for further basic information on isotope fractionation, see Galimov 1985 and Hayes et al. 1982).

3.3.2 Isotopic Fractionation in Physical Processes

During photosynthesis diffusion processes are involved at different stages and components of isotopic fractionations. The heavier isotopes diffuse slowly than lighter ones. The diffusion of $^{12}CO_2$ and $^{13}CO_2$ in air shows a fractionation of 4.4‰ (O'Leary 1981; Hersterberg and Siegenthaler 1991) while diffusion of dissolved CO_2 in water is slow and shows fractionation of 0.7‰.

3.3.3 Isotopic Fractionation in Enzymatic Processes

Isotopic fractionation associated with enzyme-catalyzed reactions has been measured for a wide variety of enzymatic systems. The enzyme Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) which catalyzes the first step in CO_2 fixation in C_3 plants is a major source of isotopic fractionation in C_3 plants. A consistent value of 29‰, compared with dissolved CO_2 at 25°C, was found by several investigators using a variety of methods (Cleland 1987; O'Leary 1988; Cook 1991). The initial carboxylation in C_4 plants is carried out by PEP carboxylase, and the measured isotopic fractionation is 2‰ (Whelan et al. 1973; O'Leary 1981). However, this is the fractionation starting from HCO_3^-, whereas in plants the reaction begins with CO_2. When referred to gaseous CO_2 the isotope fractionation becomes 5.7‰.

3.3.4 Environmental Effects on Fractionation

The values of fractionation can slightly change with species, CO_2 concentration, or other natural variables. Isotopic fractionation in physical and diffusive processes

should not be affected by the range of environmental conditions or CO_2 concentration encountered during normal plant growth. The isotope fractionation due to RuBisCO is independent of CO_2 concentration and almost independent of pH (Roeske and O'Leary 1984). The fractionation associated with the CO_2 hydration equilibrium has a small temperature effect (Mook et al. 1974). The species effects on the fractionation due to RuBisCO is still controversial. The enzymes from *Rhodospirillum rubrum* (Roeske and O'Leary 1985) and *Anacystis nidulans* (Guy et al. 1987) show fractionation significantly different from spinach enzyme.

4 Carbon Isotope Discrimination and Photosynthetic Types in Plants

Following the first observation of slight depletion of heavy ^{13}C in plants with respect to inorganic carbonaceous material (Nier and Gulbransen 1939), Murphey and Nier (1941) reported a wide variation in ^{13}C among different plant species. From early surveys of $^{13}C/^{12}C$ in plants, Wickman (1952) suggested that ^{13}C depletion was a cyclic process and that the degree of ^{13}C depletion in plants depended on the amount of recycling of soil derived CO_2. Craig (1954) disagreed and suggested that fractionation processes within leaves accounted for the variation in ^{13}C composition and that the magnitude of fractionation was environment-dependent. Meanwhile, Baertschi (1953) showed that no fractionation occurred during the respiration of bean seedlings and found 26‰ ^{13}C depletion during CO_2 assimilation. The similarity of the fractionation factor found by Urey (1948) (29‰) and by Weigl and Calvin (1948) (27‰) suggested that fractionation process was almost the same for different plants and growth conditions. The values reported by these authors incidentally corresponded closely to the maximum fractionation factor for photosynthesis in nature. Park and Epstein (1961) proposed a three step model to explain the observed differences in ^{13}C composition in leaves relative to atmospheric CO_2, suggesting some additional photosynthetic and diffusion fractionations.

Wickman (1952) found an aquatic grass with high ^{13}C content compared to known values. The connection between ^{13}C contents in terrestrial plants and C_4 syndrome became clear when Kortshak et al. (1965) and Hatch and Slack (1966) discovered the C_4 pathways in tropical grasses. Based upon isotopic studies, Bender (1968, 1971) and Smith and Epstein (1971) postulated a parallel evolution for C_3 and C_4 plants rather than one common origin. At that time some succulents with anomalous ^{13}C values (Bender 1971) having the ability to fix CO_2 at night were linked to crassulacean acid metabolism (CAM), a separate photosynthetic type group (Bender et al. 1973).

C_3 and C_4 processes involve different isotopic fractionation. C_3 plants convert atmospheric CO_2 to a phosphoglycerate compound with three C atoms. Carbon isotopes are strongly fractionated by photosynthesis. C_3 plants show $\delta^{13}C$ values ranging from −32‰ to −20‰ with an average value of −27‰. C_4 plants convert CO_2 to dicarboxylic acid, a four-C compound and have higher $\delta^{13}C$ values than C_3

plants, ranging from −17‰ to −9‰ with a mean of −13‰ relative to PDB. The difference in $^{13}C/^{12}C$ ratio between C_3 and C_4 is correlated with isotopic fractionation present between the ribulose biphosphate carboxylase (RuBP) activity in C_3 plants and phosphoenolpyruvate (PEP) carboxylase activity in C_4 plants. RuBP discriminates more against ^{13}C than PEP (Christeller et al. 1976). Plants exhibiting C_3 pathways are consequently the most depleted in 13C, and plants endowed with C_4 pathways are the least depleted. Plants with crassulacean acid metabolism show intermediate value (Bender et al. 1973). The ^{13}C value is a standard method for distinguishing the C_3 and C_4 plant groups.

4.1 Discrimination in C_3 Plants

Over 80% of crop plants are C_3 and most terrestrial plants are C_3. All forest communities and most temperate zone plant communities are dominated by C_3 plants. The native crops of North America and Europe are almost exclusively C_3.

There are several processes that contribute to isotope fractionation measured in plant dry matter of C_3 species (Farquhar et al. 1989; Brugnoli and Farquhar 2000) and carbon isotope discrimination can be calculated as:

$$\Delta^{13}C = [a + (b - a)C_i / C_a - d] \tag{15.7}$$

where a is the discrimination due to diffusion (4.4‰), b is the discrimination due to carboxylation (30‰ when corrected for the equilibrium effect on CO_2 dissolution), C_i is the internal gas-phase pressure of CO_2, C_a is the external CO_2 pressure and the term d involves contributions from respiration, liquid-phase diffusion, isotopic changes due to carbon export, CO_2 fixation in C_3 plants by PEP carboxylase and a variety of other factors (Farquhar et al. 1989). The value of d is usually small and variations in d can be generally ignored. The respired carbon is isotopically different from leaf carbon but most available evidence suggests that the IF associated with respiration is small (O'Leary 1981; Farquhar et al. 1989). The IF associated with photorespiration in soybean (*Glycine max*) 7‰ (Rooney 1988).

The following approximate expression has consequently been developed by Farquhar and Richards (1984) to rely Δ and C_i/C_a:

$$\Delta^{13}C = a + (b - a)C_i / C_a \tag{15.8}$$

It means that the isotope fractionation in a C_3 plant mainly occurs in two steps. In the first step, CO_2 diffuses through the stomata to the site of carboxylation; in the second step, this CO_2 is taken up irreversibly by the appropriate carboxylase. The fractionation associated with diffusion is 4.4‰ and with carboxylation is 29‰ in C_3 plants (30‰ when CO_2 dissolution is included). Fractionation may be lower than 4.4‰ when internal CO_2 concentration is low (e.g., when stomata are nearly closed) and $\delta^{13}C$ can approach −2‰. If stomata are open, the internal CO_2 concentration

approaches the external CO_2 concentration, and the observed fractionation approaches the carboxylation fractionation, and leaf $\delta^{13}C$ would then approach −38‰.where a is the fractionations associated with diffusion of CO_2 into the intercellular air spaces ($a = 4.4$) and b the fractionation associated with carboxylation of CO_2 into the first products of photosynthesis by Rubisco ($b = 28$). Substituting the numerical values indicated into Eq. 15.8 generates the following:

$$\Delta^{13}C \approx 4.4 + 23.6\, C_i / C_a \tag{15.9}$$

This simple equation shows that $\Delta^{13}C$ is positively related to the ratio C_i/C_a.

4.2 Relationship Between Carbon Isotope Discrimination and TE in C_3 Plants

TE, the ratio of net photosynthesis to transpiration (at the leaf level) can be mathematically expressed as below (Condon et al. 2004):

$$A / T \approx 0.6\, Ca\left(1 - Ci\right) / Ca) / \left(Wi - Wa\right) \tag{15.10}$$

Equation 15.10 indicates that *TE* depends on two factors. One is C_i/C_a, which decrease enhance the value of $(1 - C_i/C_a)$ and consequently *TE*. The other is $(W_i - W_a)$. Higher the gradient driving transpirational water loss, higher is *TE* (Condon et al. 2004).

It is now accepted that relative differences in C_i/C_a, at least within C_3 species, may be evaluated indirectly by measuring carbon isotope composition of plant dry matter (Farquhar et al. 1989).

However, earlier it was noted that A/T was negatively related to C_i/C_a (Eq. 15.7). Therefore, $\Delta^{13}C$ and *TE* are negatively associated in C_3 plants (Hall et al. 1994; Hubick and Farquhar 1989).

4.3 Discrimination in C_4 Plants

C_4 plants are characteristically found in hot, arid environments. A selective advantage of C_4 photosynthesis is a more efficient use of water. Some crops of big economical importance are C_4 plants: maize, sorghum, millet, and sugar cane.

Carbon isotope discrimination in C_4 plants can be calculated as:

$$\Delta = a + (b4 + b\,3\phi - a)\, C_i / C_a \tag{15.11}$$

where a is the fractionation due to diffusion (4.4‰), b4 is the fractionation from gaseous CO_2 through PEP carboxylase (−6.7‰), b3 is the fractionation due to

RuBisCo (30‰), and ϕ is the fraction of CO_2 released in the bundle sheath that leaks to the mesophyll, where it may be either taken up by PEP carboxylase or released to the atmosphere. Stomatal opening causes opposite effect in C_3 and C_4 plants (compare above two equations). Increasing relative humidity under controlled environments causes an increase in $\delta^{13}C$ in C_4 plants but a decrease in C_3 plants (Madhavan et al. 1991).

The bases of carbon isotope discrimination in C_4 plants are consequently more complex than in C_3 plants. The variation in carbon isotopic discrimination in C_4 plants depends not only on C_i/C_a (as in C_3 plants), but also on leakiness, ϕ (Farquhar 1983), the proportion of CO_2 released into the bundle sheath which is not fixed by Rubisco and leaks back to the mesophyll. ϕ is a measure of the extent to which PEP carboxylation exceed Rubisco carboxylation (Henderson et al. 1998) and has been estimated using a variety of techniques (Hatch et al. 1995). Some authors suggested that for a variety of C_4 species Φ could range from 0.2 to 0.6 (Sandquist and Ehleringer 1995; Saliendra et al. 1996; Meinzer and Zhu 1998). Henderson et al. (1992) and Williams et al. (2001) however found Φ to be constant and equal to 0.2 in *Sorghum bicolor* (L.) Moench, cultivated under a wide range of irradiances, temperatures, and partial pressures of CO_2. Krall and Edwards (1990) also found ϕ to be 0.2 in a number of C_4 species. Since there is less variation in ϕ in a given species, Δ is linearly related to C_i/C_a as in C_3 species, and consequently positively related to TE (Henderson et al. 1992, 1998). Since the slope of the relationship between Δ and C_i/C_a is lower than in C_3 plants (Henderson et al. 1998) genetic variation of C_i/C_a lead to much smaller variation in Δ than in C_3 plants. On the other hand, temporal and environmental variations in C_i/C_a give smaller variations in Δ than in C_3 species.

4.4 Discrimination in CAM Plants

The succulents that have the ability to utilize the crassulacean acid metabolism (CAM) have the ability to fix CO_2 at night as malate along the C_4 pathway. The assimilated carbon is then further processed by ribulose bisphosphate (RuBP) carboxylase on the following day, when the stomata can remain closed and restrict transpiration. Those succulents that have a strong tendency to operate in the CAM mode and assimilate only small amounts of CO_2 during the day via the C_3 pathway have isotope rations similar to those of C_4 plants. On the other extreme, such plants that have only a weak affinity for the CAM syndrome tend to have isotope ratios in the C_3 range. The latter group can usually be induced to fix carbon at night by subjecting the plants to water stress (Osmond et al. 1976) and the isotope ratios then become intermediate. The genus Aloe with 63 species has C_3-like and C_4-like species with some intermediate ones (Vogel 1980). CAM plants are distinguished from C_4 plants by anatomy (succulence) or physiology (diurnal malic acid cycling).

5 Processes Affecting Carbon Isotope Composition in Plants

5.1 Carbon Isotope Composition of the Atmosphere

Measurements at various stations show that the $\delta^{13}C$ of atmospheric CO_2 is currently close to −8‰ (Goodman and Francey 1988). This value is slowly decreasing (Keeling et al. 1979) at an average rate of 0.028‰ per year as the atmosphere becomes depleted in $^{13}CO_2$ relative to $^{12}CO_2$. This depletion occurs as a consequence of the anthropogenic burning of fossil fuels and deforestation and isotopic composition typical of C_3 plant matter (−26‰). The ice core record shows that δa was about −6.4‰ prior to the industrial revolution (Friedly et al. 1986). A latitudinal gradient in δa with 0.2‰ more negative at 60°N than at 60°S has been reported (Keeling et al. 1979). Seasonal variation in δa are also observed, being the greatest at high northern latitudes and these changes in δa correlate well with seasonal changes in the atmospheric partial pressure of CO_2.

5.2 Isotope Composition of Air Within Plant Canopies

Variations in δ_a may occur within or just above, plant canopies. The canopy layer does not need to be precisely defined in each case, but it must be close enough to vegetation for diurnal cycles in nearby plants and soil to be measured.

5.3 Flow of CO_2 Across the Laminar Boundary Layer and Within the Leaf

The theoretical value for boundary layer discrimination is 2.9‰ (Kays 1966). Diffusion across the stomata and the discrimination against $^{13}CO_2$ in free air has a theoretical value of 4.4‰.

5.4 Transport of CO_2 Within the Leaf

Discrimination against $^{13}CO_2$ in free air has a theoretical value of 4.4‰. Diffusion across the stomata varies and at low stomatal apertures (less than about 0.5 μm) the nature of the diffusion of gases is markedly different than that at greater apertures of in free air. For stomatal pores whose diameter is much greater than this value, the rate of diffusion is dependent on the concentration gradient and on the frequency and type of molecular collision and conventional isotope discrimination. The discrimination associated with this diffusion is 11.3‰, which is markedly different from the 4.4‰

value for continuous flow. Diffusion through intercellular air spaces and isotope fractionations associated with diffusion from sub-stomatal cavity to the cell wall is significant. Vitousek et al. (1990) found increases in diffusion resistance as a consequence of increases in leaf thickness.

The pathway of flow from the cell wall surface to the sites of carboxylation, sometimes referred to as the liquid phase, involves several resistances in series and total fractionation associated with this pathway is normally taken as the sum of the equilibrium fractionation as CO_2 enters solution (1.1‰ at 25°C; Mook et al. 1974; Evans et al. 1986) and the discrimination occurring during diffusion of dissolved CO_2 in water (0.7‰) (O'Leary 1984). The carbonic anhydrase interconverts CO_2 and bicarbonate and isotope fractionation for the equilibrium reaction is temperature dependent, being −9‰ at 25°C (Mook et al. 1974).

5.5 Fractionation Associated with Carboxylation

A major kinetic fractionation (30‰) occurs during the fixation of CO_2 by Ribulose −1,5 Bisphosphate Carboxylase Oxygenase (RuBisCO) (Roeske and O'Leary 1984). Phosphoenolpyruvate (PEP) discriminates against ^{13}C and at 25°C, fractionation is usually considered to be ~5.7‰ (O'Leary 1981). Fractionation associated with PEP must be taken into account for C_3 as well as for C_4 plants (Farquhar and Richards 1984), as a proportion of the carbon fixed is associated with anaplerotic reactions. Plants contain many other carboxylases, most of which function in biosynthetic pathways. However, these carboxylases probably contribute less than 0.05% of the total plant C (Raven and Farquhar 1990).

6 Carbon Isotope Discrimination and Its Relationship with Grain Yield

6.1 C_3 Crops

Carbon isotope discrimination (Δ) has been proposed by several authors as a predictive selection criterion for grain yield of wheat under drought stress (Monneveux et al. 2005). Wheat grain yield was found to be positively correlated to grain Δ under the conditions of South Australia (Condon et al. 1987), California (Ehdaie et al. 1991), Italy (Morgan et al. 1993), Morocco (Bazza 1996), Spain (Araus et al. 1998), South of France (Merah et al. 2001b), Greece (Tsialtas et al. 2001), Pakistan (Akhter et al. 2008) and Algeria (Hafsi et al. 2001).

It has been observed that the association between grain Δ and yield was quite stable over growing seasons under those Mediterranean-type climatic conditions (Merah et al. 2001b; Monneveux et al. 2005), characterized by post-anthesis water

deficit and referred by Rajaram et al. (1995) as wheat mega-environment ME4A. Few exceptions were reported by Hafsi et al. (2003) and Araus et al. (2003), under very severe drought conditions, in Algeria and southern Spain, respectively. In some cases, grain yield was also positively correlated to leaf Δ, assessed at anthesis (Merah et al. 1999, 2001c). Under semi arid climate Akhter et al. (2008) reported significant positive correlations of grain yield and WUE with both grain Δ and early leaf Δ in bread wheat, grown under different water regimes.

Different hypotheses can explain the association between grain Δ and grain yield and its stability under terminal drought conditions. Firstly, high grain Δ reflects an ability of the plant to maintain open stomata after anthesis (Morgan et al. 1993; Sayre et al. 1995; Merah et al. 1999, 2001a). The association often reported between grain yield and leaf Δ (Merah et al. 1999, 2001c) agrees with this hypothesis. Secondly, high grain Δ characterizes genotypes more dependent on pre-anthesis reserves for grain filling. Under severe post-anthesis water stress, photosynthesis is more reduced than translocation. For translocation, plants mainly use assimilates from preanthesis reserves accumulated during periods of reduced stress (Loss and Siddique 1994) and which Δ values are higher. The significant correlation generally reported between leaf and grain Δ supports this hypothesis. Finally, high grain Δ under postanthesis water stress can be a consequence of earliness (Condon et al. 2002). Strong negative associations have been found between Δ and days to heading or anthesis in studies conducted with cereals in Mediterranean, terminal-drought environments, with low Δ genotypes flowering later than high Δ genotypes (Craufurd et al. 1991; Sayre et al. 1995).

The association between grain yield and Δ was found to be weaker and less stable under pre-anthesis water stress by Xu et al. (2007) and under residual soil moisture conditions by Misra et al. (2006). In these two environments, referred to as mega-environments ME4B and ME4C, respectively (Rajaram et al. 1995), the association between grain yield and Δ highly depended on the quantity of water stored in the soil before sowing and on the evaporative demand during the growth cycle (Monneveux et al. 2005).

Little is known concerning the association between grain yield and Δ under pre-anthesis water stress. In contrast to ME4A, the association is generally weaker and highly depends on environment. Condon et al. (2002) postulated that the dependence on stored assimilates for grain filling increases as the degree of post-anthesis water stress increases. This suggests that in ME4B conditions, a small proportion of starch originates from reserves accumulated in vegetative organs and that most of the C products are synthesized after anthesis, under more optimal water conditions. This hypothesis is supported by the weak or not significant correlations between leaf and grain Δ reported under his drought scenario (Monneveux et al. 2005; Xu et al. 2007). Under this drought scenario, yield is likely to be related to pre-anthesis growth, which may influence the source and sink capacity. Maximizing crop transpiration, in such conditions, can be achieved by a higher stomatal conductance when plants are suffering drought (i.e. before anthesis) and/or a rapid growth after water recovering (i.e., after anthesis). Consistent genotypic variation was noted in Δ during the vegetative period in this environment (Monneveux et al. 2005), reflecting

differences among genotypes in their ability to maintain open stomata (Morgan et al. 1993). The lack of correlation between grain yield and leaf Δ at anthesis in this mega-environment (Monneveux et al. 2005; Xu et al. 2007) suggests that differences in transpiration rate among genotypes become less marked with time, and poorly explain final yield differences. The correlation between grain Δ and grain yield becomes stronger as the pre-anthesis water stress is weaker, suggesting that the strength of the correlation between Δ and yield is highly dependent on the quantity of water stored in the soil at sowing.

Under residual moisture stress (ME4C), the relationship between Δ and yield was found to be weaker and less stable than under ME4A (Misra et al. 2006). According to Condon and Richards (1993), high-Δ genotypes tend to grow faster than low-Δ genotypes. By covering the ground more quickly, they are more efficient in reducing soil evaporation. Their higher biomass at anthesis and greater reserves enable them to translocate a large amount of stored assimilates to the grain. High Δ may also reflect higher stomatal conductance, particularly after anthesis, when soil moisture decreases and water stress becomes stronger. The weak association between grain Δ and grain yield suggests that differences in transpiration rate had little influence on yield differences among genotypes. At anthesis soil water depletion may have been greater for high−Δ genotypes than for low−Δ genotypes, which were more conservative in their water use. In some cases, excessive soil water depletion by high−Δ genotypes can offset the advantages described above. This could explain the negative relationships between Δ and grain yield reported by Condon et al. (2002) in the northern Australian wheat belt. In this environment, transpiration represents a high proportion of total crop water use and high TE (low Δ) can lead to better soil water conservation thus sustaining better growth until the end of the growth cycle.

Under irrigated conditions, no association is generally found between Δ and grain yield. This suggests that the potential decrease in Ci associated with increased photosynthetic capacity was already largely offset by the increase in stomatal aperture thereby restricting the variation in Δ and reducing the correlation between Δ and grain yield. Few crops are however grown in a total absence of water stress.

6.2 C_4 Crops

In C_4 crops, information concerning Δ variation in different organs of the plant and its possible association with biomass or grain yield is scarce. Substantial variation in Δ has been reported in several species, as maize (Samejima 1984), *Panicum coloratum* (Ohsugi et al. 1988), sorghum (Hubick et al. 1990) and sugarcane (Meinzer et al. 1994). The variation for Δ with abiotic stresses and the relationship between Δ and yield under drought have been poorly investigated in C_4 crops. Bowman et al. (1989) and Meinzer et al. (1994) reported an increase of Δ in leaves with salinity in *Andropogon glomeratus* and sugarcane, respectively. A comparison of two sugarcane cultivars highly differing for salinity tolerance revealed that Δ was significantly lower in the tolerant than in the susceptible cultivar (Meinzer et al. 1994).

In grasses Akhter et al. In *Atriplex confertifolia*, water stress did not affect Δ. In pearl millet, Bruck et al. (2000) found an increase of Δ with water stress but did not find differences between two cultivars highly contrasting for drought tolerance. Δ analysis conducted by Monneveux et al. (2007) at flowering, under drought and well-watered conditions, in different organs of maize (*Zea mays* L.) inbred lines differing for drought tolerance showed that leaf Δ was higher than ear and silk Δ. Drought stress significantly increased Δ in all organs. Under drought, tolerant inbred lines showed significantly higher Δ than susceptible ones. There was a significant positive correlation between leaf, ear and silk Δ, and ear dry weight at flowering, a trait closely associated to grain yield. Δ was then analyzed at flowering in leaves of a set of drought tolerant maize hybrids and checks. Drought tolerant hybrids had significantly higher grain yield and Δ than the used checks. No correlation was found, however, between Δ and grain yield within tolerant hybrids. The use of Δ appears consequently accurate for a first screening of lines or hybrids highly contrasting for drought tolerance, but not for a more advanced selection among tolerant hybrids. More studies are needed in C_4 crops to establish the causes of Δ variation and precise which organ sampled and time of sampling may allow the better precision in the screening process.

7 Carbon Isotope Discrimination and Water Use Efficiency (WUE)

As mentioned above, and as experimentally confirmed by Farquhar et al. (1982), Farquhar and Richards (1984), Heitholt (1989), Johnson et al. (1990) and Ritchie et al. (1990), Δ and TE are negatively related in C_3 crops. Agronomic water-use efficiency could be improved by exploiting variation in leaf-level water-use efficiency (Morgan and LeCain 1991). Negative correlation between Δ and WUE has been reported in several species. Çagirgan et al. (2005) reported higher WUE in barley cultivars with lower Δ values. A negative correlation was reported between WUE and Δ in rice (Impa et al. 2005) under drought conditions. Negative correlation between Δ and WUE has also been reported in other crops as peanut (Hubick et al. 1986), tomato (Martin and Thorstenson 1988), common bean (*Phaseolus vulgaris* L.), for a range grass (*Agropyrum desertorum*) (Ehleringer et al. 1990), and cowpea (Ismail and Hall 1992). A significant negative correlation has been obtained in wheat between Δ and M/T (Condon et al. 1990; Ehdaie et al. 1991). In some grasses (*Leptochloa fusca* (L.) Kunth and *Sporobolus arabicus* Boiss), leaf Δ was found to be significantly and negatively correlated with Water-use efficiency under semi-arid conditions (Akhter et al. 2003). In rice, Impa et al. (2005) also found a negative association between leaf Δ and WUE. However, due to the difficulty of directly assessing WUE components, these studies were carried out in pot experiments. Runoff and drainage were negligible and soil surface was generally covered by a plastic film to minimize soil evaporation. WUE was consequently close to M/T (see Eq. 15.2). Kirda et al. (1992), by using a water balance model (Hatfield 1990)

to evaluate evapo-transpiration and by measuring soil water with a neutron probe, reported a highly significant negative association between Δ and WUE in durum wheat brown in Austria. Condon et al. (1993), however, did not found in bread wheat cultivated in Australia a significant correlation between Δ and WUE, estimated by using a soil water balance and neglecting run off and drainage.

In conclusion, carbon isotope discrimination of plant tissues reflects the variation of photosynthesis to stomatal conductance ratio over a considerable length of time and under variable environmental conditions, and consequently represents a long-term integrative estimate of transpiration efficiency. As emphasized by Chaves et al. (2003) this may be quite different from long-term WUE defined, according to Gregory et al. (1997), as the units of dry matter synthesized divided by the units of water lost (M/T).

It is even more difficult to use *Δ* for predicting variation for agronomic WUE. WUE variation is manly driven by factors that poorly rely to plant characteristics (e.g., runoff, drainage, soil evaporation). Transpiration, which depends on the crop, generally represents a small part of the water available. Allen (1990), for example, found that transpiration represented less than 35% of total water use for barley in Syria. Similarly, in Niger, Wallace et al. (1993) reported that transpiration was less than evaporation from the soil surface. Such finding led to the conclusion that Δ poorly informs about agronomical WUE that can be improved mainly by crop management techniques. Rather, Δ can be used for improving yield of C_3 crops under drought prone conditions, with the limitations that have been previously mentioned. The systematic use of easy-to-use water balance models (e.g., the *budget* model developed by Raes et al. 2006a), would be very useful to simultaneously (i) describe the drought scenario faced by the crop and (ii) estimate the different components of agronomical WUE. This would permit, respectively, (i) to better anticipate the conditions and mega-environments in which Δ can be efficiently used as a selection trait to improve yield and to test the effect of different crop management techniques on agronomical WUE.

8 Conclusion

Many studies conducted across the globe suggest that carbon isotope discrimination has great potential to select high water-use efficient cultivars in different mega (water) environments. Correlation between Δ of plant tissues (leaf or grain) is negative with transpiration efficiency and positive with grain yield in a wide range of crops. Increasing water scarcity under erratically changing climatic conditions demands a reliable and fast screening tool for suitable plants with high WUE. The present review highlights the use of Δ in C_3 and C_4 plants. It also provides the basis of carbon discrimination in common food crops grown in irrigated and rain-fed areas. Although this technique has provided the better selection criteria to the physiologist, agronomist and breeders, still studies are required to establish a relationship of Δ (grain/leaf or specific tissue) with yield and/or WUE in the target environment for different crop plants.

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Chapter 16
Behaviour of Plant Pathogens for Crops Under Stress During the Determination of Physiological, Biochemical, and Molecular Approaches for Salt Stress Tolerance

Murat Dikilitas and Sema Karakas

Contents

Abstract Agricultural technology is the only way to meet the nutritional needs of the world's population. In the last century, crop production has significantly increased and has reached its plateau, coincident with the increase in world population. However, a large percentage of the human population still does not have enough food, therefore many are underfed and face malnourishment. The world population will continue to grow along with poverty, environmental health concerns, issues surrounding the availability of clean water sources, etc. By the middle of this century it may reach 10 billion. Therefore, scientists need to try to keep the relationship between crop production and population on an upward slope. To fully understand trends and to determine increases in crop production, many marginal areas need to be included into agricultural lands. If environmental pollution, drought, salinity, disease, and insect problems, as well as the use of irrigation water of marginal

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quality, are taken into account along with newly added low fertile agricultural lands, many crop plants, for a major part of their growth periods, are grown under adverse conditions. Therefore, now and in the future, scientists have to find physiological, biochemical, and molecular approaches to overcome negative production issues. However, the behaviour and characteristics of plant pathogens have also changed. In this chapter, recent developments in agriculture regarding the production of crops under stress conditions and the behaviour of plant pathogens are evaluated and discussed.

Keywords Plant-salt interactions • Pathogen-salt interactions • Abiotic stress tolerance • Microorganisms • Crop production • Crop improvement

Abbreviations

CMC	Carboxymethylcellulase
FOV	*Fusarium oxysporum* f.sp. *vasinfectum*
GA	Gibberellic Acid
GM	Genetically Modified
IAA	Indole Acetic Acid
NAR	Net Assimilation Rate
PAL	Phenylalanine Lyase
PG	Polygalacturanase
PL	Pectate Lyase
QTL	Quantitative Trait Locus
RAPD	Random Amplification of Polymorphic DNA.
RFLP	Restriction Fragment Length Polymorphism
RGR	Relative Growth Rate
VW	Verticillium Wilt

1 Introduction

The production of safe and sufficient amount of food of high quality in sustainable agricultural systems is a new trend in agriculture. For achieving high quality food goals, new biochemical, molecular, and physiological treatments, combined under the name of biotechnology, are the most promising approaches. In many countries around the world, organizations operating under various names such as Agriculture 2020 or 2020-European Vision for Plant Science, etc., have been working on the current major challenges in biology and plant sciences by investigating how plants of the future can provide solutions to numerous problems such as increasing demand for food, competition for land use, climate change, etc. (Cavalieri 2005; Dikilitas and Karakas 2011; www.arabidopsis.org). Ameliorating these types of

issues will partly be dependent on increasing our knowledge of plant biology and environmental sciences.

Over the last century, advances in plant sciences have been spectacular. However, in the next 20 years, in order to obtain more tolerant plant species, information regarding biochemical and molecular processes should be converted into predictive modelling. To achieve this goal, many new technologies and analytical techniques should be developed. Since abiotic factors such as cold, drought, and salt interact with biotic stress factors such as pathogens, insects, and beneficial microorganisms, their complex metabolisms need to be fully understood. The salt, heat, drought, and cold tolerant plants could play important roles in areas where crops are lost, and could be important for increasing crop yields (Ashraf and Foolad 2007). In the past, agricultural improvements, through the use of traditional breeding methods, managed to keep pace with increasing food demands. Using traditional methods, plant breeders developed new strains of crops that significantly increased yields by 3–6% a year, with method that required many years to bring together desirable and to eliminate undesirable traits (Abumhadi and Atanassov 2010). Over the next decade, scientists will use genomics and other biochemical approaches (Atanassov 2009).

Plant biotechnology offers significant improvements in every area of crop production. Recent advances in plant molecular biology have allowed us to discover and to isolate important genes that regulate yields and that tolerate environmental stress (Abumhadi and Atanassov 2010). Intensive studies in plant biotechnology began in the 1980s with the use of *Agrobacterium* (Bevan et al. 1983) and these studies are still carried out using the same organism (Harfouche et al. 2011). Recent biotechnological advances in direct gene transfer have helped engineers to develop more resistant plants with new traits from unrelated organisms such as fungi, viruses, or bacteria that are otherwise very difficult to transfer using classical breeding programs (Abumhadi and Atanassov 2010; Jauhar, 2006). Plant biotechnology using molecular markers, genomics, and proteomics are new and promising tools for improving crop plants. For example, tree genetic engineering has advanced to the point at which genes for desirable traits can now be introduced and expressed efficiently; examples include biotic and abiotic stress tolerance, improved wood properties, root formation, and phytoremediation (Harfouche et al. 2011). Also, through genetic engineering, the biological components of plants can be maintained and crop production can be increased when chemicals or irrigation water or nutrients are minimized (Sridhara 2006; Fageria et al. 2008). However, stable gene expressions are key issues that need to be resolved before transgenic plants can be used commercially.

Diseases may have an impact on crop loss, and result in high costs associated with their control through protective chemicals and drug resistance. Various high-tech approaches have been proposed to protect plants from such type of stresses. Virus resistant transgenic plants, especially, have gained attention, and fungi-, bacteria-, and nematode-resistant plants are currently in the development stage. Very few reports exist regarding the loss of tolerance or pathogenicity after a period of time in living organisms (Krokene and Solheim 2001). Engineering plants to contain increased disease resistances or stress tolerances that are durable can be a

challenge for biotechnologists. Despite much effort, most attempts using genetic engineering to develop crops that are resistant to fungal and bacterial diseases or salt tolerances have been unsuccessful in the long term. The most common reason for this is that the success of resistance (*R*) genes relies upon recognition by the encoded *R* protein of a specific avirulence (Avr) protein in the pathogen. Therefore, given the gene-for-gene relationship underlying this type of resistance, *R* genes are extremely vulnerable to a single loss-of-function mutation in the corresponding pathogen *Avr* gene, which leads to a loss of resistance (Rougon-Cardoso and Zipfel 2010).

In this chapter we attempt to underline important issues regarding the stress mediated aspects using new approaches and methods. We will emphasize the pathological impact of microorganisms such as fungi or bacteria under stress conditions, whether they evolve during the stress period and adapt themselves to conditions of crop plants, are suppressed by stress and cannot tolerate stress as crop plants, or can surpass defence barriers without exerting remarkable force. Attempt is made here to understand the possible behaviours of plant pathogens, as well as recent advances in crop production for crops that grow under stress conditions.

2 Improvements in Salinity and Disease Tolerance in Crop Plants

Salinity affects over 6% of the world's land and 20% of the world's irrigated land, posing a major threat to our future (http://www.fao.org). Increased incidences of salinity and disease stress affecting the productivity in principal crops has been common in arid and semi-arid areas. Therefore, diseases and other abiotic stress factors are interconnected in saline soils, and their combined impacts, are very complex. Strategies for obtaining maximum crop yields under stressed conditions include the following: (1) the breeding of new crop varieties, (2) the screening and the selection of existing germplasm in potential crops, (3) the production of GM crops, and (4) the exogenous use of osmoprotectants (Fageria et al. 2008; Athar and Ashraf 2009). Since conventional breeding programs are limited, molecular biology techniques have been introduced for inducing stress tolerance in economically important crops. As for other stress tolerance mechanisms, molecular biology techniques, aimed at crop improvements in salt tolerant species, have followed common biochemical and physiological pathways. However, due to toxic and osmotic effects, such as selective ion accumulation or exclusion, the control of sodium uptake and its distribution within plants, and the compartmentation of ions at the cellular or the entire plant level are important issues that should be considered (Munns and Tester 2008; Athar and Ashraf 2009).

In the first studies on molecular approaches salt tolerant genes controlling salt uptake and transport, or regulating osmotic functions in plants were considered first. However, disease resistance mechanisms and crop improvements for disease resistance were considered separately. Understanding stress metabolism in detail is crucially important since many stresses are involved and such a situation could

possibly become more complex due to the involvement of biotic stress factors in physiologically and biochemically improved salt tolerant plants (Dikilitas 2003). Therefore, it is important to identify key genes and their functions at the cellular as well as at the entire plant level using advancements in functional genomics (Athar and Ashraf 2009). However, when developing transgenic plants for salt stress tolerance, disease incidence should be considered. At present, in order to increase plant defence mechanisms and salt tolerances under adverse conditions, it is logical and appropriate to apply exogenous chemicals, plant growth regulators, or compatible solutes or microorganisms. As a result of its complexity, salinity tolerance is difficult to measure. Not only are there a number of genes controlling salinity tolerance whose impacts interact with environmental conditions, but there are also two major and distinct components of salinity tolerance that are often difficult to distinguish (Munns 2002). Screening based on growth needs to allow for two distinct mechanisms for salinity tolerance are as follows: (1) tolerance to the osmotic effect of the saline soil solution, and (2) tolerance to the salt-specific nature of the saline solution.

Significant technological advances in the field of molecular biology have been made during the past decade. For achieving salinity tolerance, molecular techniques designed to selectively introduce desired genes may provide alternative means to classical plant breeding. These techniques, based on specific traits that are controlled by one gene (e.g. a transcription factor or an important ion channel), will benefit the development of salinity-tolerant cultivars. Blumwald et al. (2000) reported the development of a salinity tolerant transgenic tomato plant for which the over-expression of the vacuolar Na^+/H^+ antiporter showed dramatic improvement in vegetative growth and fruit yield. The antiporter is the only known transporter that compartmentalizes Na^+ within the vacuole, and the extrusion of Na^+ from the cell, whereas Na^+ has little chance of toxic effects on metabolism, or transportation to younger leaves and fruits. Such studies indicate a great potential for transgenic approaches. The use of molecular markers in breeding programs is increasing rapidly. Marker-assisted selection is non-destructive and can provide information on the genotype of a single plant without exposing the plant to stress. The technology is capable of handling a large number of samples. In order to use marker-assisted selection in breeding programs, markers must be closely linked to the trait and must operate across different genetic backgrounds. In recent years the efficiency of genetic mapping has also greatly improved with the advent of high-density maps that incorporate microsatellite markers, which are overtaking tedious RFLP markers and unreliable RAPD markers. Molecular approaches for transferring genes from one plant to another have also gained popularity. Munns et al. (2003) reported that two major genes control Na^+ uptake in durum wheat. One gene retrieves Na^+ from the xylem in roots, one enhances K^+ loading in the xylem, while another retrieves Na^+ from the xylem in both the root and the shoot (James et al. 2006). Details and comprehensive information regarding crop productivity under saline conditions can be obtained from the work of Munns (2009), and Athar and Ashraf (2009). For plant disease resistance, detailed information can be obtained from the work of Knox and Clarke (2007). However, major improvements for crop production generally involve

either improvements for disease resistance or abiotic stress tolerance and these two stress factors should be handled together while crop production improvements are considered.

3 Impact of Plant Pathogens Under Salt Stress Conditions

Environmental factors such as drought, excess irrigation, and high temperatures interact with microorganisms in the air or in the soil fauna. Therefore, these factors may increase or decrease the virulence of pathogens. In a similar manner, salinity may also interact with fungi in soil fauna and may reduce or increase the effectiveness of fungi. Since many semi-arid and arid areas of the world are characterized by salinity, such a case cannot be inevitable. In the past, researchers have directed their attention either to the relationship between plants and abiotic stresses, or between plants and pathogens. Relationships also exist between abiotic stresses such as drought, heat, salinity, etc., and microorganisms. Therefore, a newer concept is the interaction between plants, pathogens, and abiotic stresses. The exposure of plants to salt stress generally begins with the exposure of roots to stress. Salt stress leads to changes in the growth, morphology, and physiology of roots that, in turn, change water and ion uptake, and the production of signals (hormones) that communicate information to the shoot. The entire plant is then affected when roots are grown in a salt medium (Grover et al. 2011). Since any plant pathogen living under these conditions may be a potential danger to crops, both stress factors should be considered together in relation to crop improvement.

Improving salt tolerance in plants becomes more complex as plants are exposed to additional environmental and biotic stress factors. Interactions of heat and drought, water logging, heavy metal toxicity, and biotic stresses have been studied (Singh et al. 1997; Obuekwe et al. 2005; Jin et al. 2010). The stress responses of many plants have been identified as multigenic traits. The largest threat to molecular and biochemical techniques that aim to obtain resistant or tolerant crops under stress conditions is adaptation of their biotic enemies, that eventually reduce crop production. Additionally, more virulent races of pathogens can possibly emerge and additional destructive consequences can be inevitable.

In general, due to the presence of other environmental factors, plant responses, in addition to salt stress, could interact with biotic stress factors (Dikilitas 2003). In nature, such environmental complexities are a usual occurrence. Both abiotic stress factors such as drought and salinity, or heat and salinity, or heavy metal toxicity and salinity lead to osmotic stress in plants and cause many similar metabolic responses, although salinity may induce additional responses (Singh et al. 1997; Dikilitas 2003; Dimartino et al. 2011). Further progressive symptoms of these stresses can make plants more prone to additional abiotic and biotic stress factors. Salt stresses have not only been studied in semi-arid and arid areas, but also in heavy metal contaminated soils. Plants near industrial vicinities, especially in developing countries, suffer from both stressors. For example, an additive effect of NaCl (6 EC) and lead

acetate (1.0 mM) on biomass accumulation has been observed in *Vigna radiata* (L.) Wilczek cv. Pusa baisakhi (Singh 1995; Xu et al. 2010).

Plants growing in the saline environments of arid zones may suffer from high salt stress and high temperature during summer and low temperatures during winter. Under these conditions, plants show an accumulation of low molecular organic osmolytes, changes in polyamine levels, the synthesis and release of ethylene, and other types of metabolite adaptations to high salt and high temperatures. In many studies, heat, high temperature, drought, and salt have resulted in the increased accumulation of carbohydrates such as sucrose, sorbitol, mannitol, glycerol, and nitrogenous compounds such as proline, putrescine, betaine, glycine, choline, and organic acids (Ramagopal 1993; Gamalero et al. 2009); and have indicated the existence of a common defense system that may operate in two or multiple stress systems. Although combined stress factors are responsible for a high loss of agricultural productivity, the development of crops resistant to combined stress factors have, so far, resulted in limited success due to the difficulties of multigene transfer. Most studies have concentrated on the cloning of genes that are responsible for the synthesis of enzymes, leading to the accumulation of osmolytes. However, the regulation of genes and gene products responsible for the responses have not been extensively studied (Ramagopal 1993). Interactions between two kinds of stresses may cause significant alterations in normal physiological processes in all plant organisms, including economically important crops, and may alter the regulation of common transport systems and metabolic events in the cell. Eventually the induction of stress adaptation mechanisms for the survival of stressed plants occurs. However, a wide range of adaptations are required to cope with stress factors.

The adaptations of microorganisms, as well as crop plants, to stress are a complex multilevel regulatory process in which many genes may be involved (Srivastava et al. 2008). In certain species such as halophilic or thermophilic organisms living in adverse conditions, enzymatic activities are normally produced without any stress symptoms (Madigen 1999; Palmero et al. 2010). However, many microorganisms develop different adaptation mechanisms in order to combat the stress (Grover et al. 2011). The susceptibility of plants to insects and diseases may be affected by the presence of nutrients in the soil. For example, salinity induces several metabolic changes in diseased plants, such as accumulation of proline and glycine betaine, and antioxidant enzymes (Bray et al. 1991; Jbir et al. 2001; Dikilitas 2003). Under the combined stress of salinity, and with the wilt pathogen *Verticillium albo-atrum*, metabolic changes in lucerne plants, such as the accumulation of proline and antioxidant enzymes, were significantly effected and their accumulation drastically and unexpectedly decreased (Dikilitas 2003). These findings clearly indicate that the pathogen managed to survive and to infect the host plant after a period of adaptation. Therefore, genetic manipulation of improved crop cultivars for salt tolerance should be coupled with improved disease resistance if we really want to improve crop production under such conditions. The possible interactions between plants and microbes and their possible behaviours under stress conditions are presented in Fig. 16.1.

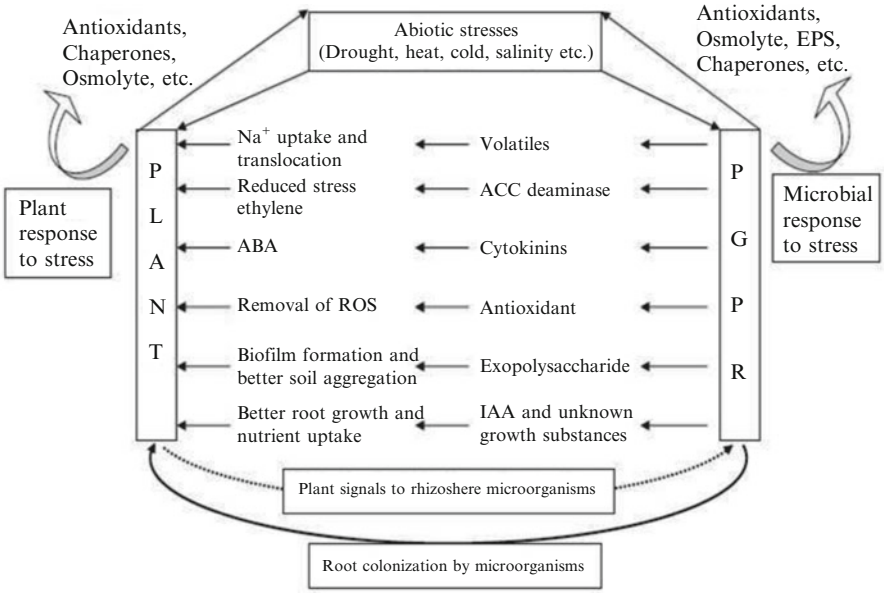

Fig. 16.1 A conceptual diagram of plant-microbe interactions under abiotic stress (Grover et al. 2011)

3.1 Interactive Effects of Salinity and Plant Diseases on Crop Productivity

The irrigation waters rich in salts in nature effect the distribution and severity of diseases. One of the earliest studies regarding interactions between salt and pathogens was made by Holmes (1976) on maple trees; where young trees were badly affected by high NaCl stress while low salt concentration alone only induced a foliar scorch. However, with the combined effect of NaCl and *V. albo-atrum*, the limitation of crop production was more pronounced. Similar reports were published by Nachmias et al. (1993, 1994) on potato plants irrigated with a salt solution ($NaCl:CaCl_2$; 4:1) equivalent to 5 mmhos cm^{-1}. Drought stress was also induced by reducing irrigation to 25%. The treatments increased symptom severity and eventually reduced crop production, while increasing pathogen growth in the vascular system. Again, Orenstein et al. (1995) simulated field conditions in potato crop plants by irrigating with a salt solution equivalent to 10.2 mmhos cm^{-1} under a drought stress induced by 10% polyethylene glycol (PEG). Potato growth was significantly reduced while the conidi forming unit (c.f.u) count of *V. dahliae* increased when a comparison was made with that of the control. Similarly, irrigation with saline water (EC = 3.2) resulted in a build up of a *Fusarium oxysporum* f.sp. *radicis-lycopersici* population at the root zone of tomatoes in soil (Triky-Dotan et al. 2005). Howell et al. (1994) reported that *V. albo-atrum* inoculation alone; on disease resistant lucerne plants

(cv NK-89786); had a minimal impact on foliage yield. However, when the resistant cultivar was treated with salt at 3, 5, and 7.6 dS m^{-1}, the yield was reduced when compared with non-inoculated controls. *In vitro* studies on *V. dahliae*, cultured on potato-based solid or liquid media containing 0.05–0.35 M NaCl, showed that the growth of the fungus was depressed with an increase in the NaCl concentration, but that the production and behaviour of the conidia were not impaired, and conidial differentiation and germination ability gradually increased as the concentration of salt increased (Danti and Broggio 1997; Goudarzi et al. 2008). High soil salinity or saline irrigation has also been reported to increase the severity of stem rot caused by *Phytophthora citrophthora* in citrus roots (Sulistyowati and Keane 1992; Grattan and Grieve 1999; Syvertsen and Levy 2005). Although it did not stimulate growth of the pathogen *in vitro*, increases in disease under saline conditions were attributed to a direct effect in reducing resistance in the host. Besri (1990) and Triky-Dotan et al. (2005) also reported that high salt stress in irrigation water resulted in the breakdown of resistance in tomato cultivars. Similar results on the effect of salinity stress on development of plant diseases have been reported for phytophthora root rot in chrysanthemum and citrus (Blaker and MacDonald 1986; MacDonald, 1984); for pythium blight in penncross creeping bentgrass (*Agrostis palustris* Huds. 'Penncross') (Rasmussen and Stanghellini 1988); for olive trees infected with *Verticillium dahliae* (Levin et al. 2003); and for pistachio trees infected with *V. dahliae* (Mohammadi et al. 2007). Increases in disease severity under saline conditions have also sometimes been attributed to an increase in the virulence of a pathogen (Ragazzi et al. 1994, 1996). For example, salinity increased the development of the motile zoosporangia *Perkinsus* sp., and developmental rates increased with increasing salinity (Ahn and Kim 2001). Similar findings were made by Ragazzi et al. (1994, 1996), who reported that *Fusarium oxysporum* f.sp. *vasinfectum* chlamydospores and their germ tubes grew better in a saline medium and that the pathogen was more virulent when it had been cultured on a saline-enriched medium.

The results of Dikilitas (2003) showed that the growth and sporulation of *V. albo-atrum* isolates, from lucerne (V1-strong pathogen) and tomato (V2-weak pathogen), decreased after the threshold level of 150 mM NaCl. Under non-saline conditions, the fastest growing isolate V2 was the most affected isolate under saline conditions, especially when accounting for sporulation. Above 150 mM NaCl fungal isolates still continued to grow and to produce an ample amount of spores that were capable of infecting host plants. It has been suggested that the highly virulent isolate (V1), showing a high salt tolerance, also has a high adaptation capacity in relation to salinity, since the appearance of the colonies of both isolates, especially the V1 isolate, became more feathery with increasing NaCl concentrations (Dikilitas 2003). At low osmotic potentials, the morphological switch to feathery patterns in some mycelial fungi has been attributed to their ability to adapt to drought conditions (Griffith and Boddy 1991; Llamas et al. 2008). For example, Hasan (2002) stated that the mycelial growth of *F. oxysporum* significantly increased with 1–10% (0.17 – 1.70 M) concentrations of NaCl, although the production of GA (Gibberellic acid) and IAA (Indole acetic acid) by the fungus decreased above 1% NaCl. On the other hand, Amir et al. (1996) reported that concentrations of NaCl over 1% (170 mM)

negatively influenced the radial growth of *F. oxysporum* f. sp. *lini*. In general, a narrower range of environmental conditions are required for sporulation than for mycelial growth. Gao and Shain (1995) reported that the conidial germination of *Cryphonectria parasitica* (Murr.), chestnut blight fungus, was more sensitive than mycelial growth to NaCl at a −2.0 MPa osmotic potential.

A study containing the salt-adapted wilt fungus *V. albo-atrum* showed that the growth of isolates of the non-salt-adapted (V1 and V2) and salt-adapted strains (V1-, V2-150; V1-, V2-200), continuously grown by sub-culturing (in their respective conditions) under NaCl stress for at least 8 months, showed similar growth patterns in Petri plates although the salt-adapted strains of V1-200 and V2-200 produced less spores (Dikilitas 2003). When the non-salt-adapted lines (V1 or V2) were cultured on 150 and 200 mM NaCl containing media, their growth was lower than those of the corresponding salt-adapted lines. Although salt-adaptation resulted in the ability to maintain better growth rates under elevated concentrations of NaCl than strains that had not been adapted, adaptation did coincide with a decrease in the ability to form spores, in all of the salt-adapted isolates the effect however, was more marked in V1- and V2-200 than in the corresponding -150 strains, which may impact the ability of *V. albo-atrum* to infect plants under saline conditions. The lower rate of sporulation under saline conditions may still be efficient enough to cause pathogenicity, and pathogen may eventually develop an adaptation mechanism. For example, findings of Llamas et al. (2008) have indicated that the marine strains of *F. solani* have a physiological mechanism that permits survival in environments with low water potential. Many fungal species, such as *Aspergillus*, *Penicillium*, *Rhizopus*, and *Fusarium*, produce increased amount of hormones, such as GA or IAA, or both, under salinity (0.5% and 1%, NaCl) stress, suggesting that an increase in the level of hormones may act as an adaptive response that maintains the stability of the fungus. However, at higher concentrations of NaCl (above 1%), the content of GA and IAA decreased over time.

Salinity not only exerts stress on fungal metabolism but also reduces the resistance of cultivars. Under such circumstances a lower germination rate for conidia may still result in pathogenicity. MacDonald (1984) reported that the root systems of hydroponically grown chrysanthemums exposed to salinity following inoculation with *Phytophthora cryptogea* developed more severe symptoms than plants that were not exposed to salinity. Also, the hyphae of *P. cryptogea* rapidly colonized salinity-stressed roots causing extensive necrosis after 12 h of inoculation, indicating a salt-induced change from resistant to a highly susceptible condition. Similarly, Turco et al. (2002) reported that a nutrient solution with an EC of 20 mS cm^{-1} increased the severity of *Fusarium* wilt by approximately 34% in the cotton cultivars 'Coker 304' and 'Acala SJ2', as compared to the non-saline test group. Recently, Al-Sadi et al. (2010) reported that increasing irrigation-water salinity from 0.01 to 5.0 dS m^{-1} significantly increased mortality in cucumber seedlings inoculated with *Pythium. aphanidermatum*. *In vitro* tests in culture media amended with various concentrations of NaCl also showed that the growth of *P. aphanidermatum*, *P. spinosum*, and *P. splendens* isolates were either stimulated or unaffected at salinity levels that were stressful for cucumber (electrical conductivity = 5 dS m^{-1}). The isolates of

P. aphanidermatum from greenhouses with no salinity problems were as tolerant to salinity as the isolates obtained from salinity-affected greenhouses, suggesting that increased mortality in cucumber seedlings at higher salinity levels may imply a synergistic interaction between salinity stress and salinity tolerant *Pythium* species in cucumber seedlings, resulting in greater seedling losses.

A reduction in the growth of plants under saline conditions can largely be attributed to reduced water absorption caused by reduced water potential in the root environment. Under saline conditions, Na^+ and Cl^- ions are taken up at high rates, and may lead to excessive accumulation in tissues, which may inhibit the uptake of other ions into the root and their transportation to the shoot (Mer et al. 2000). As reported by Rasmussen and Stanghellini (1988) (who stated that increased salinity levels predisposed Penncross creeping bentgrass to cotton blight caused by *Pythium aphanidermatum* and accelerate the onset and development of disease) may predispose the condition of crop plants. The combined effect of both salt (even low concentrations) and the pathogen may cause more serious problems than those of the salt or the pathogen alone. For such conditions, additional energy may be required in order to cope with disease stress (Dikilitas et al. 2009). The resistance of plants to a pathogen may depend on the speed and the extent of synthesis of the enzymes induced in the host by the pathogen. PAL (Phenylalanine ammonia lyase) is a key enzyme in the production of the basic molecule used for the biosynthesis of most phenolics, including phytoalexins and lignin (Tang 2001; Neves et al. 2010). The accumulation of PAL in response to *V. albo-atrum*, isolate V2, and/or NaCl was studied in cell suspension cultures of lucerne cv. Kabul. Isolate V2, or NaCl, or both, were effective in inducing PAL accumulation in cell cultures *in vitro* (22 °C). The combined effect of NaCl (50 mM) and of the elicitor, derived from the isolate V2 of *V. albo-atrum* (0.05 or 0.1 mg ml^{-1}) resulted in a further increase in PAL activity. However, PAL activity was inversely correlated with a further increase in the concentration of NaCl (200 mM) and the elicitor (0.1 mg ml^{-1}). Similarly, Jbir et al. (2001) reported that salt stress increased PAL activity in wheat plants. On the other hand, Dunn et al. (1998) reported that after 30 days of high salinity (0.1 M NaCl), citrus plants grew more slowly and produced a lower PAL activity and, as a result, became more susceptible to nematode (*Tylenchulus semipenetrans*) attacks. The recovery of tomato plants from the effects of *V. albo-atrum* or salt was quicker and earlier than the combined effect of salt and fungus. Therefore, the results suggest that *V. albo-atrum* was still pathogenic under saline conditions (50 mM) and delayed the recovery of plants, regardless of their age. This is in good agreement with the results of Swiecki and MacDonald (1991) who reported that the exposure of tomato plants (*L. esculentum* Mill.) to salinity stress, either before or after inoculation with *Phytophthora parasitica*, increased root and crown rot severity relative to non-stressed controls. The synergy between salinity and *P. parasitica* was most pronounced on young (pre-bloom) plants and less pronounced on older (post-bloom) plants. For example, the interaction between salinity and *P. parasitica* significantly increased root necrosis, reduced top weight, and caused a higher incidence of crown infection than for the corresponding non-stressed plants. A well known outcome is that plants exposed to salinity show marginal Ca^{2+} deficiency (Kostandi and Soliman 1998;

El-Iklil et al. 2002). Under such conditions, the membrane becomes fragile, permitting the continuous out flow of assimilates (sugars and amino acids) that facilitate fungal growth (Hancock and Huisman 1981).

Although pathogenic fungi living under adverse conditions act as pathogens, they may still be pathogens when adverse conditions turn into optimal conditions for pathogen growth. Salt-adapted strains of V2 (V2-150 or V2-200) were still pathogenic to tomatoes even under non-saline conditions, suggesting that the fungus did not lose its pathogenicity over a period of time, even when sub-cultured under saline conditions (Dikilitas 2003). A tomato isolate of *Phytophthora. parasitica* has been recovered from saline soil and was found to be more tolerant to salinity than one recovered from non-saline soils (Bouchibi et al. 1990). Similarly, Ragazzi et al. (1994, 1996) reported that mycelia of *F. oxysporum* f. sp. *vasinfectum* (FOV) from non-saline medium and from saline-enriched medium both produced wilt symptoms. However, symptoms for a medium-resistant cotton, cv. GSC 20, appeared earlier and advanced rapidly with mycelium from the saline-enriched culture. Furthermore, Ragazzi and Vecchio (1992) reported that chlamydspore viability and the pathogen virulence of FOV was enhanced when sub-cultured on NaCl-enriched media. Turco et al. (1999) also reported that FOV had a greater enzymatic activity in saline environments, especially with regard to pectate lyase (PL) and polygalacturonase (PG) enzymes. However, high salt concentrations (85–250 mM) may strongly inhibit the enzymatic activities (cellulase, PL, and PG) of soil-borne fungi, as well as growth-promoting fungi, as is the case with *Cladosporium cladosporioides*, *F. moniliforme*, *F. oxysporum*, *F. solani*, *Rhizoctonia solani*, and *Macrophominia phaseolina* on the nodules of *Vicia faba* L (Omar and Abd-Alla 2000). The crop plants in areas where soil salinity is a problem could undergo an increase in susceptibility to pathogens and suffer a greater yield loss, as a result of combined stresses or increased pathogenicity due to the adaptation of the fungus.

Although plants may not show stress symptoms and may metabolize normally under low to moderate salinity levels, additional energy may be required to cope with disease, due to the stress caused by microorganisms such as fungi. For example, *Verticillium* wilt (VW) disease generally depends on the conditions in which plants are grown, and it has been reported that the irrigation regime may increase the incidence of wilt (Jefferson and Gossen 2002). If irrigation water is associated with salinity, detrimental effects on plants would be inevitable. In fact, *Verticillium* wilt has now become more prevalent in many areas where an increase in the salinity of irrigation water poses its greatest threat (Nachmias et al. 1993; Saadatmand et al. 2008). It is known that the reaction to infection by disease can be modified by environmental factors, the potential for interactions between salinity and disease is a realistic possibility that must be considered. It has been reported that abiotic stresses may increase symptoms by directly impacting the pathogen or its host (Dimartino et al. 2011). Similar results have been reported by Snapp and Shennan (1994) who found that a tomato cv. UC82B became vulnerable to infection by *Phytophthora. parasitica* when subjected to salt stress, and produced thinner roots and higher root-senescence rates when compared to the non-stress control group. Various researchers

also report that soil salinity markedly increased the incidence of *Phytophthora* root rot in tomato and chili peppers under field and greenhouse conditions (Snapp et al. 1991; Sanogo 2004). The combination of salinity and enhanced disease severity may also lead to significant reductions in fresh fruit yields, fruit size, and total above-ground biomass. On the other hand, salinity decreases the resistance of crop plants against soil-borne saprophytes such as *Pythium ultimum.*

The effect of salinity on microorganisms is quite similar to that of crop plants. High salinity concentrations lower the production of mycelia and the sporulation of fungi, like reduced germination capacity and rooting ability of crop plants. A reduction in germination under saline conditions may be attributed both to the toxic and osmotic effects of NaCl. Chandler et al. (1994) reported that reduced osmotic potentials decrease germination and radial extension in *Verticillium* species. Similar findings have been recorded by McQuilken et al. (1992) and Goudarzi et al. (2008), who found that a decrease in osmotic potential caused a reduction in mycelial growth and the germination of oospores of *Pythium oligandrum* and *Macrophomina phaseolina* respectively. A decrease in the growth of *Phytophthora* sp. caused by salinity was also reported by Wilkens and Field (1993) and DiLeo et al. (2010). The lower sporulation rate resulting from higher concentrations of NaCl may affect the pathogenicity of the fungus, and another factor may also influence infectivity of the germination rate of those spores that are produced, since germination rate either decreases or increases depending on the concentration of NaCl.

It is not clear how salinity stress has such a pronounced influence on disease severity in plants, while having relatively less effect on non-inoculated plants. The mechanism is not fully understood, but may result from an impairment of normal host defence mechanisms. The plants respond to pathogenic invasion in numerous ways that function to block, slow, or prevent the pathogen from successful establishment or spread in the host's tissue. Many defence responses involve the biosynthesis of compounds that are toxic to invading pathogens. However, under salinity stress many defense responses and symptoms, including changes in membrane permeability, necrotic leaf margins, leaf drop, wilt, the ultra structure of organelles, and the synthesis of DNA, RNA, proteins, carbohydrates, and ultra low molecular weight antimicrobial substances, such as the accumulation of phytoalaexins, are severely affected (Jbir et al. 2001; Sanogo 2004). The host is predisposed to disease by the decreased availability of water and the accumulation of toxic ions in tissues is also a possibility. As a result, the plant may have lower photosynthetic activity and a lower daily growth (Brugnoli and Bjorkman 1992; Saadatmand et al. 2008). Inhibitions of these processes in dehydrated tissues have been reported to reduce plant resistance, through the inhibition of protein synthesis that may contribute to increased host susceptibility by preventing the synthesis of important enzymes and the low molecular weight of antimicrobial substances for disease resistance in the host (Turco et al. 2007). However, little work has been done on the enzyme synthesis for disease resistance under salinity or dehydrating conditions (Boyer 1995; Turco et al. 2007).

Under saline conditions fungi may become more aggressive. For example, El-Abyad et al. (1992) reported that, when compared to non-saline conditions, the activities of the cell wall enzymes (xylanase and galactanase) of *Sclerotium rolfsii*

increased with increased salt concentrations. Dikilitas (2003) suggested that low levels of NaCl (50 mM) have a positive influence on fungal growth. Turco et al. (1999, 2002) determined that the virulence of the pathogen, surmised by the increased production of cell wall enzymes and conidia, increased under 50 mM NaCl. Under non-saline conditions, the impact of the fungus was more destructive than pathogen infection. Similarly, Regragui et al. (2003) stated that tomato plants of var Marmande became highly susceptible to infection by the virulent Moroccan isolate P80 of *Verticillium albo-atrum*, and was slightly susceptible to the avirulent isolate P(3) A of the same fungus. Repeated watering of infected plants with saline solution enriched by NaCl (80 mM) induces an aggravation of *Verticillium* symptoms caused by the aggressive isolate, and a gain of new pathogenic aptitude for the non-pathogenic isolate. The impact of salinity on the fungus developed on CMC (carboxymethylcellulase) medium produced a slight decrease in mycelial growth and a significant increase in CMC activity, *in vitro*. Dikilitas (2003) has reported that even non-pathogens may act as pathogens under saline conditions. For example, weak pathogenic isolates (VL, VS, and VF) of *V. albo-atrum* cause significant reductions in the relative growth rate (RGR), the net assimilation rate (NAR), and the height of lucerne plants under 50 mM NaCl (Dikilitas 2003).

3.2 *The Response of Salt Tolerant Plants to the Effect of Plant Pathogens Under Normal or Salinity Stress*

Determination of the degree of resistance of crop plants under saline conditions, as well as testing salt tolerant crop plants for resistance to diseases, is very important. Using this approach, we can know the threshold level of salt tolerance or disease resistance under combined stress. The breaking point of disease resistance or salt tolerance under combined stress is also possible. A study carried out on the pathogenicity of *V. albo-atrum*, isolate V1, on various disease-resistant and salt-tolerant plants (13R Supreme, Vertus, and Bilensoy-80); and disease susceptible and salt susceptible (Rambler) or disease susceptible and salt tolerant (R-350-N) cultivars of lucerne has revealed that cvs. 13R Supreme, Vertus, and Bilensoy-80 showed resistance to V1 under non-saline and saline conditions (50 mM NaCl), whereas Rambler and R-350-N both showed susceptibility under saline or non-saline conditions, as indicated by height, RGR, and NAR measurements, compared to corresponding controls. This result indicates that a low level of salinity is not enough to alter the resistance of disease-resistant and salt tolerant cvs. 13R Supreme, Vertus, and Bilensoy-80 to the pathogenicity of *V. albo-atrum*. However, when cultivars are tested under high salinity stress (100 mM NaCl) against *V. albo-atrum*, the resistance of cultivars is surpassed (Dikilitas 2003). Similarly, Besri (1990) reported that high salt levels in irrigation water in Morocco caused a total breakdown in the resistance of tomato cultivars that were normally resistant to race 1 of *V. dahliae*. He also reported that cultivars that were resistant to race 2 became susceptible with increasing soil salinity.

Regenerating salt tolerant plants through biochemical or biotechnological means is remarkable. However, when salt tolerant plants are regenerated, one should keep in mind that these plants can face other stress factors, in nature, other than salinity. If the stress factor is biotic, complex biochemical mechanisms may emerge. For example, biochemically and physiologically salt-tolerant improved lucerne *M. media* cv. Rambler displayed the highest degree of susceptibility to *V. albo-atrum*, under both normal and saline conditions, and the susceptibility of salt tolerant plants increased with an increasing level of tolerance to salinity. The salt tolerant-generated lucerne cultivar (*M. media* strain R-350-N); which thrives under 350 mM NaCl; showed a great susceptibility to the pathogen *V. albo-atrum* under normal and even low levels of salinity (Dikilitas 2003). The salt tolerant or disease resistant, or both salt tolerant and disease resistant, plants may lose their potential tolerance or resistance mechanisms to stress agents is important. However, it is critical to point out that under adverse conditions plant pathogens increase their virulence, while alone, they become more aggressive and produce more virulent strains and races in non-polluted environments. Also, disease-susceptible cultivars, whether they are salt tolerant or not, under low saline conditions, show a susceptibility to *V. albo-atrum*. Therefore, if we need to improve crop production under stress conditions, we definitely cannot ignore the weakest side of crop plants with regard to the potential danger of such stress. Although much work has been done in the past regarding breeding crop plants with a tolerance to salinity, we suggest that salt tolerant and disease resistant cultivars need to be included in breeding programs in order to minimize further yield declines in areas where the two factors occur together.

Salinity may have a positive effect on fungal growth, and may increase the development of motile zoosporangia, spores, conidia, and the germ tubes of fungi. In such a case, fungi may grow better and become more virulent. After an adaptation period, halophytic races of fungi may appear and seriously threaten crop production in non-saline soils. Palmero et al. (2010) has reported that the increase of mycelium growth of *Fusarium oxysporum* strains under moderate saline conditions was simulated with KCl or NaCl. These findings indicate that *F. oxysporum* was well adapted to exist in moderate saline conditions. Although this feature is not directly related to the pathogenicity of the isolate, the experimental results indicate that the pathogen has the capacity of growing at low osmotic pressures, which could explain the prevalence of the *Fusarium* genus in soils in semiarid areas.

On the other hand, the negative effect of salinity could prevent pathogen propagules from reaching root infection sites. In this manner, the effectiveness of microorganisms in saline soils may also be reduced. Amir et al. (1996) have reported that salinity induced soil suppressiveness to vascular fusariois. Salinity reduced the sporulation and germination of *Fusarium oxysporum*. Engel and Grey (1991) stated that chloride fertilizers increased the yield of winter wheat and reduced the severity of root diseases caused by *Fusarium culmorum*. Similar findings were made by Kostandi and Soliman (1998), who found that the effect of saline irrigation water containing NaCl or Na2SO4 reduced susceptibility to smut by 22.7% and 10.8% respectively. The wheat and barley seedlings irrigated with saline water in the Arnon and Hoagland (1940) solution, showed an accumulation of Na^+ and Cl^- ions, as well

as metabolites such as glycine betaine, in leaves, decreasing the population and the growth rate of the aphids *Schizaphis graminum* and *Rhopalosiphum padi* feeding on the plants (Araya et al. 1991). Araya et al. (1991) concluded that the accumulation of NaCl in the plants was the real cause for the reduction in the survival and reproduction of aphids. However, *in vitro* studies indicate that the accumulation of higher glycine-betaine was not deleterious to the insects. Therefore, salt tolerant barley accumulating higher amounts of glycine-betaine under salt stress made the barley prone to insect attacks. However, it should be noted that crop plants displaying a resistance to pathogen or insect attack should be very tolerant to high salt stress. The low or high concentrations of salt tolerated by attacking microorganisms or insects will have drastic negative consequences on crop plants.

The negative effect of salt on microorganisms has inspired many workers and led them to use salt as a fungicide. For example, the studies of Mecteau et al. (2002; 2008) and Mill et al. (2004) showed that several salts (0.2 M) completely inhibited mycelial growth and the spore germination of *Fusarium sambucinum*, a casual agent of potato dry rot under *in vitro* conditions. The authors suggested that controlling dry rot may be possible with the application of various salts such as sodium benzoate, sodium metabisulfite, potassium sorbate, and ammonium salts since the post-harvest application of thiabendazole, an effective fungicide, has become less effective to many strains of *F. sambucinum*, which has become resistant to this fungicide. Among the salts tested, aluminium chloride and sodium metabisulfite were able to significantly reduce the development of potato dry rot. Recently, similar reports have been made by El-Mougy and Abdel-Kader (2009) for potato early blight disease caused by *Alternaria solani*. However, for the effective application of salts, there is a need to understand the chemical and biochemical basis of pathogen-plant-salt interactions. However, the fungi may also develop a resistance to salts, which have curative or preventive impacts, as they have developed a resistance to fungicides.

4 Reducing the Impacts of Salinity and Disease Incidence via Bioamelioration for Crop Improvement

Many studies related to understanding the stress metabolism of plants have been designed to keep one stress in mind and to concentrate on its biochemical and molecular metabolism. In nature, however, many abiotic and biotic stress factors are interconnected. Under saline conditions the impacts of pathogens have more detrimental effects than that of each stress factor separately. In order to reduce the impact of both salt stress and pathogens, crop plants resistant to both or more stress factors obtained via molecular or biochemical techniques are thought to be the only solution. However, the reduction of at least salt stress via bio-amelioration, using either halophytes or microorganisms, could partly reduce the impact of salt stress. Additionally, microorganisms that have high bio-control potentials under saline stress could well be the best and the least expensive solution for the combined effects of stress. In most cases, excessive salt concentrations in soils cannot be

reduced with time by routine irrigation and crop management practices. Such soils should be ameliorated, for agricultural purposes, in order to meet increasing food demands. The movement of excess soluble salts via leaching, the surface flushing of salts from soils, and the biological reduction of salts from the soil by halophytes or microorganisms are some commonly used methods (Qadir et al. 2000; Dikilitas and Karakas 2010). In recent years, there has been increased interest in the bio-control of pathogens and the phytoremediation of saline soils, especially in arid and semi-arid regions of the world as a result of increase in pollution and soil salinity via excessive irrigation. To date, the control of plant pathogens or the remediation of saline soils have been considered to be separate issues. However, these two stress factors may be present in one field or even on one single crop plant. Considering these two stress issues together through physiological, biochemical, or molecular approaches is important for crop improvement. But every approach related to crop improvement should be evaluated based on cost and efficacy, as well as practical application.

Various plant and microorganism species exist for bioremediation. In fact, for the bio-control of various important plant pathogens, competitive microorganisms also exist. The important issue here is to test and to increase the efficacy of competitive microorganisms under adverse conditions. The potential bio-control agents or fungi isolated from saline soils are better adapted to such conditions and, therefore, are more efficient in plant growth promotion, and may better control plant pathogens under salt stress (Gamalero et al. 2009; Urja and Meenu 2010). Many microorganisms; especially nitrogen fixers; have been shown to change the properties of salt-affected soils, resulting in the bioremediation of saline soils (Zahran 1991; Al-Abed et al. 2004; Egamberdieva 2011). The major groups of salt tolerant microorganisms reported from many salt-affected soils include free nitrogen fixing bacteria (e.g. *Azotobacter*, *Alcaligens*, *Azospirillum*); cyanobacteria (e.g. *Anabaena*, *Nodularia*, *Nostoc*, etc.); and symbiotic *Rhizobia* species (Zahran 1991). Recently Urja and Meenu (2010) reported that inoculation with fungal isolates of *Aspergillus sp*. (S-11) and *Penicillium sp*. (S12B) on chickpea plants led to an increase in plant growth, total chlorophyll content, and the fresh and dry weight of plants with 2% NaCl salinity as compared to non-inoculated controls. Obuekwe et al. (2005) reported hydrocarbon degradation by desert fungi in crude oil-affected areas, and found that fungi such as *Fusarium lateritium* and *Drechslera sp*. grew in the presence of 10% NaCl, while *Papulaspora sp*. grew in 5% NaCl. However, growth was dependent on the nutritional status of the growth medium. Similarly, Al-Abed et al. (2004) found that the EC level of soil was markedly reduced with aerobic bacteria. The result could be a good sign for the use of bio-control agents which cannot tolerate high salt levels. The bio-control agents such as *Trichoderma harzianum*; obtained at low or moderate salt concentrations; have been applied for the biological control of plant pathogens on saline soils (Regraui and Lahlou 2005). In areas where soil salinity aggravates plant pathogens, research on new strains of *Trichoderma* will help to select more salt tolerant strains. Determining the adaptive conditions of these strains to saline soils and their interactions with other abiotic and biotic factors will be useful. Recently, a new approach for developing improved

bio-control strains of fungi by increasing salinity tolerance phenotypes using mutation techniques has been determined (Mohammad and Haggag 2006). Such an approach could be useful for enhancing salt tolerance, metabolic production and bio-control ability against plant pathogens under non-saline and saline conditions, and may efficiently protect crop plants, as well as increase crop production. Mohamed and Haggag (2006) stated that exposing a wild-type culture of *Trichoderma harzianum* to gamma irradiation induced two stable salt-tolerant mutants (Th50M6 and Th50M11). Under saline conditions, both mutants greatly surpassed their wild type strain in growth rate, sporulation, and biological proficiency against *Fusarium oxysporum*, the causal agent of tomato wilt disease. *Trichoderma* mutants that produce a higher content of chitinases, cellulases, as well as antibiotics, significantly reduce wilt disease incidence and improve the yield and mineral content of tomato plants under both saline and non-saline soil conditions. In some crops such as onions and bell peppers, vesicular-arbuscular mycorizhal (VAM) fungi also seem to increase salt tolerance (Borde et al. 2011). However, to date, the use of mycorrhizae is still controversial.

5 Future Prospects

The development of crop varieties with an increased salt tolerance is crucial for providing a long-term solution to the problem of salinization. However, the problem of improving salt tolerance in plants becomes more complex when plants are exposed to other environmental and biotic factors. The disease-resistant plants should be generated from suspension cells that have been adapted to higher salt concentrations. It is possible to regenerate 50 mM NaCl-tolerant plants from a disease resistant crop plant. However, it is not known whether or not an increase in the level of salt tolerance in the resistant cultivar will decrease the resistance of the plants towards fungi under non-saline and saline conditions. Commercially available drought or salt tolerant plants should be considered in order to maximize crop production and marketable fruit yields. Changes in gene expression have been reported in various crops and microorganisms when they were subjected to various environmental stresses (Katsuhara and Kawasaki, 1996; personal communication with Dr. Tinley Basset, Swansea University, Wales-UK), as such, salt-adapted strains of the fungus should be examined on a molecular level to determine if any changes resulting from the accumulation of NaCl have been made in the gene-sequence. If changes have been made, the genes should be characterized.

A further objective for future research should be to assess the long-term effects of salt accumulation within the soil profile and ground water. In order to achieve the proposed future objectives for long-term improvements in crop production in saline environments, research must be carried out in a manner that promotes close associations between scientists, commercial growers, retailers, and consumers. Plant scientists, including plant physiologists, molecular biologists, geneticists, and plant pathologists should work together to improve agricultural productivity.

Potential bio-control agents for plant diseases should be improved, for controlling diseases under normal and saline conditions, through biochemical or molecular means because efficient resource management and crop improvements for evolving better breeds can help us overcome diseases to some extent. However, such strategies take time and are cost intensive. Therefore, in the short-term, there is a need to develop simple and low cost biological methods for the management of abiotic and biotic stresses (Grover et al. 2011). Using plant growth promoting bacteria or beneficial fungi under salt stress conditions instead of GM plants is advantageous when considering a large number of plants and their cultivars, as well as the multiplicity of genes that would need to be engineered into plants in order to ensure salt tolerance (Gamalero et al. 2009). The technology is easier to access for farmers. In fact, generating more salt tolerant halophytes or microorganisms with high bio-control ability would be much easier and logical than generating genetically improved salt tolerant and disease resistant plants. The increased levels of salinity are frequently associated with the irrigation practices of agricultural land which pose a threat to crop production, especially where water quality is marginal. Under such conditions of poor water quality, plants are not only stressed but may also be more susceptible to various pathogens. One strategy for maintaining yields in such situations or increasing yields in marginal areas is to develop salt tolerant crop strains. In this environment, such strains should also be resistant to pathogens, including halophytic races of pathogens that may have adapted to living in higher salt concentrations. Therefore, understanding the interactions between crop plants and potential pathogens under salinity is an important part of any project aimed at developing strategies for disease control for crops grown in saline soils, which may have a minimal impact in stressed environments.

Now-a-days, in many countries, regenerated or modified plants with resistances to stress are available for increasing crop production even on marginal land. Although these developments are promising, only one factor is considered to be important when plants are developed against stress. However, by acting synergistically, many stress factors can deteriorate plant development. In near future, due to a number of reasons, irrigated lands may be exposed to salinity. Therefore, salt-tolerant plants that tolerate high levels of salt may be economically important (Dikilitas 1997, 2003; Al-Rawahy 2000). However, stress factors other than salinity may still have the potential to damage plants that tolerate the effects of salt. Dikilitas and Smith (1999) have shown that salt tolerant lucerne strains generated for saline conditions, when attacked by microorganisms, become more susceptible than those of non-salt tolerant parental cultivars. Since a living organism is involved, adaptation, or in the long term, the evolution of the pathogen may create larger problems for agricultural lands; or, in the short term, the tolerance level of salt by regenerated plants may be less than that of the plant pathogen. So, the problem is not yet resolved. Since the use of arable land is decreasing, it is important to understand the physiological responses and the defense mechanisms of plants required to be grown under stress conditions. The economically important crop plants should be bred for their resistance to disease and non-pathogenic stress agents, and should be made commercially available.

6 Conclusion

The conclusion that can be drawn from this overview is that when saline water is used to irrigate crop plants in areas where infecting soil microorganisms may be present, soil salinity should be maintained and controlled in order to prevent infecting microorganisms from reaching a degree of virulence that would put even medium resistant cultivars at risk. For crop plants, different stressors may interact, both in their occurrence and impact. So, real progress in crop improvement is likely to depend on a better understanding of these interactions and their consequences for plants and ecosystems. We must keep in mind that there are two contributing factors, resistance of the plant and virulence of the pathogen. Furthermore, studies at the cellular and molecular level are needed in order to understand the overall response of plants. The plant pathogenic fungi develop an enormous array of strategies under salt stress conditions in order to infect their host as discussed above, such as production of extracellular enzymes, conidia, and mycelium growth.

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Chapter 17
Biochemical and Molecular Aspects of Drought Tolerance in Wheat *Triticum* L. Genotypes

Irada Mammad Huseynova, Samira Mahammadrahim Rustamova, Saftar Yusif Suleymanov, and Jalal Alirza Aliyev

Contents

Abstract Wheat (*Triticum aestivum* L.) cultivars contrasting in genetic makeup and differing in drought-resistance were grown in field conditions in a wide area under normal water supply and severe water deficit. One of the genotypes (Azamatli-95) was short-stemmed, with vertically oriented small leaves and drought-tolerant while

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the other genotype (Giymatli-2/17) was short-stemmed, with broad and drooping leaves and drought-sensitive. It was found out that the content of CP I (M_r 115 kD) and apoprotein of P700 with M_r 63 kD, also LHC II polypeptides insignificantly increased in the drought-resistant cv. Azamatli-95 under extreme water supply condition while their content decreased in drought-sensitive cv. Giymatli-2/17. The intensity of synthesis of α- and β-subunits of CF_1 (55 and 53.5 kD) and 33–30.5 kD proteins also decreased in the sensitive genotype. The intensity of short wavelength peaks at 687 and 695 nm sharply increased in the fluorescence spectra (77 K) of chloroplasts from Giymatli-2/17 under water deficiency and there was a stimulation of the ratio of fluorescence band intensity F687/F740. After exposure to drought, cv Giymatli-2/17 showed a larger reduction in the actual PS II photochemical efficiency of chloroplasts than cv. Azamatli-95. Activities of catalase, ascorbate peroxidase, superoxide dismutase and glutathione reductase, as well as photochemical activity of photosystem I and photosystem II were studied in leaves of durum and bread wheat genotypes in ontogenesis. It was found out that dynamics of catalase and ascorbate peroxidase functioning in well-watered plants through ontogenesis practically did not change both among durum and among bread wheat cultivars. Functioning of these enzymes during ontogenesis under water deficit differed. Catalase activity increased in all stressed genotypes: in durum wheat cultivars maximal activity was observed in the milk ripeness and in bread wheat cultivars at the end of flowering. Ascorbate peroxidase activity also increased under water deficit: in tolerant wheat genotypes maximal activity occurred at the end of flowering, and in the sensitive ones at the end of ear formation. The maximum activity of glutathione reductase both as in the control, as well as in drought-subjected plants was observed in the anthesis stage. Superoxide dismutase activity was lower than the control during ontogenesis, except in the last stages. It should be noted that PS I and PS II photochemical activities were also high in genotypes subjected to drought both at the end of ear formation and flowering stages. Drought resistance was checked by RAPD-PCR as a quick and easy method for durum (*Triticum durum* L.) and bread (*Triticum aestivum* L.) wheat genotypes contrasting in tolerance. P6 primer (5′ TCGGCGGTTC 3′) produced 920 bp band mainly in drought tolerant genotypes. It was found that P7 (5′ TCGGCGGTTC 3′) primer produced 750 bp band was not absolutely universal for *Triticum* L. genotypes. In order to identify *DREB1* genes in these genotypes PCR-analysis was carried out using functional markers specific for A, B, and D genomes. It was found that *DREB 1* gene was located on chromosome 3A in all genotypes, excepting one semi-tolerant genotype Tale-38. In comparison with other genotypes, a 717 bp PCR product of *DREB -B1* gene was located on B genome in drought-tolerant Barakatli-95. The results reported here provide an entry point and a reference to future analysis of gene expression during drought. In addition, these results can suggest possible targets for the enhancement of stress tolerance in crops by genetic engineering. The data presented here might be used for monitoring environmental stresses in field-grown plants and selecting stress-resistant varieties for growth under unfavorable conditions.

Keywords Wheat genotypes • Antioxidant enzymes • Thylakoid membrane polypeptides • *DREB* genes • Functional markers • PCR analysis • Chlorophyll • Fluorescence • Photosystems

Abbreviations

CAT	Catalase
Chl	Chlorophyll
DCIP	2,6-dichlorophenolindophenol
DREB	Drought resistance element binding
F	Fluorescence
LHC	Light-harvesting chlorophyll a/b-protein complex
MV	Methyl viologen
PCR	Polymerase Chain Reaction
POD	Peroxidase
PS I	Photosystem I
PS II	Photosystem II
RAPD	Random Amplified Polymorphic DNA
ROS	Reactive oxygen species
RWC	Relative water content
SDS	Sodium dodesyl sulphat
SOD	Superoxiddismutase

1 Introduction

Plants are subjected to a range of abiotic and biotic stresses that affect their growth and development. In particular, it is predicted that water deficit will continue to be a major abiotic factor affecting global crop yields (Sharma and Lavanya 2002). One third of the world's population resides in water-stressed regions, and with elevated CO_2 levels in the atmosphere and climatic changes predicted in the future, drought could become more frequent and severe.

Wheat is a staple food crop for more than 35% of the world population and also one of the widely cultivated crops in Azerbaijan, where drought is the main abiotic stress limiting its grain yield. Wheat is counted the 'big three' cereal crops, with over 600 million tons being harvested annually. Wheat is unrivalled in its range of cultivation, from 67° in Scandinavia and Russia to 45° in Argentina, including elevated regions in the tropics and sub-tropics (Shewry 2009). In view of a projection by Rajaram (2001) more than 50% of the 237 million ha area in the world under wheat cultivation is affected by periodic drought. So study of the wheat anti-drought mechanism is of great importance wheat production and biological breeding for the sake of coping with abiotic and biotic conditions. Much research is involved in this

hot topic, but the pace of progress is not so large because of drought resistance being a multiple-gene-control quantitative character and wheat genome being larger (16,000 Mb) than those of most other crops (Shao et al. 2005). However, despite all the recent technological breakthroughs, the overall contribution of genomics-assisted breeding to the release of drought-resilient wheat cultivars has so far been marginal. The elucidation of genomic regions associated with the expression of traits involved in drought adaptation, the novel genes discovery or the determination of their expression patterns in response to drought stress will provide the basis of effective engineering strategies leading to enhanced wheat germplasm for specific agro-ecological niches. For any molecular assessment to be performed, it is paramount to firstly establish the plant adaptation strategy to overcome drought (Zhao et al. 2008).

To cope with highly variable environmental stresses plants have to set a series of adaptation mechanisms ranging from cellular metabolism to physiological and developmental responses (Zaidi et al. 2010). These may be classified into three groups: drought escape, drought avoidance and drought tolerance (Turne et al. 2001).

In response to stress, plants activate a number of defense mechanisms that function to increase tolerance to adverse conditions. The response to drought stress, which involves a number of biochemical-molecular mechanisms, is complex (Seki et al. 2003, 2007; Zheng et al. 2010). The application of this emerging understanding to the genetic engineering of food crops has already led to examples of improved drought tolerance and increased yield under drought (Hu et al. 2006, 2008; Nakashima et al. 2007).

Extreme circumstances can limit CO_2 fixation and enhance the generation of reactive oxygen species (ROS), such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$). Increased levels of ROS cause damage to various cellular components, such as enzyme inhibition, protein degradation, DNA and RNA damage, and membrane lipid peroxidation, which ultimately culminate in cell death (Ishikawa et al. 2010). The plants have developed different scavenging mechanisms to control the level of ROS. Antioxidant resistance mechanisms may provide a strategy to enhance tolerance, and processes underlying antioxidant responses to stress must be clearly understood (Ashraf 2009).

Production of ROS and other radicals increases dramatically during water deficiency and in plants enhanced levels of reactive oxygen species are generated in various intracellular compartments that may cause oxidative damage or act as signals (Apel and Hirt 2004; Gechev et al. 2006; Laloi et al. 2004). Although ROS in plants are produced under normal growth conditions, their concentration remains low (Polle 2001). Water stress causes stomatal closure, which reduces the CO_2/O_2 ratio in leaves and inhibits photosynthesis. These conditions increase the rate of ROS, particularly in chloroplast and mitochondria, via enhanced leakage of electrons to oxygen (Moussa and Abdel-Aziz 2008). The superoxide radicals and their dismutation product, hydrogen peroxide, can directly attack membrane lipid and inactivate SH containing enzymes (Sairam et al. 2001; Mishra and Singhal 1992). The enhanced production of ROS in chloroplasts and peroxisomes has been correlated with drastic changes in nuclear gene expression (Gadjev et al. 2006), that reveals the transfer of $^1O_2$-derived signals from the plastids to the nucleus. Many of

the $^1O_2$-responsive genes are different from those activated by superoxide (O_2^-) or H_2O_2, suggesting that O_2^-/H_2O_2- and $^1O_2$-dependent signaling occurs via distinct pathways. These pathways could act independently or may interact with each other (Baruah et al. 2009). Plants protect cellular and sub-cellular systems from the cytotoxic effects of active oxygen radicals with anti-oxidative enzymes such as SOD, POD and CAT as well as metabolites like glutathione, ascorbic acid, tocopherols and carotenoids (Moussa and Abdel-Aziz 2008).

The most important phenomenon in drought stress is the deleterious effect that can be seen in the photosynthetic machinery of the plant cells. Photosynthetic electron transfer reactions that take place in thylakoid membranes have been shown to be remarkably resistant to dehydration (Cornic et al. 1992; Kaiser 1987), suggesting that higher plants develop adaptive mechanisms conferring dehydration tolerance to the photosynthetic apparatus. In response to drought, the adaptation shown by many plants could partly be due to changes in membrane composition and phase behavior, which optimizes the fluidity (Navari-Izzo et al. 1993). Indeed, models for thylakoid membrane function require mobility of protein components and redox carriers. Membrane proteins are particularly important for the functionality of the photosynthetic apparatus (Duxbury et al. 1991; Friso et al. 2004). The function of membrane proteins are influenced by the lipids matrix in which they are embedded (Horvath et al. 1989). Within the thylakoids the memrane lipids have an important role to play in stabilizing the structural arrangement and, via the lipid-protein complexes and possibly in maintaining their spatial distribution (Li et al. 1989). Alterations in bulk membrane lipids perturb all functions by inducing changes in the structure and function of thylakoid membrane protein complexes (Caldwell and Whitman 1987). Until now, very few drought-induced chloroplastic proteins have been identified in detail and the molecular basis of drought-stress tolerance is still unknown.

To develop crop plants with enhanced tolerance of drought stress, a basic understanding of gene regulatory network is essential (Shinozaki and Yamaguchi-Shinozaki 2002; Valliyodan and Nguyen 2006; Zhao et al. 2008). Responses to drought stress are extremely different according to the complexity of the genetic background of most crops (Rampino et al. 2006). Hundreds of genes and their products respond to these stresses at transcriptional and translational level. Understanding the functions of these stress-inducible genes helps unravel the possible mechanisms of stress tolerance (Sreenivasulu et al. 2007). Transcriptome analysis using microarray technology (Bohnert et al. 2001; Seki et al. 2001, 2002; Zhu et al. 2001) has revealed that genes induced by stress could be categorized into two groups according to the functions of their products. The first group consists of functional proteins such as membrane proteins that maintain water movement through membranes (water channel proteins and membrane transporters); key enzymes for osmolyte biosynthesis (proline, betaine and sugars, etc.); the detoxification enzymes enabling cellular, physiological or biochemical metabolism to maintain a normal level (glutathione *S*-transferase, hydrolase, catalase, superoxide dismutase and ascorbate peroxidase, etc.); and other proteins for the protection of macromolecules (LEA protein, osmotin, antifreeze proteins, chaperons and mRNA binding protein, etc.). Tolerance to drought or high salinity can be improved by introduction of genes encoding LEA

proteins, proline synthetase or betaine synthetase, etc. The second group comprises regulatory proteins, i.e. transcription factors (bZIP, MYC, MYB and *DREB*, etc.), protein kinases (MAP kinase, CDP kinase, receptor protein kinase, ribosomal-protein kinase and transcription-regulation protein kinase, etc.) and proteinases (phosphoesterases and phospholipase C, etc.) involved in the regulation of signal transduction and gene expression (Agarwal et al. 2006).

Many genes whose expressions are induced by drought have been isolated and characterized at the cellular and molecular level (Wei et al. 2009; Zhu 2002; Shinozaki et al. 2002). Genetic engineering of plants for tolerance to extreme abiotic stresses could be achieved by the regulated expression of stress-induced transcription factors, which in turn would regulate the expression of a large number of relevant downstream genes. Thus, transcription factors are powerful tools for genetic engineering as their overexpression can lead to the up-regulation of a whole array of genes under their control (Agarwal et al. 2006). Dehydration-responsive element binding (*DREB*) proteins constitute a large family of transcription factors that are induced by abiotic stresses. They regulate a large number of functional genes related to drought, high-salinity and low temperature (Wei et al. 2009). The *DREB* transcription factors could be dichotomized as *DREB1* and *DREB2*, which are involved in two separate signal transduction pathways. To date, full-length sequences of *DREB* genes have been cloned from wheat (Shen et al. 2003a), rice (Chen et al. 2003), corn (Qin et al. 2007), Arabidopsis (Liu et al. 1998), and the halophyte *Atriplex hortensis* (Shen et al. 2003b).

Marker-assisted selection (MAS) provides a strategy for accelerating the process of wheat breeding (Ashraf 2010). Through marker-assisted breeding (MAB) it is now possible to examine the usefulness of thousands of genomic regions of a crop germplasm under water limited regimes, which was, in fact, previously not possible. Marker-assisted breeding approach is a prospective alternative to traditional breeding, because of being less time-consuming and labor- and cost-effective.

The Random Amplified Polymorphic DNA-PCR (RAPD-PCR) technology is a powerful tool in quickly identifying markers related to drought tolerance and RAPD technique was found to be quite effective in determining the genetic variation among wheat genotypes and could be utilized as DNA fingerprinting for variety identification and for the establishment of plant breeder rights (Iqbal et al. 2007).

However, conventional markers, such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs), used in common wheat, are usually not developed from the genes themselves, because the cloning of genes in wheat is complicated by its allohexaploid ($2n=6x=42$) nature and large genome size. In contrast, functional markers (FMs) are usually designed from polymorphisms within transcribed regions of functional genes. Such markers are completely correlated with gene function (Andersen and Lübberstedt 2003). Therefore, FMs can dramatically facilitate accurate selection of target genes (Wei et al. 2009).

The aim of this study was to investigate structural and functional characteristics of thylakoid membrane and antioxidant enzymes that ensure resistance of plant organisms to water deficit. For this purpose wheat (*Triticum* L.) genotypes with

contrasting genetic architecture and different genetically stipulated sensitivities to drought were used. Such approach allows to identify not only precise limits of variation of plant reaction to stress, but also to reveal specific features typical for high-resistant genotypes that may be taken into consideration in crop breeding practice for development of drought tolerant varieties. At the same time, RAPD and functional markers were used to confirm drought tolerance in 12 wheat genotypes.

2 Materials and Methods

2.1 Plant Material and Dehydration Conditions

A total of 12 wheat genotypes, three tetraploid (*Triticum durum* L., AABB, $2n=4x=28$) and nine hexaploid cultivars (*Triticum aestivum* L., AABBDD, $2n=6x=42$) including tolerant, moderately-tolerant and non-tolerant to drought were used (Table 17.1). Different sensitivities of these cultivars to drought had been determined during a few years in different regions of Azerbaijan based on grain yield (Aliev 1998, 2001). The plants were provided by the Experimental Station of the Research Institute of Agriculture (Baku, Azerbaijan). All genotypes were grown in field conditions in wide area under normal water supply and dryland conditions from November to May. A group of plants was cultivated under optimum irrigation condition (control), and another set of plants was subjected to water deficit. Dehydration was imposed by withholding water supply. Samples were collected from control and stressed plants at grain filling period up 9:30 a.m. to 10:30 a.m. Roots and shoots were separated, their fresh weights recorded and then these samples were dried in an oven for dry weight measurements. Three different samples for each treatment were taken and analyzed twice.

2.2 Relative Water Content

Leaf relative water content (RWC) was estimated gravimetrically following Tambussi et al. (2005).

2.3 Isolation of Thylakoid Membranes

Leaves were homogenized with a Waring blender at full speed four times for 20 s each in an ice-cold grinding chloroplast isolation medium (1:6 w/v) containing 0.4 M sucrose, 20 mM Tris, 10 mM NaCl, 1 mM EDTA (sodium salt), 5 mM sodium ascorbate, and 0.1% polyethylene glycol, at pH 7.8 following the procedure of Aliyev et al. (1992). The homogenate was filtered through four layers of cheesecloth twice. The filtrate was centrifuged at 200 x*g* for 5 min and then the supernatant

Table 17.1 Wheat genotypes and their drought tolerance status

Genotype name	Reaction to drought
Triticum durum L. (Tetraploid AABB)	
Barakatli-95	Tolerant
Garagylchyg-2	Sensitive
Gyrmyzy bugda	Tolerant
Triticum aestivum L. (Hexaploid AABBDD)	
Azamatli-95	Tolerant
Giymatly-2/17	Semi-tolerant
Gobustan	Tolerant
Gyrmyzy gul	Semi-tolerant
Tale 38	Semi-tolerant
Ruzi 84	Tolerant
12 nd FAWWON No 97 (130/21)	Sensitive
4 tn FEFWSN No 50 (130/32)	Semi-tolerant
Saratovskaya	Tolerant

centrifuged at 1,000 x*g* for 10 min. The chloroplast pellet was suspended for 30 min in a hypotonic buffer consisting of 5 mM Tris-HCl (pH 8.0) and 1 mM $MgCl_2$, and centrifuged at 5,000 x*g* for 20 min. The pelleted thylakoid membranes were resuspended in 5 mM Tris-HCl (pH 8.0). All steps were executed at 4°C.

2.4 Chlorophyll and Protein Determinations

The chlorophyll concentration was determined in 80% acetone extract (Mc-Kinney 1941). Protein determination was performed according to Sedmak and Grossberg (1977). All samples were frozen in liquid nitrogen and stored at −80°C until required.

2.5 Electrophoresis Analysis

For polypeptide analysis, the samples of thylakoid membranes were separated under denaturing conditions at 2–3°C in the presence of 0.1% (w/v) SDS using a 10–25% (w/v) linear gradient polyacrylamide gel (acrylamide: methylenebisacrylamide ratio = 30:0.8) in combination with the Laemmli buffer system (Laemmli 1970) as described previously (Guseynova et al. 2001). To each slot, 20–45 µl of samples (an equal Chl content) were applied. The gels were stained for 30 min with 0.04% (w/v) Coomassie brilliant blue G-250 (France) prepared in 3.5% perchloric acid ($HClO_4$). Immediately after electrophoresis the gels were scanned using an Ultroscan 2202 densitometer (LKB, Sweden) with a 633 nm laser as the light source. A set of standard proteins (kD) consisting of bovine serum albumin (66), glyceraldehyde-3-phosphate dehydrogenase (36), carbonic anhydrase (29), trypsinogen (24), trypsin inhibitor (20.1), and lactalbumin (14.2) (Sigma, USA) were used for the determination of molecular masses of the polypeptides.

2.6 Fluorescence of Chloroplasts

The measurements of fluorescence (F) at 77 K were performed using a Hitachi-850 (Japan) fluorescence spectrophotometer as reported previously (Asadov et al. 1986). Fluorescence emission spectra were corrected for the spectral sensitivity of the spectrophotometer using rhodamine B. Chlorophyll fluorescence was excited by dark blue light with wavelength of 440 nm. The samples on quartz glass fiber were quickly frozen at 77 K by dipping the glass fiber into liquid nitrogen.

2.7 Assay of Electron Transport Activities

Electron transport activities of chloroplasts isolated from control and drought-stressed plants were determined polarographically as O_2 evolution or uptake at 20°C using a water-jacketed Clark type oxygen electrode chamber under illumination with saturating white actinic light (850 μE m^{-2} s^{-1}), according to Aliyev et al. (1992). Chloroplast concentrations equivalent to 100 μg Chl were used for all measurements. Artificial donors and acceptors were added immediately before or during illumination. The following electron transport activities were assayed in μmol $O_2 \cdot mg^{-1}$ Chl·h^{-1}. PS II activity ($H_2O \rightarrow K_3Fe(CN)_6$) was measured in a medium containing 330 mM sorbitol, 40 mM Hepes-NaOH, pH 7.6, 10 mM NaCl, and 5 mM $MgCl_2$ using 0.5 mM $K_3Fe(CN)_6$ as terminal electron acceptor. PS I activity was assayed in the reaction mixture contained in 2 ml, 80 mM sucrose, 30 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM sodium ascorbate, and 2 μM 3-(3-4-dichlorophenyl)-1,1-dimethylurea (in order to block electron transport from PS II), using 0.3 mM 2,6-dichlorophenolindophenol as electron donor and 50 μM methylviolgen as electron acceptor.

Measurements of photoinduced changes of fluorescence yield from F_o level to F_{max} were carried out at room temperature using laboratory-built set-up as described earlier (Klimov et al. 1982). Potential to the formula:

$$\Phi_p = F_v/F_m = (F_m - F_o)/F_m$$

2.8 Enzyme Extraction and Activity Determination

Enzyme extract was prepared by homogenizing leaf material (1 g f. wt.) with a pestle in an ice-cold mortar with 0.05 M Na_2HPO_4/NaH_2PO_4 (pH 7.0) buffer. The homogenates were filtered through four layers of cheesecloth and then centrifuged at 4°C. The supernatant was collected and used for the assays of enzymatic activities.

2.9 Catalase (CAT)

The activity of catalase was determined as a decrease in absorbance at 240 nm for 1 min following the decomposition of H_2O_2 as described by Kumar and Knowles (Kumar and Knowles 1993). The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2 and the reaction was initiated by adding enzyme extract.

2.10 Ascorbat Peroxidase (APO)

The activity of ascorbat peroxidase was assayed according to Nakano and Asada (Nakano and Asada 1981). The assay mixture consisted of 0.05 mM ASA, 0.1 mM H_2O_2, 0.1 mM EDTA, 50 mM sodium phosphate buffer (pH 7.6), and 0.3 mL enzyme extract. The activity was measured as a decrease in absorbance at 290 nm for 30 s.

2.11 Glutathione Reductase (GR)

Glutathione reductase activity was determined at 340 nm for 10 min in the reaction mixture containing 100 mM potassium phosphate buffer (pH 7,8), 1 mM EDTA, 0,2 mM NADPH and 0.5 mM GSSG (Yannarelli and Fernandez-Alvarez 2007).

2.12 Superoxide Dismutase (SOD)

Superoxide dismutase activity was estimated by using SOD Assay Kit-WST (Sigma-Aldrich, USA). The absorbance was recorded at 450 nm and one enzyme unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction. Protein content was determined according to Sedmak (Sedmak and Grossberg 1977) using bovine serum albumin as a standard.

2.13 DNA Extraction

DNA was extracted from leaves using CTAB method (Murray and Thompson 1980) with some modifications. In the growth room, 5–7 cm long pieces of fresh leaf material was cut from the plants and the leaf tissues were ground in a preheated 2×CTAB extraction buffer (100 mM Tris, pH 8, 1.4 M sodium chloride, and 20 mM EDTA, pH 8.0). Liquid nitrogen ground samples were also processed with CTAB buffer. The samples were incubated for 60 min in 60°C water bath with occasional vigorous shaking. The samples were mixed gently after adding 400 µl of chloroform

and placed on an orbital shaker for 20 min at room temperature. After centrifugation at 5,000 rpm, an equal volume of cold absolute isopropanol was added to the supernatant. The solution was well mixed and incubated for 60 min at 20°C. The sample was centrifuged for 5 min at 5,000 rpm to pellet the DNA, which was followed by washing with 70% alcohol and then dried at 56°C for 5 min. DNA was resuspended by adding 300 µl TE buffer (10 mM Tris, 1 mM EDTA at pH 8.0).

2.14 DNA Quantification

After diluting, the DNA was quantified by taking the optical density (OD) at λ = 260 nm with a spectrophotometer ULTROSPEC 3300 PRO ("AMERSHAM", USA). The purity of genomic DNA was determined by the A260/A280 absorbance ratio. The quality was also examined by running the extracted DNA samples on 0.8% agarose gel stained with 10 mg/ml ethidium bromide in 1 × TBE (Tris base, Boric acid, EDTA) buffer. The gel was visualized and photographed under UV light.

2.15 Polymerase Chain Reaction Conditions for RAPD

PCR was carried out essentially as described by Williams (Williams et al. 1990). Two 10-mer oligonucleotide primers (Eurogentec S.A., Belgique) were used for DNA amplification (Table 17.2). Amplifications were performed in "Applied Biosystems 2720 Thermal Cycler" as follows: first 4 min at 94°C followed by 10 cycles of: 1 min at 94°C, 1 min at 36°C and 1 min at 72°C. After that, for next 35 cycles, 0.2°C was added to annealing temperature. After the final cycle, samples were incubated at 72°C for 15 min and then held at 4°C prior to analysis.

2.16 Polymerase Chain Reaction Conditions for Functional Markers

PCR was carried out essentially, as described by Wei et al. (2009). Five pairs of genome-specific primers designed for the wheat *DREB 1* genes using the Primer Premier 5.0 software (http://www.premierbiosoft.com) were used for DNA amplification (Table 17.3). Genome-specific PCR was performed in a total volume of 20 µl containing 80 ng of genomic DNA, 1 × PCR reaction buffer, 0.25 µM of each primer, 0.45 mM of each deoxyribonucleotide, 4.0 mM $MgCl_2$ and 1.6 U of Taq DNA polymerase (Sigma, USA).

The PCR was carried out using "Applied Biosystems 2720 Thermal Cycler" as follows: initial denaturation at 94°C for 3 min; 34 cycles of 94°C for 1 min, an annealing step at variable annealing temperatures depending on the primer pairs for

Table 17.2 Primer nucleotide sequence used to amplify DNA

Primer designation	Sequence 5′→3′
P1	TCGGCGGTTC
P2	CTGCATCGTG

Table 17.3 Genome-specific primers of the wheat *DREB 1* genes used for PCR reactions

Primers	Sequences (5′→3′)	Chromosome location	Expected size (bp)	Ann. temp. (°C)
P18F	CCCAACCCAAGTGATAATAATCT	3B	717	50
P18R	TTGTGCTCCTCATGGGTACTT			
P20F	TCGTCCCTCTTCTCGCTCCAT	3D	1,193	63
P20R	GCGGTTGCCCCATTAGACATAG			
P21F	CGGAACCACTCCCTCCATCTC	3A	1,113	63
P21R	CGGTTGCCCCATTAGACGTAA			
P22F	CTGGCACCTCCATTGCCGCT	3D	596	63
P25F	CTGGCACCTCCATTGCTGCC	3A	596	57
PRa[a]	AGTACATGAACTCAACGCACAGGACAAC			

[a]PR is a public primer matched with P22F and P25F, respectively

1 min, 72°C for 1.5 min; and a final extension at 72°C for 10 min and then held at 4°C prior to analysis. The PCR products were electrophoresed on 2.5% agarose gels, stained with ethidium bromide and visualized under UV light by "Gel Documentation System UVITEK' .

3 Results and Discussion

The investigated genotypes responded to water deficit through various changes in physiological and biochemical processes. Significant differences in relative water content (RWC) were observed between normally irrigated plants and those subjected to water stress (Fig. 17.1). Genotype Giymatli-2/17 grown in normal water supply condition showed the higher RWC in the leaves. Drought-stress conditions induced a slightly larger decrease in RWC in the sensitive cv. Giymatli-2/17 than in the tolerant cv. Azamatli-95; dehydration decreased the RWC by 14% in comparison with fully irrigated plants. The rate of water loss during drought was low in Azamatli-95. The RWC lowered from 83.9% to 72.1% following stress. Exposure to drought caused a reduction in dry weight accumulation in Giymatli-2/17 plants, whereas it had smaller, insignificant effects in cv. Azamatli-95, even though both cultivars showed a certain drop in RWC.

A reduction in the total chlorophyll content and Chl a/b ratio occurred during drought stress (Fig. 17.2). This pattern of change was not evident in tolerant genotype Azamatli-95, in which these parameters did not change statistically, whereas

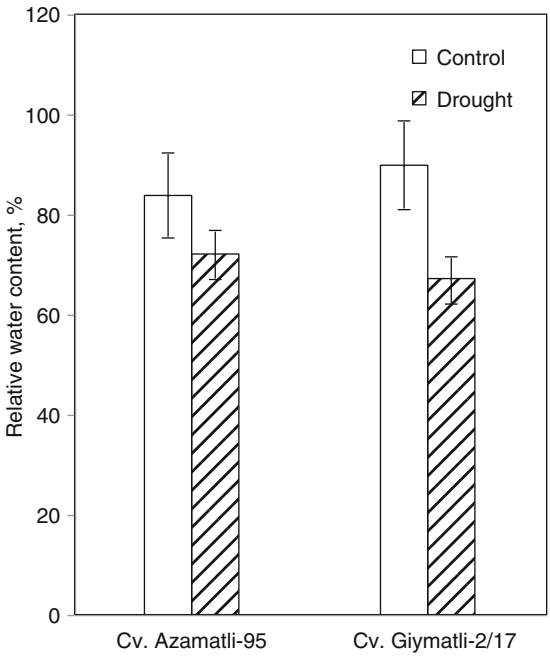

Fig. 17.1 Effect of water stress on relative water content of leaves in normally irrigated and drought stressed plants of *Triticum aestivum* wheat. Vertical bars represent SE of mean, $n=6$

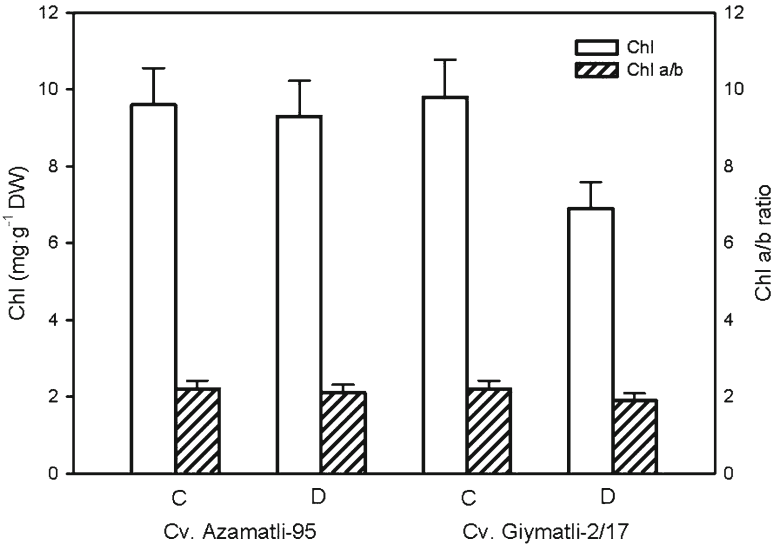

Fig. 17.2 Chlorophyll content of leaves of the wheat genotypes Azamatli-95 and Giymatli-2/17 during water deficit; C-control; D drought stress. Results are the means of three repetitions of two independent experiments SE ± ($n=6$)

the difference was significant in sensitive cv. Giymatli-2/17. A drought-induced decrease in pigment contents was previously reported in several plant species, including pea (Moran et al. 1994), durum wheat (Loggini et al. 1999) and *Boea hydroscopica* (Navari-Izzo et al. 2000). The drought-sensitive cv. Giymatli-2/17 showed a slight increase in the pool size of xantophyll-cycle components, but such effect was not shown in the tolerant cv. Azamatli-95, which may be explained by its higher rate of electron transport compared with cv. Giymatli-2/17 (Guseynova et al. 2006).

Total protein synthesis was slightly reduced by water deficit in these experiments. The decrease in thylakoid proteins observed during dehydration may be associated with degradation of lipoprotein thylakoid membrane structure. In addition, the photosynthetic apparatus may show acclimation responses such as changes in the relative proportion of stacked and unstacked membrane domains (Anderson and Aro 1994). At the ultrastructural level the thylakoid system of hydrated chloroplasts was organized in several well-defined and regularly distributed grana connected by parallel stroma lamellae. The increased thylakoid stacking in dried chloroplasts could be a consequence of membrane and/or environmental changes leading to a weakening of the repulsive force between the membrane surfaces. Another influential factor might be the rise due to water loss in the stroma ionic charge screening the repulsive force between thylakoids (Barber 1982).

The protein profiles of thylakoid membranes in nonstressed and water-stressed plants were analysed. Figure 17.3 shows density patterns from Coomassie blue staining SDS-PAGE analysis of membrane proteins of two wheat genotypes with differential tolerance to drought. As shown in Fig. 17.3, thylakoid membranes isolated from the wheat genotypes grown under normal water supply appeared to have about 26 polypeptides with M_r from 115 to 11 kD. It was found that Giymatli-2/17 genotype with broad and lodging leaves and drought-sensitive is characterized by low content of chlorophyll a-protein of photosystem (PS) I core (CP I) and β-subunit of CF_1 ATP-synthase complex, the high content of proteins in the 33–30.5 kD region and the relative high amount of polypeptides of light-harvesting complex (LHC) under normal irrigation in comparison with drought-tolerant genotype Azamatli-2/17, having vertically oriented small leaves. Drought stress caused significant changes in the content and composition of thylakoid membrane proteins. The content of CP I (115 kD) and apoprotein of P700 (63 kD) were maintained at relatively high levels in tolerant genotype Azamatli-95, but were slightly little affected by drought in more sensitive cv. Giymatli-2/17. It is interesting to note that the intensity of 60 kD polypeptide strongly increased (about 2-fold higher) in the drought-resistant genotype Azamatli-95. However, a detection of this polypeptide was not available in the experiments with seedlings of wheat grown in a growth chamber under controlled environment conditions (Guseynova et al. 2006). On the basis of the results of the present study and literature data, it is possible to suggest that this protein is related to dehydrins (PCA 60). Seasonal expression of dehydrins has been noted in several species (Wisnewski et al. 1999). The dehydrin family of proteins is induced by environmental stresses that result in cellular dehydration (Close 1997). All these protein groups are characterized with high hygrophilous

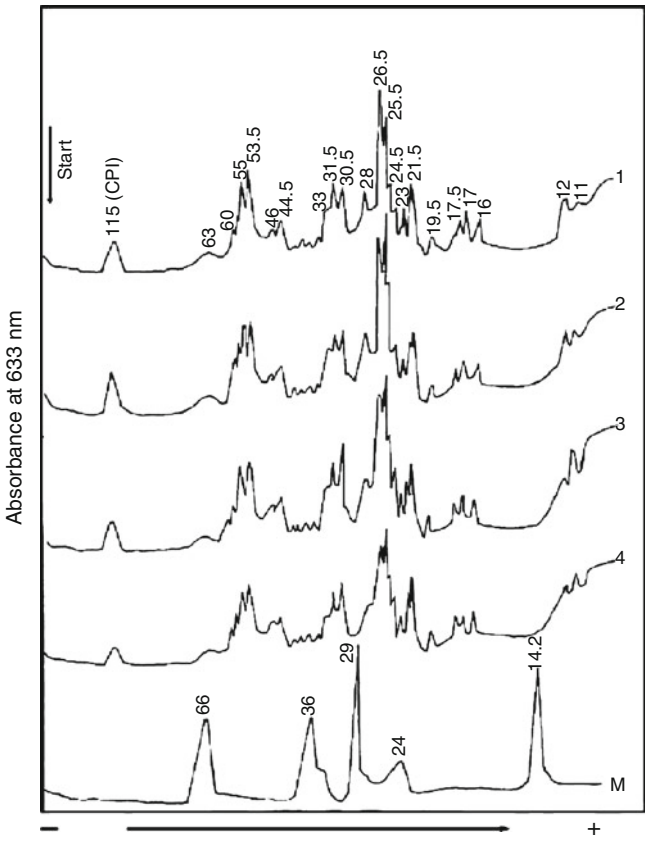

Fig. 17.3 Density patterns from Coomassie blue staining SDS-PAGE (10–25% gel) analysis of thylakoid membrane proteins from wheat plants grown in field conditions under normal water supply (Azamatli-95) (*1*) and Giymatli-2/17 (*3*) and drought stress (Azamatli-95) (*2*) and Giymatli-2/17 (*4*). M, standard proteins (kD): bovine serum albumin (66), glyceraldehydes-3-phosthate dehydrogenase (36), carbonic anhydrase (29), trypsinogen (24), and α-lactalbumin (14.2). Samples on an equal Chl content (25 μg) were placed on the gel. The samples were applied to each slot corresponding to 50 μg of protein

protein molecules. During dehydration of cells they prevent water loss on account of high hydrophilic capacity and stabilize cell proteins. PCA 60 was freely distributed in the cytosol, plastid, and nucleus. Although the functional role of dehydrins remains speculative, the data support the hypothesis that it plays a role in preventing denaturation of proteins exposed to dehydrative stresses in a manner similar to chaperones.

The synthesis of α- and β-subunits of CF_1 ATP synthase complex (55 and 53.5 kD, respectively) tended to increase slightly in stressed plants of Azamatli-95 and decrease in cv. Giymatli-2/17. The low content of β-subunits of CF_1 ATP synthase complex has been also shown in pea plants subjected to water deficit at high light

exposure (Giardi et al. 1995; Guseynova et al. 2006). Steady-state levels of the core antenna of PS II (CP 47 and CP 43) serving as the connecting antenna between the main light harvesting complex LHC II and reaction center of PS II remained more or less unchanged in both genotypes. These results agree with the data reported earlier (Masojidek et al. 1991; Giardi et al. 1995; Guseynova et al. 2006).

The most striking change was the appearance of protein with molecular mass of 40.5 kD in tolerant genotype Azamatli-95. It is absent in leaves from nonstressed plants, but at a lower level it was detected in only tolerant genotypes, subjected to water deficit. According to the current literature, C 40.4 protein shares high sequence homology with CDSP (termed CDSP for chloroplastic drought-induced stress protein), the previously described accumulation of a 34 kD thylakoid protein in tomato in response to drought (Pruvot et al. 1996). Substantial increases in CDSP 34 transcript and protein abundance were also observed in potato plants, subjected to high illumination (Gillet et al. 1998). The accumulation of two chloroplastic nuclear-encoded proteins in water-stressed *Solanum tuberosum* plants were reported (Quartacci et al. 1995). A stromal protein of 32 kD related to thioredoxins, was suggested to maintain the redox state of chloroplastic proteins upon drought stress (Rey et al. 1998). Another protein of 34 kD, named CDSP 34 protein is proposed to participate in structural stabilization of thylakoids upon environmental constraints and prevent damage resulting from osmotic or oxidative stress (Pruvot et al. 1996). It is supposed that C40.4 protein is closely bound to LHC II and has functional role by modeling photosynthetic effectiveness and light dissipation of excess absorbed light energy inside antenna complex (Monte et al. 1999).

At the same time, in the sensitive genotype Giymatli-2/17 there was a considerable decrease in the amount of proteins in 33–30.5 kD region. The decrease in the content of 31.5 kD protein in thylakoid membrane from water-stressed plants (especially in cv. Giymatli-2/17) seems to be due, in part to its enhanced degradation rate (Guseynova et al. 2006; Sippola et al. 1998). High rate of D_1-protein turnover provides stability of thylakoid membranes and their electron transport chain to damaging action of free radical forms under stress conditions. On the other hand, thylakoid membranes from stressed plants showed an increased level of LHC polypeptides (28–24.5 kD) in tolerant cv. Azamatli-95 compared to Giymatli-2/17, at which the levels of these units decreased.

A slight increase in 21.5 kD polypeptide (according to literature it is related to WSCP-water-soluble chlorophyll proteins) was also observed in both genotypes under drought. Such effect was found in our previous studies with durum wheat seedlings under water stress (Guseynova et al. 2006). It is supposed that this protein might involve in the decrease of protease activity in leaf senescence.

Drought also caused a decrease in the synthesis of low molecular weight polypeptides of 17.5–12 kD in both genotypes under extreme conditions of water supply. The intensity of 11 kD polypeptide slightly increased in cv. Azamatli, but significantly decreased in cv. Giymatli-2/17. Correlation between tolerance and overexpression of some proteins including 60, 40.5, and 28–24.5 kD assumes that changes in expression of these polypeptide genes can be functionally involved in the ability of plants to survive and grow under water deficiency.

Table 17.4 Photosynthetic membrane proteins from wheat chloroplasts subjected to changes under drought stress

Samples	Molecular mass of proteins, kD[a]					
Azamatli-95 (control)	CP I, 115 (PS I core)	60	55 and 53.5 α and β sub. CF_1	33–30.5	28–24.5 (proteins of LHC)	21.5
Azamatli-95 (drought)	0	+	+	0	+	+
Giymatli-2/17 (control)	CP I, 115 (PS I core)	60	55 and 53.5 α and β- sub. CF_1	33–30.5	28–24.5 (proteins of LHC)	21.5
Giymatli-2/17 (drought)	–	0	–	–	–	+

[a](+) protein content is increasing, (−) content is decreasing, 0 not changes

According to the current literature, there is a cycle of PS II reparation during which the most damaged 32 kD protein (D_1) of reaction center of PS II is replaced (Melis 1998). Selective proteolysis is involved, inactive form of D_1-protein is removed, and newly synthesized D_1-polypeptide is integrated into the PS II holo-complex (Sippola et al. 1998; Chaloub et al. 2003). High rate of D_1-protein turnover provides stability of thylakoid membranes and their electron-transport chain to damaging action of free radicals formed under stress conditions (Guseynova et al. 2006). Thus, the literature and our results suggest that the biochemical response at the level of D_1-turnover and intensive synthesis of polypeptides 60, 40.5 and 28–24.5 kD could act as a general adaptation signal for the plant in response to water stress. Table 17.4 presents the data of membrane proteins in which quantitative changes are significant under drought stress.

In parallel the fluorescence emission spectra (77 K) of chloroplasts from normally irrigated and drought-stressed plants were also measured. As shown in Fig. 17.4, chloroplasts from drought-sensitive genotype Giymatli-2/17 has more intensive fluorescence at 740 nm from PS I under normal water supply. The F687/F740 ratio of control (non-drought stressed) chloroplasts of genotype Azamatli-95 was close to 0.38 and for genotype Giymatli-2/17-0.35. The shift of the main peak from 742 to 740 (in Azamatli-95) and from 740 to 738 nm (in Giymatli-2/17) was observed in both genotypes grown under water deficit. According to the data on the contents of pigments in leaves with normal irrigation and in the plants subjected to water deficit, a short wavelength shift of the main maximum in the fluorescence spectra was coupled with a decrease in the amount of chlorophyll in PS I antenna (see Fig. 17.2). The fluorescence intensity at 740 also slightly increased. The short wavelength peaks at 687 and 695 nm (fluorescence from the PS II core complex CP 47 and CP 43) remained and their fluorescence intensities started to increase sharply under water deficit. It is especially observed in drought-sensitive genotype Giymatli-2/17. At the same time, in chloroplasts from stressed plants, the F687/F740 ratio increased compared with the control plants; the lowest value was that of the Azamatli-95 (F687/F740 = 0.45) and the highest of Giymatli-2/17 (F687/F740 = 0.77), suggesting again that the most detrimental influence of drought stress occurred in Giymatli-2/17. The results suggest that antenna system of the photosynthetic

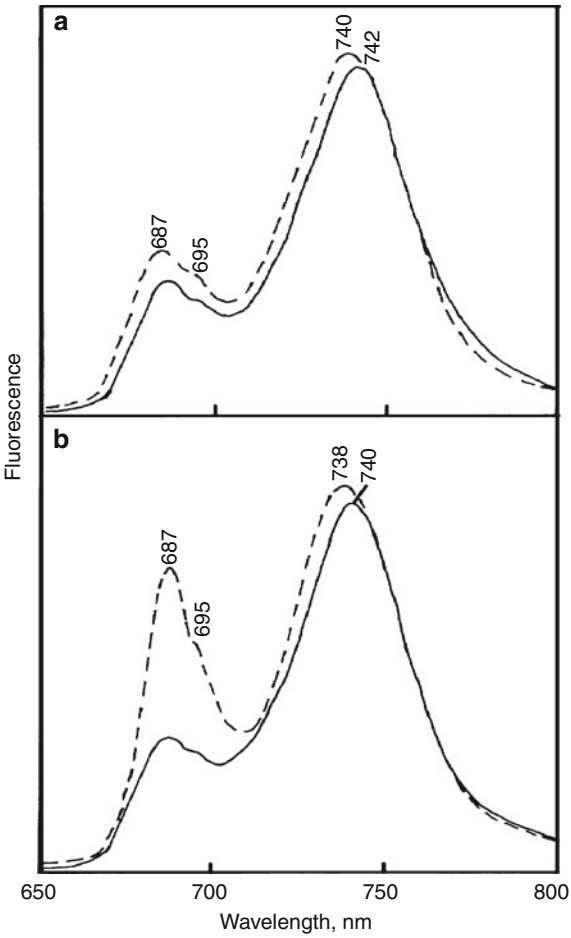

Fig. 17.4 Fluorescence emission spectra at 77 K of chloroplasts from drought-tolerant Azamatli-95 (**a**) and drought-sensitive Giymatli-2/17 (**b**) genotypes grown under normal water supply (*solid curves*) or drought conditions (*dashed curves*)

apparatus in the drought-tolerant genotype Azamatli-95 was rapidly reorganized, and plants began to adapt to environmental stress. More frequently changes in F687/740 ratio may be explained by redistribution of excitation light energy between PS II and PS I. This rise in short wave fluorescence intensity may have been due to the lower content of RC′s of PS II synthesized under water deficit.

Significant differences were found in functional activity of photosynthetic apparatus at the level of photochemical reactions of chloroplasts in comparative studies of genotypes distinguishing by the genetic architecture and drought resistance. In our experiments, the highest PS II activity (oxygen evolution rate) of irrigated plants was found in drought-sensitive genotype Giymatli-2/17 with broad and drooping leaves (Table 17.5). Drought stress caused a significant change in the photochemical activity of chloroplasts in both genotypes. The electron transport activities of all stressed plants were lower than those in the control plants. However, the activity of PS II was significantly affected by dehydration in cv. Giymatli-2/17 only 41% of

Table 17.5 The Photosystem II and Photosystem I activity in chloroplasts from wheat genotypes subjected to drought stress (μmol $O_2 \cdot mg^{-1}$ chlorophyll$\cdot h^{-1}$)

Genotypes	Photosystem II $H_2O \rightarrow K_3Fe(CN)_6$	In %	Photosystem I DCIP$\cdot$H $\rightarrow$ MV	In %
Azamatli-95 (control)	45 ± 4	100	250 ± 12	100
Azamatli-95 (drought)	35 ± 3	78	225 ± 9	90
Giymatli-2/17 (control)	85 ± 7	100	190 ± 8	100
Giymatli-2/17 (drought)	35 ± 4	41	150 ± 4	79

control values. In drought-stressed cv. Azamatli-95 leaves the photochemical activity of PS II was about 78% of the control value. The case of PS II inactivation in both genotypes may be due to the suppression of synthesis of 32 kD protein (D_1-protein of RC of PS II), which is carrier of photochemical active form of Chl a P680, or breach of electron transfer from pheophytin intermediate electron carrier on quinone acceptor (Q_A) in non-cyclic transport of electrons. It is likely that, desiccation inhibited the energy transfer from the Chl molecules anchor to PS II core complexes. PS I activity (O_2 uptake rate), however, was affected much less under drought stress. It can be caused by a higher ability of PS I to adapt to dehydration.

Concerning the other drought stress actions, several authors reported that PS II photochemistry is predisposed by drought stress to photoinhibitory damage (Björkman and Powles 1984; Peltier et al. 1995). In contradiction, Genty et al. (1987) concluded that PS I-mediated electron transport was inhibited by drought, whereas PS II electron transport remained the unaffected. During rehydration, PS II activities recovered slowly, but PS I complexes recovered their functional forms very quickly (within 1 min) (Hirai et al. 2004).

It is known that fluorescence yield is minimal (F_o), when primary electron acceptor of PSII, i.e. plastoquinone (Q_A), is oxidized. Reduction of Q_A results in rise of chlorophyll fluorescence, approximately 3–5 times, up to F_m level. Rise in chlorophyll fluorescence yield from initial (F_o) to maximal (F_m) level, i.e. appearance of variable fluorescence (F_v, where $F_v = F_m - F_o$), reflects process of accumulation of reaction centers (RC) of PSII in "closed" state with reduced primary quinone acceptor (Q_A). Values of fluorescent parameters that characterize the functional state of photosynthetic apparatus of winter wheat plants grown under different conditions of water regime are shown in Table 17.6. Potential quantum yield of photochemical reactions of PSII (F_v/F_m ratio) in chloroplasts from control (non-drought stressed) plants was 0.74 for Azamatli-95 and 0.81 for Giymatli-2/17, that is typical for normally grown plants. As it is evident from Table 17.6, the state of PSII in dehydration process was significantly changed. Potential yield of photochemical reactions of PSII underwent appreciable changes in comparison with control plants; the highest value of F_v/F_m was in Azamatli-95 ($F_v/F_m = 0.71$) and the lowest in Giymatli-2/17 ($F_v/F_m = 0.69$). It is interesting to note, that chloroplasts from non-drought-tolerant genotype Giymatli-2/17 had higher value of photochemical efficiency of PSII under regular irrigation conditions of growth. However, low ratio of F_v/F_m again confirms that strong effect of drought appeared in genotype Giymatli-2/17 (genotype

Table 17.6 Change of parameters of chlorophyll fluorescence in chloroplasts isolated from wheat leaves after drought. Fluorescence components: F_o – constant fluorescence; F_v – variable fluorescence; F_m – maximal fluorescence[a]

Variant	Control	Drought	% from control
Azamatli-95			
F_o	29.0±1.2	30.0±2.8	103
F_v	85.0±6.1	74.5±5.4	87
F_v/F_m	0.74	0.71	96
Giymatli-2/17			
F_o	27.0±1.1	28.5±2.9	106
F_v	118.0±6.5	63.0±4.3	54
F_v/F_m	0.81	0.69	85

[a]Average arithmetic and standard mistakes from three independent experiments, each of which was carried out in double biological frequency are shown in the table

Giymatli-2/17 is strongly affected by drought). Decreasing of a photochemical efficiency (F_v/F_m) under severe drought can be considered as the main reason of damage of photosynthetic reaction centers.

Both Q_B-reducing and Q_B-non-reducing complexes of PSII make a contribution in variable fluorescence (F_v). Charge separation is realized in Q_B-non-reducing complexes of PSII, but electrons are not transported to plastoquinone pool. Q_B-reducing complexes of PSII in active state are able to realize electron transport between Q_A and Q_B. They lose this ability when D_1-protein is damaged and turn to Q_B-non-reducing complexes (Pshibytko et al. 2003). In optimal conditions due to reactions of reparation cycle the constant ratio between these types of complexes of PSII is supported. Probably, dehydration induces disruption of reactions at the acceptor side of PSII, expressed in increasing a number of Q_B-non-reducing centers.

Under water deficit, linear electron transport is suppressed by the accumulation of plastoquinones caused from difficulty of lateral diffusion of plastoquinones because of increased viscosity of lipid bilayer (Hirai et al. 2004), that could be caused by increasing of reduced level of plastoquinone pool, damage of Q_B-binding site with D_1-protein and deterioration of conditions of damaged D_1-protein reparation (Melis 1998).

The results presented here show that functioning dynamics of CAT and APX in well-watered plants through ontogenesis practically did not change both among durum and among bread wheat cultivars. In Barakatli-95 and Garagylchyg-2 both enzymes exhibited a maximal activity in the end of flowering, in Azamatli and Giymatli-2/17 – in the end of ear formation (Fig. 17.5). Functioning of these enzymes during ontogenesis under water deficit differed: CAT activity increased in all stressed genotypes as compared with control: in durum wheat cultivars maximal activity was observed in the milk ripeness and in bread wheat cultivars at the end of flowering. In drought-tolerant genotypes Barakatli-95 and Azamatli-95 CAT activity increased more substantially as compared to that in the sensitive ones. The increase in CAT activity in plants has been reported in other studies under water stress (Mukherjee and Choudhuri 1983; Quartacci and Navari-Izzo 1992). It is

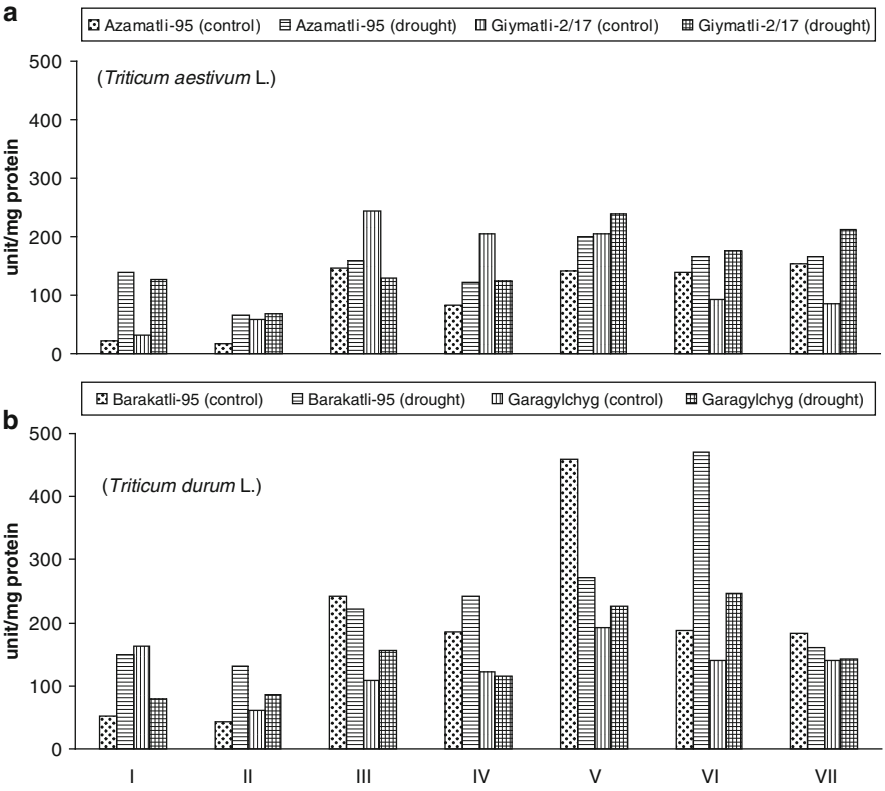

Fig. 17.5 Effect of water stress at different ontogenesis stages on CAT activity (unit/mg protein). (*I* – stalk emergence, *II* – beginning of earing, *III* – end of earing, *IV* – flowering, *V* – end of flowering, *VI* – milky ripeness, *VII* – wax ripeness)

known that catalase reacts with H_2O_2 directly to form water and oxygen (Smirnoff 1993). The decrease in CAT activity in the end of ontogenesis could indicate its inactivation by the accumulated hydrogen peroxide by water shortage and could be explained partly by photoinactivation of the enzyme. When plants are not exposed to water stress, resynthesis of CAT compensates for the loss of total activity caused by irradiance. Inhibition of protein synthesis induced by water stress (Badiani et al. 1990) conceivably could impair resynthesis and partly account for the marked decrease in CAT activity in plants subjected to water stress in the light.

APX activity also increased under water deficit: in tolerant wheat genotypes maximal activity occurred at the end of flowering, and in the sensitive ones in the end of ear formation (Fig. 17.6). An increase in POD activity was also observed by different authors during drought and salt stress (Badiani et al. 1990; Siegel 1993). It indicates the formation of large amounts of H_2O_2 during water stress. Increased activity can explain with some assumptions: elevated H_2O_2 concentrations could release POD from membrane structures, with which it is normally associated. POD

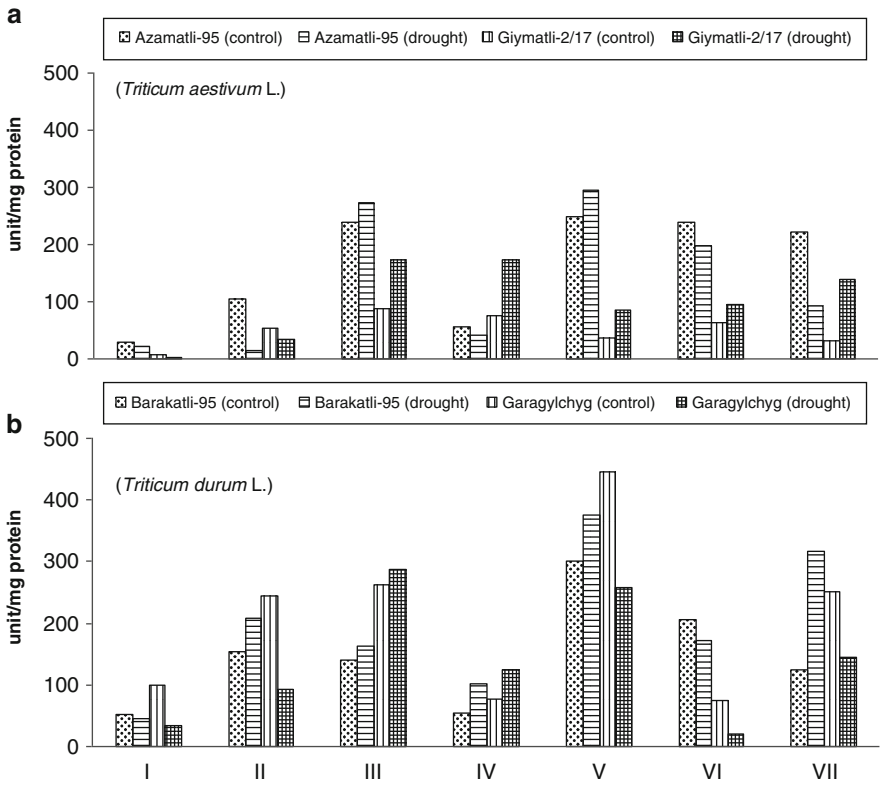

Fig. 17.6 Effect of water stress at different ontogenesis stages on APX activity (unit/mg protein). (*I* – stalk emergence, *II* – beginning of earing, *III* – end of earing, *IV* – flowering, *V* – end of flowering, *VI* – milky ripeness, *VII* – wax ripeness)

could be synthesized *de novo* at least in some cases. Water stress could increase the accumulation of POD substrates, such as glutathione, ascorbate, and phenolic compounds, which, in turn, are scavengers of activated oxygen species (Winston 1990). Also it is known, that H_2O_2 participates in signal transduction at development of oxidizing stress, inducing genes of cytosolic POD. The maximum activity of GR both in the control, as well as in drought-subjected plants was observed at the anthesis stage. GR activity in drought-tolerant durum wheat Barakatli-95 and resistant bread wheat Azamatli-95 was higher than the control in all stages of ontogenesis (Fig. 17.7).

SOD functioning dynamics through ontogenesis differed from CAT and APX. Interestingly, SOD activity was lower than the control during ontogenesis, except at the last stages. In drought-tolerant Barakatli-95 and Azamatli-95 it increased against the control only at wax ripeness stage, when the effect of drought was the highest (Fig. 17.8). Many authors specify the key role SOD in antioxidative protection (Raychaudhuri 2000; Alscher et al. 2002). However, the results reported here on

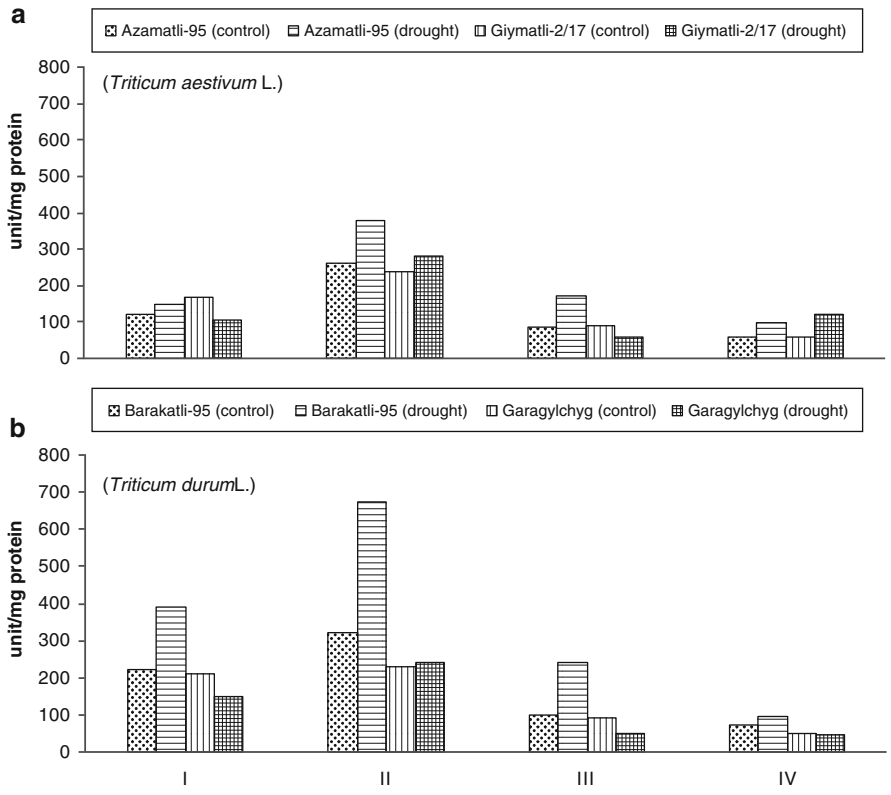

Fig. 17.7 Effect of water stress at different ontogenesis stages on GR activity (unit/mg protein). (*I* – earing, *II* – flowering, *III* – milky ripeness, *IV* – wax ripeness)

SOD activity differed partly from the literature. Nevertheless, it is known that plant cells contain a little isoform of SOD which probably unequally reacts to water deficiency. The study of these SOD isoforms during water stress induction revealed differential regulation of their activities: MnSOD and FeSOD activities increased rapidly while Cu/ZnSOD activities decreased in cowpea plants (Brou et al. 2007). Also direct correlation between APX activity and carotenoid content was observed through ontogenesis. It should be noted that in plants subjected to drought PS II and PS I activities were also high both at the end of ear formation and flowering stages (Tables 17.7 and 17.8).

RAPD-PCR analysis was carried out using seedlings of 12 wheat genotypes *Triticum* L. with different levels of drought tolerance (Table 17.1). For this purpose, P6 and P7 RAPD markers associated with drought tolerance were used (Pakniyat and Tavakol 2007). According to the literature data in tolerant genotypes these primers should produce appropriate fragments.

Figure 17.9 shows the electrophoretic patterns of PCR products, obtained by applying P6 primer (5′ TCGGCGGTTC 3′). This primer produces 920 bp band. As

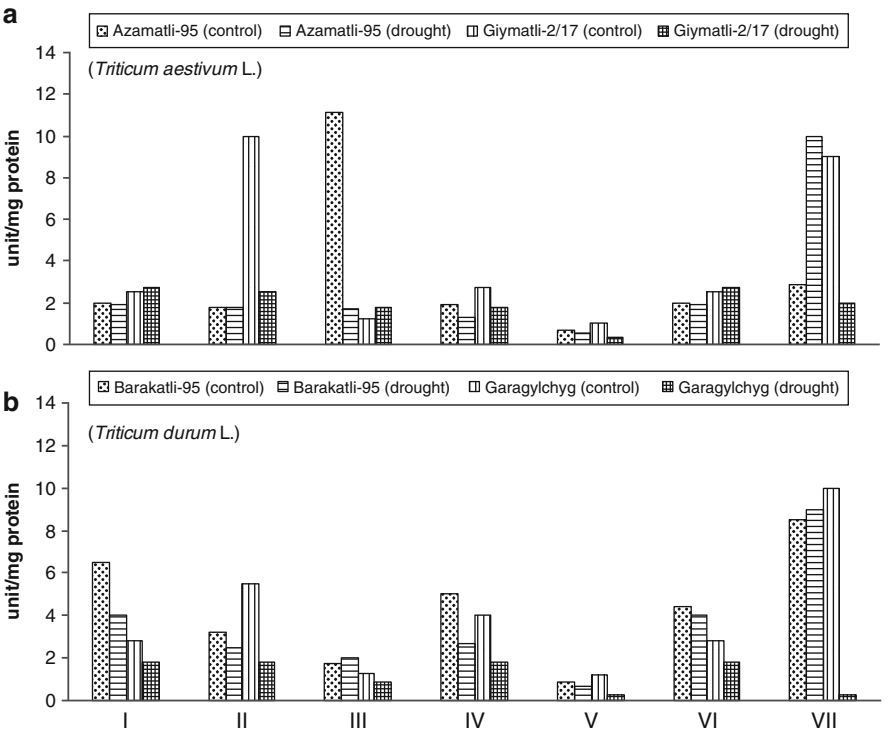

Fig. 17.8 Effect of water stress at different ontogenesis stages on SOD activity (unit/mg protein). (*I* – stalk emergence, *II* – beginning of earing, *III* – end of earing, *IV* – flowering, *V* – end of flowering, *VI* – milky ripeness, *VII* – wax ripeness)

Table 17.7 Photosystem II activity in chloroplasts from wheat genotypes at different stages of ontogenesis (μmol $O_2 \cdot mg^{-1}$ chlorophyll$\cdot h^{-1}$)

Genotypes	Stalk emergence	Beginning of earing	End of earing	Flowering	End of Flowering	Milky ripeness	Wax ripeness
Barakatli-95 (control)	20	24	120	100	84	40	12
Barakatli-95 (stress)	60	60	160	92	100	45	26
Garagylchyg (control)	80	108	84	120	100	24	20
Garagylchyg (stress)	72	72	120	112	108	27	24
Azamatli-95 (control)	88	100	120	88	100	28	40
Azamatli-95 (stress)	104	72	136	120	92	16	8
Giymatli-2/17 (control)	140	72	140	112	120	20	12
Giymatli-2/17 (stress)	120	96	160	104	120	22	8

Table 17.8 Photosystem I activity in chloroplasts from wheat genotypes at different stages of ontogenesis (μmol $O_2 \cdot mg^{-1}$ chlorophyll$\cdot h^{-1}$)

Genotypes	Stalk emergence	Beginning of earing	End of earing	Flowering	End of flowering	Milky ripeness	Wax ripeness
Barakatli-95 (control)	200	600	820	600	740	680	560
Barakatli-95 (stress)	540	400	840	500	520	500	280
Garagylchyg (control)	420	300	540	500	460	360	280
Garagylchyg (stress)	320	100	560	440	440	360	280
Azamatli-95 (control)	400	180	640	400	500	400	240
Azamatli-95 (stress)	400	180	640	440	520	440	160
Giymatli-2/17 (control)	400	240	740	420	440	440	120
Giymatli-2/17 (stress)	360	180	760	420	440	440	160

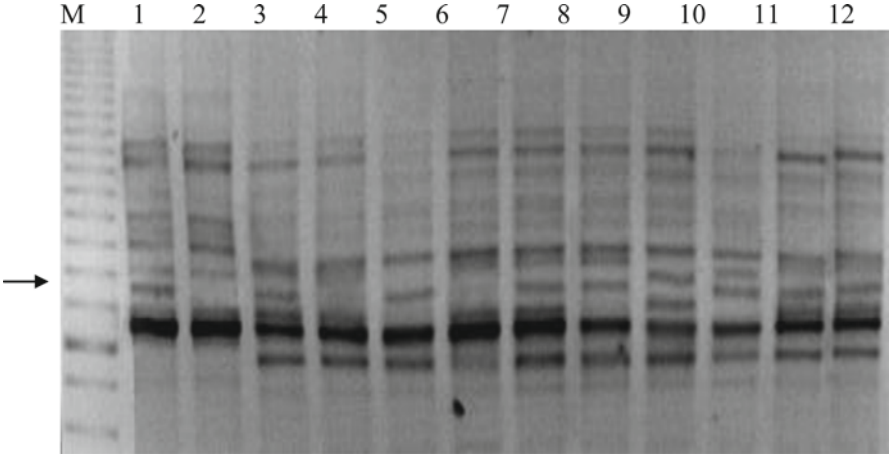

Fig. 17.9 PCR amplification profiles of *Triticum* L. wheat genotypes using P6 primer (5′ TCGGCGGTTC 3′). The arrow shows 920 bp DNA fragment, present in drought tolerant varieties and absent in non-tolerant. *M* – DNA ladder 100. *1* – Barakatli-95, *2* – Garagylchyg-2, *3* – Azamatli-95, *4* – Giymatli-2/17, *5* – Gyrmyzy bugda, *6* – Gyrmyzy gul, *7* – Tale 38, *8* – Ruzi 84, *9* – 12 nd FAWWON No 97 (130/21), *10* – 4 tn FEFWSN No 50 (130/32), *11* – Nurlu-99, *12* – Gobustan

it follows from figure, in drought-sensitive durum wheat genotype Garagylchyg-2 and moderately-tolerant bread wheat genotypes Giymatli-2/17 and Qyrmyzy gul 920 bp bands were not revealed. This locus is well seen on PCR amplification profiles from all other genotypes supporting their drought tolerance. It is noticeable

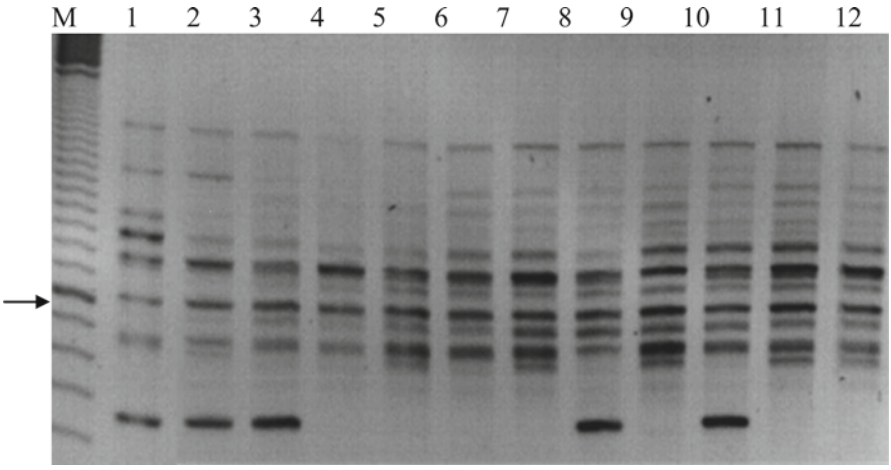

Fig. 17.10 PCR amplification profiles of wheat genotypes *Triticum* L. using a primer P7 (5′ TCGGCGGTTC 3′). The arrow shows 750 bp DNA fragments. *M* – DNA ladder 100. *1* – Barakatli-95, *2* – Garagylchyg-2, *3* – Azamatli-95, *4* – Giymatli-2/17, *5* – Gyrmyzy bugda, *6* – Gyrmyzy gul, *7* – Tale 38, *8* – Ruzi 84, *9* – 12 nd FAWWON No 97 (130/21), *10* – 4 tn FEFWSN No 50 (130/32), *11* – Nurlu-99, *12* – Gobustan

that 12nd FAWWON No 97 (130/21) is estimated as non-tolerant to drought. However, as the electrophoretic patterns indicate, 920 bp locus is also present in 12nd FAWWON No 97 (130/21). In terms of genetics it must have a potential for tolerance, but for some reasons expression of these genes not come true. The second RAPD primer in our experiments was P7 marker (5′ TCGGCGGTTC 3′). This primer produces 750 bp band. Figure 17.10 demonstrates that this band has occurred neither in tolerant variety Barakatli-95, nor in non-tolerant varieties Garagylchyq-2 and Giymatli-2/17. Meanwhile, in drought sensitive variety Gyrmyzy gul this locus has occurred. Therefore from the PCR results carried out with this primer it can be conclude that P7 RAPD marker is not absolutely universal for drought tolerance.

At the same time PCR analysis was carried out to identify *DREB 1* genes, which was responsive for drought tolerance in 12 wheat *Triticum* L. genotypes with different levels of resistance to drought. For this purpose functional markers for *DREB 1* genes, especially synthesized for A, B and D wheat genomes, were used. P25F/PR was designed to amplify a 596-bp DNA fragment downstream of *DREB-A1* in the A genome. P21F/P21R was selected to amplify an upstream region (1113-bp DNA fragment) of the same gene. Similarly, P22F/PR and P20F/P20R were designed to amplify sequences from the D genome, with the amplifications resulting in 596 and 1193-bp DNA fragments, respectively. The P18F/P18R primers, which amplify a 717-bp DNA fragment, were designed as a B genome-specific primer pair (Table 17.3).

Figure 17.11 demonstrates gel electrophoresis of PCR profiles of amplified DNA from *Triticum* L. using the primer pair P21F/P21R. As shown in Fig. 17.11, fragment amplified with this marker in 1113-bp region is present in the high drought

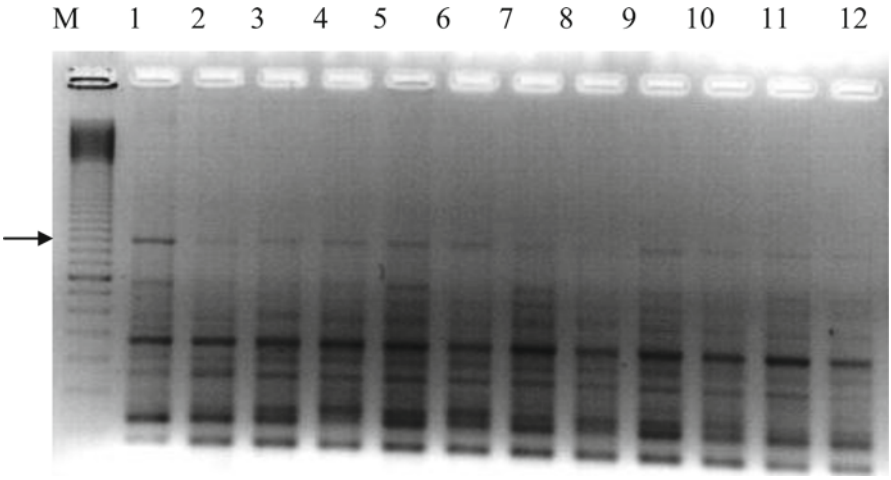

Fig. 17.11 PCR amplification profiles of wheat genotypes *Triticum* L. using an A genome-specific primer pair P21F/P21R. The *arrow* shows 1113 bp DNA fragments. *M* DNA ladder 100. *1* – Barakatli-95, *2* – Garagylchyg-2, *3* – Gyrmyzy bugda, *4* – Azamatli-95, *5* – Giymatli-2/17, *6* – Gobustan, *7* – Gyrmyzy gul, *8* – Tale-38, *9* – Ruzi-84, *10* – 12nd FAWWON No 97 (130/21), *11* – 4tn FEFWSN No 50 (130/32), *12* – Saratovskaya

tolerant durum wheat genotype Barakatli-95. Except in moderately-tolerant bread wheat genotype, *DREB 1*-responsive fragment was obtained in both drought tolerant and sensitive genotypes. This indicates that in these genotypes *DREB 1* gene responsive for tolerance to drought as well as other abiotic stresses is in the third chromosome of A genome (Wei et al. 2009). The second primer for *DREB 1* gene in A genome is a P25F/PR that amplifies fragments of 596-bp.

As it seemed from Fig. 17.12, 596-bp fragments were not synthesized in selected genotypes. Absence of these fragments can be explained by some mutations that, probably, took place in *DREB 1* gene region, complementary to this primer. Thus the presence of *DREB 1* gene in A genome is confirmed by PCR results using the primer pair P21F/P21R.

The results obtained using primer pair P18F/P18R, specific for *DREB 1* gene in B genome, are shown in Fig. 17.13. As it seemed from Fig. 17.3, fragment pair 717–789 bp revealed only in Barakatli-95. This shows that *DREB 1* gene also occurred in Barakatli-95 B genome. It should be noted that in our experiments this genotype also shows its high drought tolerance in other parameters (Aliev 1998, 2001). As to the absence of this pair of fragments in other wheat genotypes it can be explained from the literature that the *DREB1* proteins showed the most specific variations in the B genome, including three single amino acid mutations (amino acids 46, 140 and 200) and a deletion of 24 amino acids in a region rich in Ser and Thr in the orthologous A and D genomes.

PCR analysis with P22F/PR and P20F/P20R primers specific for D genome was also carried out. As it is known D genome occurs in hexaploid genotypes *Triticum*

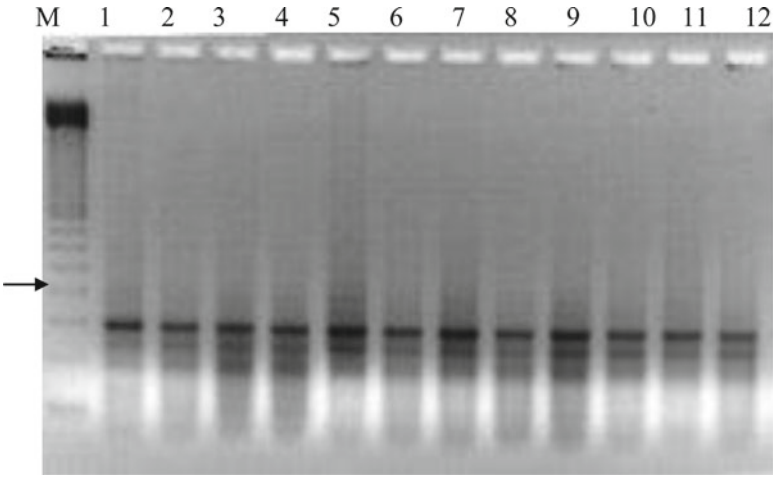

Fig. 17.12 PCR amplification profiles of wheat genotypes *Triticum* L. using an A genom-specific primer pair P25F/PR. The *arrow* shows 596-bp DNA fragment. *M* DNA ladder 100. *1* – Barakatli-95, *2* – Garagylchyg-2, *3* – Gyrmyzy bugda, *4* – Azamatli-95, 5 – Giymatli-2/17, *6* – Gobustan, *7* – Gyrmyzy gul, *8* – Tale-38, *9* – Ruzi-84, *10* – 12nd FAWWON No 97 (130/21), *11* – 4tn FEFWSN No 50 (130/32), *12* – Saratovskaya

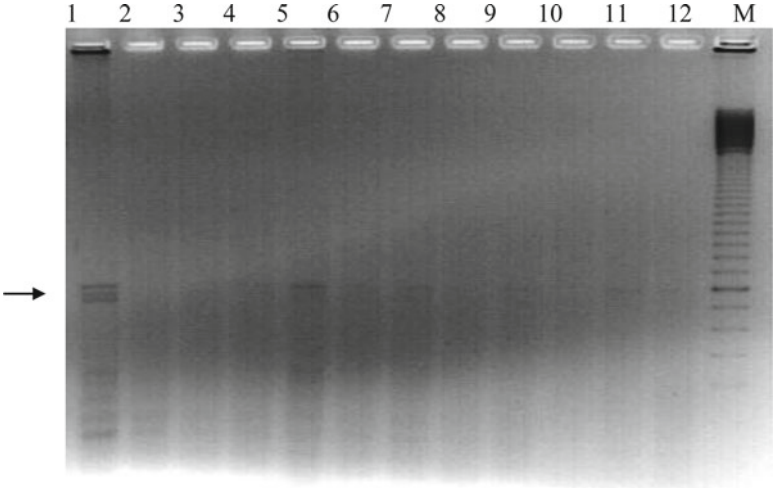

Fig. 17.13 PCR amplification profiles of wheat genotypes *Triticum* L. using a B genome-specific primer pair P18F/P18R. The *arrow* shows 717–789 bp DNA fragment. *M* DNA ladder 100. *1* – Barakatli-95, *2* – Garagylchyg-2, *3* – Gyrmyzy bugda, 4 – Azamatli-95, *5* – Giymatli-2/17, *6* – Gobustan, *7* – Gyrmyzy gul, *8* – Tale-38, *9* – Ruzi-84, *10* – 12nd FAWWON No 97 (130/21), *11* – 4tn FEFWSN No 50 (130/32), *12* – Saratovskaya

aestivum L. In our experiments there were no appropriate fragments responsive to these markers. This allows us to suppose that the hexaploid wheat varieties used here were possibly nulliosomics, i.e. chromosome pair possibly a *DREB 1* gene is absent from the D genomes.

4 Conclusion

So, dehydration involves many changes resulting in disrupted membrane integrity, suppression of many photosynthetic genes, decreased activities of photosystems, etc. The higher water content and its better distribution in the stressed cv. Azamatli-95 permitted the plants to retain a higher turgor in comparison with cv. Giymatli-2/17, which resulted in the maintenance of growth. Drought induced photoinhibition and photodestruction of pigments and pigment-protein complexes and destabilization of photosynthetic membrane. In cv. Azamatli-95 the lack of changes in pigment content and composition following drought indicated the capacity to preserve the photosynthetic apparatus. At the same time, the decline in PS II activity induced by water deficit was more marked in sensitive cv. Giymatli-2/17 than in tolerant cv. Azamatli-95. The drought-sensitive genotype Giymatli-2/17 responded to a period of stress by reducing photosynthetic efficiency and biomass accumulation. In this genotype, the defense mechanisms prevent plants from suffering irreversible damages during drought. Therefore, in cv. Azamatli-95 the photosynthetic electron transport was probably sufficient to preclude the buildup of excess energy in PS II. On the other hand, drought tolerant genotype Azamatli-95 seems able to avoid drought stress by maintaining a high photosynthetic activity, and does not suffer an oxidative stress high enough to trigger the defense mechanisms active in the genotype Giymatli-2/17. Dynamics of changes of anti-oxidative enzymes activity in wheat genotypes under normal water supply and deficiency of water in all phases of ontogenesis reveal that drought differently changes a balance between production of free radicals and enzymes involved in antioxidative defence mechanism.

In the drought-affected tolerant wheat the lower degree of changes in membrane in comparison with those in the sensitive genotype, is probably due to the presence of a more fluid bilayer, since neither non-bilayer-forming lipids of thylakoid membrane nor free fatty acids (FFA) accumulated following drought. Changes in fatty acid saturation are required to preserve an appropriative balance of bilayer and non-bilayer forming lipids in the membrane. In drought adaptation, it is probably the occurrence of bilayer/nonbilayer transformations and their influence on the packaging of proteins that are of primary importance. The larger capacity of the charged lipids to swell with water may also have increased the ability to bind water in the tolerant cultivar.

Thus, results obtained from RAPD-PCR analysis are promising beginning for further research of plant tolerance to water stress on molecular-genetic basis. Marker-assisted selection based on genotype will greatly increase breeding efficiency (Manavalan et al. 2009). Recent advances in wheat research, ranging from breeding

programs to genome sequencing and genomics technologies, provide unprecedented opportunities to understand global patterns of gene expression and their association with the development of specific phenotypes, as well as promising tools for the genetic improvement of plants cultivated in adverse environments by molecular breeding or transgenic approaches. The presence of DREB 1 gene in A genome of 3 tetraploid and 8 hexaploid wheat genotypes was identified using functional markers. Unlike other genotypes, this gene was also found to be present in B genome of tetraploid genotype Barakatli-95. Understanding of the functions of these stress-inducible genes helps unravel the possible mechanisms of stress tolerance. Marker-assisted selection was also employed to improve the staygreen trait involved in drought tolerance of wheat. The results presented here open an excellent opportunity to develop stress tolerant crops in future.

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Chapter 18
Molecular Basis of Disease Resistance in Cereal Crops: An Overview

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Abstract Deep insight into the molecular mechanism of disease resistance in plants is essential for devising sophisticated breeding strategies leading towards crop protection. Most of the disease resistance (*R*) genes show genetic and structural similarity even though their target pathogens are extremely diverged in strain types, mode of action, virulence and target plant part. The search for a unified model operating for disease resistance in cereals may provide improved control of devastating pathogens which is critical for global food security. A super family of '*R*' genes encoding NBS-LRR (Nucleotide binding site- Leucine rich repeats) with its several sub-classes, recently identified ABC (ATP binding cassette) transporter and kinase START genes confer resistance to numerous pathogens. These molecular motifs activate a wide array of metabolic responses under tight genetic control as the plant detects a prospective invader. Some of the '*R*' genes require additional genes for the expression of resistance. Investigations into the structure of '*R*' genes, protein products, their location and mechanism of interaction with pathogen elicitor molecules and future prospects are discussed in this review.

Keywords Cereals • '*R*' genes • Defense mechanism • Guard hypothesis

1 Introduction

Cereal crops are an important source of food for population. Currently, protecting crops from different diseases and environmental stresses is a pressing issue. Different approaches are applied to reduce the effects of pathogens and environmental stresses. Chemical control measure is common in this regard. Having serious environmental and health hazards, this approach is discouraged. However, use of chemicals in agriculture is common in developed countries, but it is not affordable in developing countries. Unlike chemical method, use of host genetic resistance is convenient, affordable and environment-friendly. In recent times, much effort has been directed towards understanding defense mechanism of diseases in cereal crops. Findings of Flor (1956) leading to gene-for-gene hypothesis provided a basis for predicting the molecular basis of disease resistance. In view of molecular interpretation of the Flor's findings, avirulent (*Avr*) genes encode signal transduction that is perceived by the products of plant *R* genes. These are regarded as foundation concept in disease resistance. The *R*-gene/avirulence factor complex is thought to instigate a series of signaling cascades leading to disease resistance. Rapid oxidative bursts, cell wall strengthening, induction of defense gene expression and rapid cell death at the site of infection are the downstream cellular events that confer resistance state (Morel and Dangl 1997). In a more elaborated form, the direct or indirect recognition of pathogens by host '*R*' genes leads to a resistance response known as effector-triggered immunity (ETI), which includes localized programmed cell death (PCD), known as the hypersensitive response (HR) which ultimately restrict pathogen growth (Dangl et al. 1996). R proteins serve to recognize pathogen effectors either through direct interaction or as guards for target molecules, and are known to confer resistance to

bacteria, viruses, nematodes, oomycetes, insects, and biotrophic fungi (Martin et al. 2003). Genes for resistance, their protein products, and underlying mechanisms are being investigated (Hammond-Kosack and Jones 1996). Sufficient progress has been made in these aspects that will facilitate developing effective control strategies.

2 *R* Gene Classes

Several classes of *R* genes investigated in cereals are depicted in Table 18.1. Brief description about their mechanics and structural features is given below.

2.1 NBS-LRR Genes

The majority of *R* genes which had been investigated so far by gene cloning encode nucleotide binding site (NBS) and a leucine-rich-repeat (LRR) region. These NBS-LRR types of genes are abundant in plant species (Meyers et al. 1998). These plant *R* genes encode proteins that have a putative amino-terminal signaling domain, a nucleotide binding site and a series of carboxy-terminal leucine repeats (Meyers et al. 2005). There are two classes of NBS-LRR proteins. One major class had an amino-terminal TIR (Toll/interleukin receptor) domain also called TIR-NBS-LRR or TNL proteins. Other class includes the genes which encode an amino-terminal coiled-coiled motif (CC-NBS-LRR or CNL proteins). The mechanism of resistance induced by *Pm3a* and its other allelic forms in wheat (Feng et al. 2010) and by *Pb1* against rice blast clearly exemplifies that the single amino acid residue at the final position of the kinase-2 motif is the characteristic of coiled-coil (CC) motif while the tryptophan (W) and aspartic acid (D) are the characteristics of TIR-type proteins. The details of the molecular functions of these protein domains and their interacting partners are still being established. However, the consistent identification of this class of proteins across diverse plant species demonstrates that the NBS-LRR genes are a pillar of plant defense against pathogens. The majority of the *R* genes in *Arabidopsis* are TNL genes; however, they have not yet been reported in cereals. Approximately, 1,500 NBS coding sequences encoding NBS gene analyzed in rice have no TIR binding domain (Zhou et al. 2004). *Lr1* from wheat encoding resistance against *Puccinia triticina* has a CNL type domain. Same is the case of *Lr10, Lr21,* and *Pmb3b*, from wheat *R*-genes cloned to date (Feuillet et al. 2003; Huang et al. 2003; Yahiaoui et al. 2004).

2.2 Protein Kinase

The other smaller, class of *R* proteins are those composed of serine/threonine protein kinase (S/TPK) domains such as the barley Rpg1 stem rust *R* gene

Table 18.1 Comprehensive information of cereal disease resistance genes cloned so far

Crop	Disease	Pathogen	Gene	Proteins encoded	Reference
Wheat	Leaf rust	*Puccinia triticina*	*Lr10*	NBS-LRR	Feuillet et al. (2003)
			Lr1	NBS-LRR	Cloutier et al. (2007)
			Lr21	NBS-LRR	Huang et al. (2003)
			Lr34	ABC transporter	Karttinger et al. (2009)
	Powdery mildew	*Blumeria graminis*	*Pm3*	NBS-LRR	Yahiaoui et al. (2004)
			Pm3a,b,d,f	NBS-LRR	Feng et al. (2010)
	Stripe rust	*Puccinia striiformis*	*Yr36*	Kinase START	Fu et al. (2009)
			Yr18	ABC transporter	Karttinger et al. (2009)
	Cereal cyst nematodes	*Heterodera avenae*	*Cre3*	NBS-LRR	Lagudah et al. (1997)
Rice	Bacteria blight	*Xanthomonas oryzae*	*Xa1*	NBS-LRR	Yushimura et al. (1998)
			Xa21	Receptor like protein kinase	Song et al. (1995)
			Xa26	Receptor like protein kinase	Sun et al. (2004)
	Rice blast	*Magnaporthe grisea*	*Pi-b*	NBS-LRR	Wang et al. (1999)
			Pi-9	NBS-LRR	Qu et al. (2006)
			Pi-37	NBS-LRR	Lin et al. (2007)
			Pi-36	NBS-LRR	Liu et al. (2007)
			Pb-1	NBS-LRR	Hirabayashi et al. (2010)
			OsWAKI1	Wall-associated receptor like protein kinase	Li et al. (2009)
			Pi-ta	NBS-LRR	Bryan et al. (2000)
Barley	Powdery mildew	*Blumeria graminis*	*mlo*	Mutant seven transmembrane	Freialdenhoven et al. (1996)
			Mla1	NBS-LRR	Zhou et al. (2001)
			Mla6	NBS-LRR	Halterman et al. (2001)
	Stem rust	*Puccinia graminis*	*Rpg1*	Protein kinase	Brueggeman et al. (2002)
			Rpg4/Rpg5	NBS-LRR and protein kinase	Brueggeman et al. (2008)
Maize	Leaf rust	*Puccinia sorghi*	*Rp1-D*	NBS-LRR	Collins et al. (1999)
			Rp3	NBS-LRR	Webb et al. (2002)
	Southern corn leaf blight	*Cochliobolus carbonum*	*Hm1*	HC toxin reductase	Johal and Briggs (1992)

(Brueggeman et al. 2002). Resistance to bacterial pathogen *Pseudomonas syringae* conferred by tomato *pto* gene also encodes the same protein kinase but it additionally requires an NBS-LRR gene, *prf*, for defense activation (Salmeron et al. 1996). The barley Rpg5 stem rust *R* gene characterizes a unique structure among *R* genes in that it contains an N-terminal NBS-LRR and a C-terminal S/TPK (Brueggeman et al. 2008). This receptor-like protein kinase (RLK) super-family consists of trans-membrane proteins that recognize stimuli by their extracellular domains and transmit the signals through their cytoplasmic kinase domains (Morris and Walker 2003). These distinctive structural features give RLKs the mandate to play significant roles in plant defense activities. In fact, a few plant RLK members have been found involved in defense activation. In rice, two genes *Xa21* and *Xa26* act as resistance (*R*) genes and play a key role in plant cultivar-specific *R* protein-dependent resistance mediated by a receptor like protein kinase (Song et al. 1995; Sun et al. 2004). Recent advancements have suggested that RLKs are ubiquitous throughout the plant kingdom (Shiu et al. 2004), but the precise function for most of the RLKs has not been established. Cell wall is the first line of plant defense in response to external stimuli, but little is known about what communication might occur between the cell wall and plasma membrane. Additionally, when a pathogen attacks it is not clearly known which types of proteins are integrated in mediating contact between the cell wall and plasma membrane and the subsequent signal transduction. Recently, an identified member of cell wall-associated receptor kinases (WAKs) gene in rice designated as *OsWAK1* has been reported to play an important role in rice blast disease resistance (Li et al. 2009). WAKs differ from other kinds of plant RLKs in the epidermal growth factor (EGF) like repeats in the extracellular domain (Shiu and Bleecker 2001). WAKs physically link the plasma membrane to the cell wall matrix through the extracellular domain and have the potential to directly signal cellular events through their cytoplasmic kinase domain.

2.3 Kinase START Gene

START (steroidogenic acute regulatory protein-related lipid transfer) domain proteins in humans have been recognized to participate in lipid trafficking, metabolism, and sensing; and their binding with sterols and ceramides and they cause protein conformational changes (Alpy and Tomasetto 2005). The recent development by the positional cloning of the high-temperature stripe rust resistance gene *Yr36* revealed that two pairs of duplicate genes are responsible to encode a START kinase (Fu et al. 2009) This domain had the ability to bind lipids from PST at high temperature and change its conformation resulting in initiation of signaling cascade leading to programmed cell death. The first pair of duplicated genes had two short putative genes (*1BR1 and 1BR2*) with an in-between ring finger domain. The other pair of duplicated genes designated as WKS1 and WKS2 encode 86% identical proteins that have a predicted kinase domain followed by a predicted START domain. This combination of the kinase and START domains in *WKS1* apparently is the result of a novel domain shuffling, because these two domains are not found together in other

organisms (Fu et al. 2009). The *WKS1* kinase has high resemblance to several *Arabidopsis* WAK-like kinases but *WKS1* lacks the additional domains characteristic of WAK-like kinases (Verica and He 2002). The *WKS1* kinase belongs to the non-RD kinases, which are frequently involved in the early steps of the innate immune response (Dardick and Ronald 2006).

2.4 ATP Binding Cassette Transporter

Resistance to multiple fungal pathogens in cereals through durable/non-race specific genes, have saved the cereal production since decades. Sequence analysis of the most important durable rust resistance gene *Lr34* revealed the presence of a gene rich island containing eight open reading frames predicted to encode proteins with homologies to a hexose carrier, an ATP-binding cassette (ABC) transporter, two cytochromes P450, two lectin receptor kinases, a cysteine proteinase, and a glycosyl transferase (Krattinger et al. 2009). The nucleotide sequence of *Lr34* spanned about 11,805 bp. *Lr34* consisted of 24 exons as revealed by sequencing of full length cDNA. The predicted 1401-amino acid protein belongs to the pleiotropic drug resistance subfamily of ABC transporters. Pleiotropic drug resistance transporters share a common basic structure containing two cytosolic nucleotide binding domains and two hydrophobic trans-membrane domains. In the genome of *Arabidopsis*, 15 pleiotropic drug resistance like genes have been diagnosed and additionally 23 members have been described in rice. The closest *Lr34* homolog in rice is *OsPDR23*, which is closest homolog of *Lr34* in rice showed 86% amino acid similarity and in *Arabidopsis*; the closest homologs are 56% identical to *Lr34* at the amino acid level. The knowledge about substrate specificity of these pleiotropic drug resistance transporters is very limited but these are known to confer resistance to various drugs (Rogers et al. 2001). In *Arabidopsis*, it has previously been reported that PEN3/PDR8 contributes to resistance toward non-host pathogens (Stein et al. 2006). The described model suggested that PEN3 may be involved in translocating toxic compounds derived from glucosinolates into the apoplast (Lipka et al. 2008).

2.5 HC Toxin Reductase

The maize *Hm1* gene which confers resistance against southern corn leaf blight caused by the fungal pathogen *Cochliobolus carbonum* represent a very different class of cereal resistance gene (Johal and Briggs 1992). Unlike the other *R* genes, *Hm1* encodes the enzyme HC toxin reductase which detoxifies a specific cyclic tetrapeptide toxin produced by the fungus (HC toxin) which is essential for pathogenicity. The cyclic tetrapeptide HC toxin produced by the pathogen had structure cyclo (D-Pro-L-Ala-D-Ala-L-Aeo), where *Aeo* stands for 2-amino-9,10-epoxy-8-oxodecanoic acid. The maize *Hm* gene encodes a carbonyl reductase. This reductase product uses NADPH as a cofactor to reduce the 8-carbonyl moiety

of the side chain of *Aeo* to produce the biologically inactive 8-hydroxy derivative (Meeley and Walton 1991). HC toxin reductase is found in extracts only of maize of genotype with heterozygous or homozygous dominant condition and is not detectable in maize of genotype hm/hm (Meeley et al. 1992). Therefore, detoxification of HC toxin is the basis of the specific reaction of maize to *corn* leaf blight. Therefore, cereal *R* genes are seen to encode a range of different proteins that in some cases obviously have very different functions.

3 Location of the Proteins

The site of these proteins in dicotyledonous species has been recognized as the cell cytoplasm. Few proteins products have also been identified residing on the cytoplasmic face of the cell membrane (Boyes et al. 1998). In barley resistance to stem rust operated by Rpg5 encode NBS-LRR-S/TPK domain. The location of trans-membrane domains, if functional, suggests that LRR domain may reside outside the cell and act as the pathogen receptor, whereas the NBS and PK domains are intracellular and transmit the disease resistance signaling (Brueggeman et al. 2008).

4 Mechanism of Resistance

Different mechanisms have been identified that carry out the defense process in cereals. Most important models are described as below.

4.1 Receptor- Ligand Interaction

This model proposes the mechanism based on the direct interaction between *R* proteins and *Avr* proteins. Investigations into the fungal pathogen of rice blast (*Magnaparthe grisea*), have revealed that the rice *Pi-ta* gene encodes an NBS-LRR protein that recognizes *Magnaporthe* pathotypes in a race-specific manner (Bryan et al. 2000). The pathogen produces a metalloprotease protein as a corresponding pathogen avirulence gene product (AVR-Pita) (Orbach et al. 2000). This recognition process activates the subsequent defense process. Such direct interaction between the plant resistance protein and the pathogen avirulence protein has been reported *in vivo* and *in vitro* (Jia et al. 2000). However, such evidence of receptor-ligand interaction is not common in cereals.

4.2 The Guard Hypothesis

Lack of direct interaction between the pathogen avirulence product and *R* proteins have led to the recognition of other processes involved in defense mechanism.

The model known as 'guard hypothesis' proposed by Van-der-Biezen, and Jones (1998) suggests that the avirulence products of the pathogen come into contact with the non-R factors and modify them. This interference when sensed by the *R* gene products triggers the defense mechanism (Bogdanove 2002). Furthermore, this model envisages that in cereals interaction between R proteins produced by host plant and another protein known as 'guardee' occurs. Upon invasion of the 'guardee' protein by the pathogen, the host plant activates its defense mechanism. Subsequently, the cascade signal transduction initiates to protect the host. Although direct interaction between R and Avr proteins, may not essentially happen. Evidence of this model has recently been reported in *Arabidopsis* (Mackey et al. 2002). The process has not been thoroughly understood and is under investigation (Schneider 2002; Van der Hoorn et al. 2002).

5 Durability of Disease Resistance

Durable resistance against pathogen encoded by the minor genes had attracted the researchers due to their effectiveness to protect crops from pathogens. One of the examples of durable resistance is of *Lr34/Yr18* which offer resistance against leaf and yellow rust and has been exploited by the breeders since the last 50 years. The mechanism of durable, non-race specific resistance is primarily elicited by highly conserved pathogen-associated molecular patterns (PAMPs). Plants have acquired this PAMP-triggered immunity (PTI) during co-evolution with pathogens (Zellerhoff et al. 2010). Quantitative or non-host resistance is assumed to be durable but, in the case of quantitative host resistance, it is attenuated by pathogen effector molecules that reprogram host cells for the pathogen's demand leading to susceptibility triggered by effectors known as effector-triggered susceptibility (Jones and Dangl 2006). In many cultivars the quantitative host resistance is not very effective due to weaker combinations of desirable alleles encoding PTI components that determine the accessibility of the plant for pathogen effectors or the strength of host responses. Molecular basis of PAMP-triggered immunity (PIT) in the model plant *Arabidopsis thaliana* is very well understood (Boller and Felix 2009). However, this knowledge is meager for *Triticeae,* and cereals need to be investigated for PTI. Bischof et al. (2011) reviewed the global transcriptional patterns that modify in *Triticeae* during interactions (compatible or non-compatible) with biotrophic and cell death-inducing fungal pathogens. The key factors to be identified include factors responsible for resistance and susceptibility and transcriptome-based identification of developing biological function during disease development in host plant. Among transcriptionally stress activated genes are members of the wheat induced resistance 1 (*WIR1*) family which includes the members of transcriptionally stress activated genes. On the evidence based on genomic, genetic and transcriptome, the role of *WIR1* genes in modulating quantitative resistance to powdery mildew on barley is presented by Douchkov et al. (2011). They also proposed a way beyond the description of gene-expression patterns towards gene-function analysis in *Triticeae*. Along these lines,

transgenic expression or knockdown of PTI or ETS related genes is a powerful way to demonstrate gene function in durable/quantitative resistance and might support future breeding for durable resistance in *Triticeae*. The current technical possibilities of spatial, temporal and quantitative control of transgene expression facilitated by promoters, other un-translated regulatory elements and protein signal peptides and motifs can be used for studying subcellular localization of transgene products (Hensel et al. 2011). Aghnoum and Niks (2011) developed two experimental barley lines; one had extremely low resistance to powdery mildew and the other had extremely high quantitative resistance. These barley lines which are likely to have stacked favorable alleles for quantitative resistance, for high and low efficiency of PTI might be a valuable tool for future investigations into the mechanisms of quantitative resistance and susceptibility.

6 Additional Genes Required for the Expression '*R*' Genes

In addition to *R* genes, additional genes have been reported to complement the defense mechanism operated by *R* genes. Investigations into susceptible mutants revealed that a number of additional genes are required for '*R*' gene activity. The additional genes suggest that multiple genes are actively involved in operations of multiple resistance signaling pathways. In barley, a family of *Mla* genes encodes a large number of NBS-LRR proteins. Each protein has a defense capability against a specific powdery mildew pathotype. Although, both *Mla1* and *Mla6* genes encode 91% identical proteins. The latter requires two additional genes *Rar1* and *Sgt1* to activate the defense response. The former encodes a zinc-binding protein containing a highly conserved cysteine- and histidine-rich domain (CHORD) (Shirasu et al. 1999); and *Mla6* (Azevedo et al. 2002) encodes a subunit of the SCF ubiquitin ligase complex. On the other hand, *Mla1* gene operates independently (Halterman et al. 2001; Azevedo et al. 2002). Homologues of *Rar1* and *Sgt1* gene have been observed in dicotyledonous species and known to show additional interaction with HSP90 proteins (Takahashi et al. 2003).

7 Conclusion and Future Prospects

The understanding of the molecular basis of resistance in cereals is dire need of the time to design potential measures to save the crops from insects and pathogens. Co-evolving strains of the pathogens have made cereals vulnerable threatening global food security. Although many novel insights into the defense mechanism triggered in plants to cope with the dangerous pathogens have emerged in present time, thorough understanding of the phenomena is still to be done. Facts about the structure of the '*R*' genes, protein complexes and mechanism of defense signaling are to be discovered to project the picture clearly. Discovery and candidate positional

cloning of more '*R*' genes, understanding of their protein products will have effective biotechnological applications in the field of plant protection. The application of functional genomics to disease resistance will significantly accelerate the pace of discovery and provide new insights into *Avr-R* interactions. Moreover, a detailed understanding of the structural basis of recognition will assist researchers to approach the holy grail of designing *R* proteins that recognize essential virulence factors. Considering a variety of strategies that pathogens utilize and their ability to rapidly adapt it, would be rash to predict the development of a magic bullet for durable, broad-spectrum resistance. However, it is reasonable to expect a novel array of sophisticated omics technologies that will provide efficient protection in certain situation, when they are judiciously integrated with other control measures.

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Chapter 19
Polyamines: Role in Plants Under Abiotic Stress

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Abstract Environmental changes, irrespective of source, cause a variety of stresses in plants. These stresses affect the growth and development and trigger a series of morphological, physiological, biochemical and molecular changes in plants. Abiotic stress is the primary cause of crop loss worldwide. The most challenging job before the plant biologists is the development of stress tolerant plants and maintenance of sufficient yield of crops in this changing environment. Polyamines can be of great use to enhance stress tolerance in such crop plants. Polyamines are small organic polycations present in all organisms and have a leading role in cell cycle, expression of genes, signaling, plant growth and development and tolerance to a variety of abiotic stresses. High accumulation of polyamines (putrescine, spermidine and spermine) in plants during abiotic stress has been well documented and is correlated with increased tolerance to abiotic stress. Genetic engineering of PA biosynthetic genes in crop plants is the way to create tolerance against different stresses. The present review throws light on the role of polyamines in plants.

Keywords Abiotic stress tolerance • Polyamines • ADC • ODC • SAMDC

1 Introduction

Plants are exposed continuously to a variety of adversely changing environmental factors such as heat, cold, light, drought, acidity, alkalinity, oxidative damage and metal damage, which affect plant distribution, growth, development and productivity (Ahmad et al. 2008, 2010a, b). These stressful conditions are associated with the losses in the productivity of many of the agriculturally important crops and therefore, affect the economic returns of the country. Thus, concerted efforts are underway worldwide to understand the mechanism of plant resistance against these stresses. There are several natural ways of self-defense in the plants to cope with these stressful conditions: they can induce several functional or regulatory genes (Bartels and Sunkar 2005) or can undergo different physiological or biochemical changes. The accumulation of some functional substances, such as compatible solutes and protective proteins, is an important element of the physiological and biochemical response of plants to the stressful conditions (Liu et al. 2007; Ahmad and Sharma 2008; Ahmad et al. 2010a, b, 2011). In addition to these responses by the plants, molecules known

as 'polyamines', have also been known to be an integral part of plant stress response (Bouchereau et al. 1999; Walters 2003a, b; Alcázar et al. 2006b).

Polyamines (putrescine, spermine, spermidine and cadaverine), are the widely distributed of N containing organic molecules, which were discovered more than 100 years ago and hold their significance from the minutest bacteria to multicellular pants, animals and mammals. In addition to their stabilizing effects, which they confer by binding to the intracellular anions (DNA, RNA, chromatin and proteins), they are also known to possess several regulatory functions (Igarasahi and Kashiwagi 2000; Alcázar et al. 2006b, 2010; Kusano et al. 2008). In plants, they have been associated with regulating many physiological processes, such as organogenesis, embryogenesis, floral initiation and development, leaf senescence, fruit development and ripening, and abiotic and biotic plant stress responses (Galston and Kaur-Sawhney 1990; Kumar et al. 1997; Walden et al. 1997; Malmberg et al. 1998; Bouchereau et al. 1999; Bagni and Tassoni 2001; Alcázar et al. 2006b, 2010; Kusano et al. 2008).

Several changes in concentrations of polyamines in plant cells take place while responding to the stressful conditions (Bouchereau et al. 1999; Alcázar et al. 2006b, 2010; Groppa and Benavides 2008). The importance of this process can be exemplified by the fact that the levels of Put may account for 1.2% of the dry matter, representing at least 20% of the nitrogen (Galston 1991) under stressful conditions. Though the exact mechanism of involvement of polyamines during stressful conditions is not fully understood, studies are ongoing to study the molecular mechanisms (Liu et al. 2007; Alcazar et al. 2010). Evaluating the complete genome sequence of Arabidopsis has facilitated the use of global 'omic' approaches in the identification of target genes in polyamine biosynthesis and signaling pathways (Alcazar et al. 2010). The advantages of the progress made in these directions have made possible the generation of Arabidopsis transgenic plants, which are resistant to various stresses (Alcazar et al. 2010). Efforts can be made towards the development of such varieties for the agriculturally important crops as well. Such studies add to the economic potential from the agricultural sector touched by the biotechnological advances and hence, further research in these directions is noteworthy.

2 Polyamine Biosynthesis in Different Organisms

The biosynthetic pathways of polyamines have been established for many organisms ranging from bacteria to plants to mammals (Kusano et al. 2007). The synthesis essentially starts from the two amino acid precursor molecules, L-arginine and L-methionine. An overview of the general pathway is given in Fig. 19.1.

In mammals and fungi, putrescine (Put, 1,4-diaminobutane) is produced by a single pathway catalyzed by ornithine decarboxylase (ODC, EC 4.1.1.17) whereas, in plants two alternative pathways operate, namely the ODC-catalyzed reaction, as in mammals and the second is from arginine (Arg), as a result of the action of Arg decarboxylase (ADC, EC 4.1.1.19), via agmatine. A few plant species, including *Arabidopsis thaliana*, lack the ODC pathway (Hanfrey et al. 2001; Kusano et al. 2007). The polyamines in

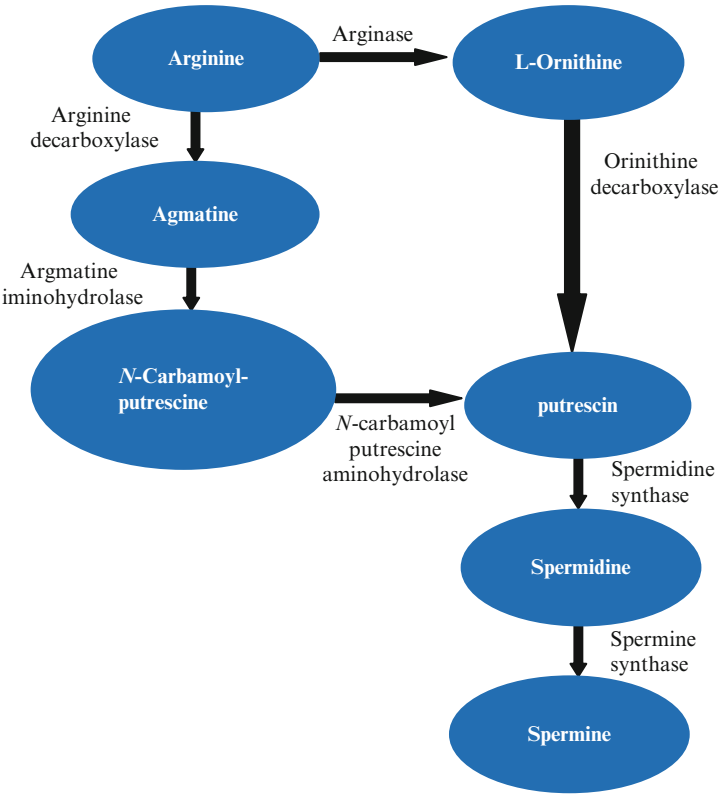

Fig. 19.1 Biosynthetic pathway of polyamines in plants

plants are not only found in the cytoplasm, but also in certain organelles like mitochondria, chloroplasts and vacuoles (Kumar et al. 1997; Kusano et al. 2008). The genes encoding enzymes for the polyamine biosynthesis pathway have been cloned and characterized from various plant species (Bell and Malmberg 1990; Michael et al. 1996; Bagni and Tassoni 2001; Liu et al. 2007; Kusano et al. 2008).

Briefly, starting from arginine, the diamine putrescine is synthesized via ornithine by arginase (EC 3.5.3.1) and ornithine decarboxylase (ODC, EC 4.1.1.17). Putrescine can also be synthesized via agmatine by three sequential reactions catalyzed by arginine decarboxylase (ADC, EC 4.1.1.19), agmatine iminohydrolase (AIH, EC 3.5.3.12), and *N*-carbamoylputrescine amidohydrolase (CPA, EC 3.5.1.53), respectively (Kusano et al. 2008). Putrescine is further transformed to Spd and Spm by successive transfers of aminopropyl groups from decarboxylated *S*-adenosylmethionine (dSAM) catalysed by specific Spd and Spm synthases. The aminopropyl groups are derived from methionine, which is first converted to *S*-adenosylmethionine (SAM) by methionine adenosyltransferase (EC 2.5.1.6), and then decarboxylated in a reaction catalyzed by *S*-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50). The resulting decarboxylated SAM is utilized as an aminopropyl donor (Fig. 19.1).

The use of the polyamine inhibitors have helped in the evaluation of their respective roles. Four commonly used inhibitors of PA synthesis are: (1) difluoromethylornithine (DFMO), an irreversible inhibitor of ODC; (2) difluoromethylarginine (DFMA), an irreversible inhibitor of ADC; (3) methylglyoxyl- bis-guanylhydrazone (MGBG), a competitive inhibitor of *S*-adenosyl-methionine decarboxylase (SAMDC); and (4) cyclohexylamine (CHA), a competitive inhibitor of spermidine synthase. Common oxidases are diamine oxidase and polyamine oxidase (PAO), Each PA has been found to be catabolized by a specific oxidase (Kaur-Sawhney et al. 2003).

3 Polyamine Catabolism

The concentrations of the polyamines in the cells is also maintained by the catabolic pathways (Bagni and Tassoni 2001; Cona et al. 2006). Copper containing diamine oxidases (CuAO, EC 1.4.3.6) and flavine-containing polyamine oxidases (PAO, EC 1.5.3.11) catalyse the oxidative de-amination of PAs. CuAO, which prefers diamine substrates, oxidizes Put and cadaverine (1,5-diaminopentane) with concomitant production of pyrroline, NH_3 and H_2O_2, and the resulting aldehyde is further metabolized to γ-aminobutyric acid via Δ^1-1-pyrroline (Bagni and Tassoni 2001; Kusano et al. 2008). On the other hand, PAO oxidizes Spd and Spm, producing 4-aminobutanal and *N*-(3-aminopropyl)-4-aminobutanal, respectively, in addition to 1,3-diaminopropane and H_2O_2 (Kusano et al. 2007). This means that plant PAOs are involved in the terminal catabolism of polyamines (Kusano et al. 2008). These enzymes are associated with the cell walls of tissues, where lignification, suberization and wall stiffening occur (Slocum 1991). Spermine oxidase (SMO), a FAD-dependent amine oxidase, which directs the back-conversion of spermine to spermidine with concomitant production of 3-aminopropanal and H_2O_2, was initially identified in mammalian cells (Wang et al. 2001; Vujcic et al. 2002; Cervelli et al. 2003; Kusano et al. 2008). Diaminopropane can be converted into β-alanine, whereas pyrroline can be further catabolized to γ-aminobutyric acid (GABA) in a reaction catalysed by pyrroline dehydrogenase (PDH). The γ-aminobutyric acid is subsequently transaminated and oxidised to succinic acid, which is incorporated into the Krebs cycle. Thus, this pathway ensures the recycling of carbon and nitrogen from Put. Far from being only a means of eliminating cellular PAs, the enzymes involved in PA catabolism and the products deriving from their action, have been demonstrated to be involved in important physiological processes (Bouchereau et al. 1999). A simple illustration of the catabolic pathways has been shown in Fig. 19.2.

4 Role of Polyamines in Plants

The first reference of the polyamines in plants can probably be dated back to 1911 when Ciamician and Ravenna demonstrated the presence of putrescine in *Datura stramonium* (Bagni and Tassoni 2001). In plant cells, the diamine putrescine (Put),

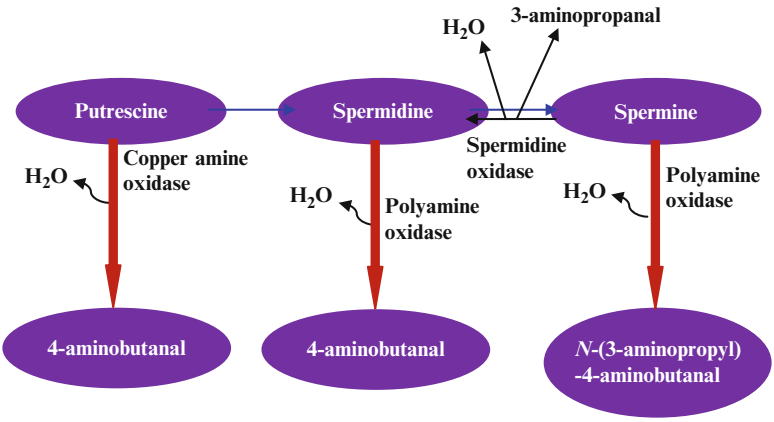

Fig. 19.2 Polyamine degradation in plants

triamine spermidine (Spd) and tetramine spermine (Spm) constitute the major PAs. Cadaverine is also present in legumes. These occur either in the free form or as conjugates bound to phenolic acids and other low molecular weight compounds or to macromolecules such as proteins and nucleic acids owing to their positive charge (Kaur-Sawhney et al. 2003). Besides stimulating DNA replication, transcription and translation, they have contributed to various biological processes in plant morphogenesis, growth, embryogenesis, organ development, leaf senescence, abiotic and biotic stress response and infection by fungi and viruses (Kumar et al. 1997; Walden et al. 1997; Malmberg et al. 1998; Bouchereau et al. 1999; Liu et al. 2000, 2010; Alcázar et al. 2006a, b; Groppa et al. 2008; Kusano et al. 2007, 2008). Their biological activity has been attributed to their cationic nature. Plant polyamines also contribute towards several characteristics of agro-economical importance, such as phytonutrient content, fruiting and fruit quality, vine life, flowering and carnation plants (Kakkar and Rai 1993; Mehta et al. 2002; Piqueras et al. 2002; Matto et al. 2006; Paschalidis and Roubelakis-Angelakis 2005).

Some of the observations suggest that PAs can act by stabilizing membranes, scavenging free radicals, affecting nucleic acids and protein synthesis, RNAse, protease and other enzyme activities, and interacting with hormones, phytochromes, and ethylene biosynthesis (Slocum et al. 1984; Galston and Tiburcio 1991). Because of these numerous biological interactions of PAs in plant systems, it has been difficult to determine their precise role in plant growth and development (Kaur-Sawhney et al. 2003). However, recent use of genomic and proteomic approaches will lead to a better understanding of the functioning in the plants (Kaur-Sawhney et al. 2003; Franceschetti et al. 2004).

The mechanisms involved in the polyamine interactions have been unveiled, at least to some extent with the help of specific polyamine inhibitors, thus explaining their physiological roles in plant growth and development. Clearly, PAs are involved in many plant developmental processes, including cell division, embryogenesis, reproductive organ development, root growth, tuberization, floral initiation and

development, fruit development and ripening as well as leaf senescence and abiotic stresses as mentioned above (Evans and Malmberg 1989; Galston et al. 1997; Bais and Ravishankar 2002; Kaur-Sawhney et al. 2003; Cona et al. 2006; Rhee et al. 2007; Groppa et al. 2008; Alcazar et al. 2010). During these developmental processes, changes in the concentrations of the free and conjugated PA's and the enzymes associated with their biosynthesis (ADC, ODC, SAMDC, etc.) take place. Many authors have reported that the increase in the PA levels and the associated biosynthetic enzymes are associated with the rapid cell division in many plant systems e.g., carrot embryogenesis (Montague et al. 1978; Feirer et al. 1984), tomato ovaries (Heimer and Mizrahi 1982; Neily et al. 2011), tobacco ovaries (Slocum and Galston 1985; Franceschetti et al. 2004), and fruit development (Kakkar and Rai 1993; Paschalidis and Roubelakis-Angelakis 2005; Falasca et al. 2010). It has been observed that cells undergoing division (apical shoots, meristems, flowers, etc) contain higher levels of PAs whereas cells undergoing expansion and elongation contain lower levels of PA synthesized via ADC (Kaur-Sawhney et al. 2003). This has been further exemplied as follows: higher levels of endogenous PAs were found in flowers and siliques as compared to their levels in leaves and bolts of certain strains of Arabidopsis; addition of the PA biosynthetic inhibitors, DFMA and CHA to the culture medium, at time of seed germination, inhibited bolting and flower formation and this was partially reversed by addition of exogenous Spd (Applewhite et al. 2000; Kaur-Sawhney et al. 2003). These results clearly show that Spd is involved in flower initiation and development. Similar results have been reported in other plants also (reviewed by Galston et al. 1997; Bais and Ravishankar 2002). The regulation of many important plant hormones such as auxins, gibberellins, ethylene, etc., which play a vital role in plant growth and developmental processes has been correlated with the changes in the PA metabolism. Of the important plant hormones, ethylene is of particular interest as PAs and ethylene are said to play antagonist roles (Kaur-Sawhney et al. 2003). While PAs inhibit senescence of leaves (Kaur-Sawhney et al. 1982), cell cultures of many monocot and dicot species (Muhitch et al. 1983) and fruit ripening (Kakkar and Rai 1993), ethylene promotes all these processes. The most commonly held view is that ethylene is an effective inhibitor of ADC and SAMDC, the key enzymes in PA biosynthetic pathway, and on the other hand, PAs tend to inhibit ethylene synthesis from SAM (Kaur-Sawhney et al. 2003). Plants are exposed to continuous and rapid changing environmental factors (biotic and abiotic) such as light, temperature, water, nutrient availability, and water. These have a major impact on plant growth and productivity and PAs play an important role in these stresses as briefly discussed below.

5 Role of Polyamines in Plant Tolerance to Abiotic Stress

Richards and Coleman (1952) observed the presence of a predominant unknown ninhydrin positive spot that accumulated in barley plants when exposed to potassium starvation. This compound was identified as putrescine. Later on, it was shown

that K-deficient shoots fed with L-^{14}C-arginine produced labeled Put in a more rapid way compared to feeding with labeled ornithine (Alcazar et al. 2010). These results suggested that decarboxylation of arginine was the main way of accumulation of Put under K deficiency (Smith and Richards 1962). The relevance of the ADC pathway in plant responses to abiotic stress was later on established by Galston et al. at Yale University (Flores and Galston 1982). It has been observed that polyamines accumulate in plants during various stressful conditions (see Bouchereau et al. 1999; Alcázar et al. 2006b, 2010; Groppa and Benavides 2008). These all reports support the fact that polyamines do play a protective role during the stressful conditions. Several examples have been quoted by Alcazar et al. (2010) in which genetic modification of the genes involved in PA biosynthetic pathway have proven useful in developing plant tolerance against abiotic stresses. The different stress factors have been briefly discussed below.

5.1 Mineral Deficiency

This is one of the most common stress related factors affecting plants almost everywhere. However, studies related to this type of stress are often performed on leaves and/or seedlings, as the external symptoms of deficiency become acute. The accumulation of Put in leaves of K-deficient barley plants was first reported by Richards and Coleman (1952) and subsequent studies by others have established that specific role of Put in maintaining a cation- anion balance in plant tissues. As a result of K starvation, this diamine accumulation (via ADC activation), is widespread among mono- and di-cotyledonous species and may well be a universal response (Bouchereau et al. 1999). The exact reason behind the increase in Put is unclear. The induced high levels of Put might be the cause of the stress injury. Put might also be beneficial for plants. Alternatively, high levels of Put could be one of the many physiological changes induced by mineral nutrient deficiency without any specific significance (Bouchereau et al. 1999). There are several other examples listing the changes in the polyamine content while responding to the mineral deficiencies (Geny et al. 1997). However, the changes differed according to the tissue and the stage of development.

5.2 Cold Stress

The injury due to cold causes alteration in the membrane structure, and the chilling injury involves phase transition in the molecular ordering of membrane lipids (Raison and Lyons 1970). This can cause several deleterious effects like increased membrane permeability and alteration of the activity of membrane proteins. Cold treatment has been reported to increase the levels of Put, and this correlates with the increase in the induction of arginine decarboxylase (ADC) genes (ADC1, ADC2

and SAMDC2) (Urano et al. 2003; Cuevas et al. 2008, 2009). On the other hand, levels of free Spd and Spm remain constant or even decrease in response to cold treatment (Alcazar et al. 2010). The absence of correlation between enhanced *SAMDC2* expression and the decrease Spm levels may be a result of increased Spm catabolism (Cuevas et al. 2008; Alcazar et al. 2010). Boucereau et al. (1999) reported that in the chilling-tolerant-cultivar, chilling induced an increase in free abscisic acid (ABA) levels first, then ADC activity and finally free Put levels. Fluridone, an inhibitor of ABA synthesis, inhibited the increase of free ABA levels, ADC activity and free Put levels in chilled seedlings of a chilling-tolerant cultivar. These effects resulted in a reduced tolerance to chilling and could be reversed by the pre-chilling treatment with ABA. All these results suggest that Put and ABA are integrated in a positive feedback loop, in which ABA and Put reciprocally promote each other's biosynthesis in response to abiotic stress (Fig. 19.1). This highlights a novel mode of action of polyamines as regulators of ABA biosynthesis (Alcazar et al. 2010).

5.3 Thermal Stress

When exposed to heat stress, plants have the ability to synthesize uncommon long chain PAs (caldine, thermine). The levels of free and bound PAs, as well as ADC and polyamine oxidases (PAO) activities, were higher in tolerant than in sensitive cultures of different crop. (Kuehn et al. 1990; Philipps and Kuehn 1991; Roy and Ghosh 1996; Bouchereau et al. 1999). The increased activities of the transglutaminases indicated the high content of the polyamines. This indicates a correlation between heat-stress tolerance, ADC, PAO and transglutaminase activities (Bouchereau et al. 1999).

5.4 Drought Stress

Certain plants during water scarcity tend to accumulate putrescine (Put) which is supported by the fact that transcript profiling under these conditions induces the expression of certain genes involved in the biosynthetic pathway. The expression of some of these genes is also induced by ABA treatment (Perez-Amador et al. 2002; Urano et al. 2003; Alcazar et al. 2010). This throws light upon the fact that up- regulation of PA-biosynthetic genes and accumulation of Put under water stress are mainly ABA-dependent responses (Alcazar et al. 2010).

5.5 Salt Stress

Differences in PA (Put, Spd, Spm) response under salt-stress have been reported among and within species. For example, according to Prakash and Prathapsenan (1988),

endogenous levels of PAs (Put, Spd and Spm) decreased in rice seedlings under NaCl stress, whereas Basu et al. (1988) reported that salinity resulted in accumulation of these compounds in the same material (Bouchereau et al. 1999). Santa-Cruz et al. (1997) reported that the (Spd + Spm):Put ratios increased with salinity in the salt-tolerant tomato species (*Lycopersicon pennellii*, Carrel D'Arcy) but not in the salt-sensitive tomato species (*L. esculentum*). In both species, stress treatments decreased the levels of Put and Spd. The Spm levels did not decrease with salinity in *L. pennellii* over the salinization period, whereas they greatly decreased in *L. esculentum*. The effects of different NaCl concentrations on maize embryogenic cells derived from immature embryo cultures of a salt-sensitive inbred line (cv. w64) and a resistant hybrid (cv, Arizona) have also been reported where increased salt concentration remarkably decreased the growth of the calluses and showed a significant increase in the total PA (Put, Spd) content, especially caused by a rise in Put. It has been reported by Bouchereau et al. (1999) that using the inhibitors of Put synthesis, the ADC pathway in tomato plants operates in both stress and control conditions, whereas the ODC pathway is stimulated only under the stress conditions. These findings are further supported by the studies of Urano et al. (2003) who concluded that the expressions of the arginine decarboxylase 2(ADC 2) and spermine synthases (SPMS) during the 24 h stress treatment maintained and hence, increased the levels of Put and Spm. Yamaguchi et al. (2006) also suggested the protective role of Spm when its addition suppressed the salt sensitivity in Spm deficient mutants. Bouchereau et al. (1999) suggested that polyamine responses to salt stress are also ABA-dependent, since both *ADC2* and *SPMS* are induced by ABA. In fact, Alcazar et al. (2006a) reported that stress-responsive, drought responsive (DRE), low temperature-responsive (LTR) and ABA-responsive elements (ABRE and/or ABRE-related motifs) are present in the promoters of the polyamine biosynthetic genes. This also reinforces the view that in response to drought and salt treatments, the expression of some of the genes involved in polyamine biosynthesis are regulated by ABA (Alcazar et al. 2010). The study of the *Arabidopsis thaliana* flowers by Tassoni et al. (2010) has also supported the hypothesis that polyamine levels (mainly Spm) increase with the increase in the salt concentration and therefore, contribute to plant tolerance during the stressful conditions.

5.6 Osmotic Stress

Osmotic treatments using sorbitol induced high levels of Put and ADC in detached oat leaves (Flores and Galston 1984), whereas, Spd and Spm show a dramatic decrease. Bouchereau et al. (1999) reported that osmotica with widely different assimilation routes, such as sorbitol, mannitol, proline, betaine and sucrose, all induce a rise in Put. These changes are coincident with measurable signs of a stress, such as wilting and protein loss. Tiburcio et al. (1995) reported that when peeled oat leaves are incubated with sorbitol in the dark, they lose chlorophyll and senescence rapidly. Senescence could be delayed by including Spm in the incubation medium.

The senescence-retarding effect of Spm was correlated with increase in the incorporation of labeled precursors into proteins, RNA and DNA. They also concluded that osmotic shock in the dark induces an activation of the pathway catalyzed by ADC. Borrell et al. (1996) have reported the regulation of ADC synthesis by Spm in osmotically-stressed oat leaves using a polyclonal antibody to oat ADC and a cDNA clone encoding oat ADC. Treatment with Spm in combination with osmotic-stress resulted in increased steady-state levels of ADC mRNA, yet the levels of ADC activity decreased. This absence of correlation has been explained by the fact that Spm inhibits processing of the ADC proenzyme, which results in increased levels of this inactive ADC form and a subsequent decrease in the ADC-processed form (Bouchereau et al. 1999). They also showed that in osmotically-stressed oat leaves, degradation of cytochrome thylakoid proteins and the enzyme rubisco can be avoided by addition of Spm to the incubation medium. Thus post-translational regulation of ADC synthesis by Spm may be important in explaining its anti-senescence properties. Interestingly, Masgrau et al. (1997) concluded that the over-expression of oat ADC in tobacco resulted in similar detrimental effects to those observed by ADC activation induced by osmotic-stress in the homologous oat leaf and stem (chlorosis and necrosis). Therefore, optimum levels of polyamines are necessary for the proper growth and development of plants (Bouchereau et al. 1999). Recently, Liu et al. (2010) have investigated the changes in the content and the form of polyamines (PAs) in the leaves of two wheat (*Triticum aestivum* L.) cultivars seedlings, differing in drought tolerance, under the osmotic stress by polyethylene glycol (PEG) treatment. The results suggested that free-Spd, -Spm and PIS-bound Put (perchloric acid insoluble bound putrescine) facilitated the osmotic stress tolerance of wheat seedlings. The important roles of reactive oxygen species in the relationship between ethylene and polyamines (PAs) have also been investigated in the leaves of spring wheat seedlings under root osmotic stress (Li et al. 2010).

5.7 Hypoxia

There has been a lot of work done by Reggiani's group on the role of polyamines under the hypoxic stress conditions. Reggiani et al. (1990) reported that there are many examples available where plant shoots and seedlings of different Gramineae species, when subjected to lack of oxygen, provide evidence of an association between tolerance and the capacity to accumulate Put. Species such as rice and barnyard grass which are adapted to germinate in an oxygen deprived environment, showed a greater capacity of Put accumulation than the anoxia-intolerant species (Reggiani and Bertani 1989). This consideration supports the hypothesis for a role of Put as a protective compound against hypoxia (Reggiani and Bertani 1990; Bouchereau et al. 1999). Reggiani et al. (1989) have reported that Put is required for the anaerobic elongation of rice coleoptiles, but it has no effect on aerobic elongation of rice coleoptiles where auxin is active. This group has also concluded that with a decrease in oxygen concentration, the conjugated Put became predominant in

comparison with the free forms (80% at 0.3% oxygen) and there is a negative correlation between Put accumulation (specially under conjugated forms) and shoot elongation (Reggiani and Bertani 1989; Bouchereau et al. 1999). On the other hand, the results of Lee et al. (1996) have indicated that increase in the activities of ADC and ODC, and Put levels are essential for the elongation of *Scirpus* shoots grown under submergence.

5.8 Ozone Stress

Ozone, the protective gas in the upper atmosphere, is known to protect us from the harmful UV rays of the sun. But it is known to have serious effects on the vegetation. Experiments are ongoing throughout the world in this respect. According to Heagle (1989) O_3-stress can lead to a significant decline in net photosynthesis, cause leaf injury and accelerate senescence, even when applied at low levels. Reaction to this stress triggers many biochemical changes in plants such as increase in ABA, peroxidases, phenolic compounds, ethylene and polyamines, which form a part of the plant self-defense mechanism. Rowland-Bamford et al. (1989) observed that the ADC activity in the ozone treated barley leaves increased before the damage became apparent. Many more examples have been quoted by Bouchereau et al. (1999) supporting the protective role of the polyamines during the ozone damage. Though the exact mechanism is not clear, there can be a possibility of PAs being involved in the free radical scavenging (Bors et al. 1989). This is also supported by the fact that the levels of superoxide radical formed enzymatically with xanthine oxidase or chemically from riboflavin or pyrogallol were inhibited *in vitro* by Put, Spd or Spm at 10–50 mM (Drolet et al. 1986). Also, superoxide radical protection was inhibited by PAs when added to microsomal membrane preparations. These findings have been also supported by the fact that PAs tend to inhibit lipid peroxidation (Tang and Newton 2005; Zhao and Yang 2008). These conclusions were, however, disputed by the findings of Langebartels et al. (1991) as mentioned by Bouchereau et al. (1999). Leaf injury, caused by O_3 in the tobacco cultivar Bel W3 could be prevented by feeding Put, Spd or Spm through the root. These exogenous treatments were correlated with a two to three-fold increase in soluble conjugated Put and Spd (monocaffeoyl forms). Conjugated Put and Spd associated with cell wall and membrane fractions were increased four to six-fold. When free PAs were assayed *in vitro* for their radical-scavenging properties, very low rate constants were found. On the other hand, PA conjugates had relatively high rate constants. It was thus concluded that free PAs could not account for the protection against O_3 damage. But assuming their role in the ozone damage, it was suggested that the protective effect of exogenous free PAs was mediated by their prior conversion to conjugated forms. Consistent with this hypothesis, it was found that monocaffeoyl Put, an effective scavenger of oxyradicals, was present in the apoplastic fluid of tobacco leaves exposed to O_3 (Dat et al. 2003). The results of Navakoudis et al. (2003) also support these findings showing that the enhanced atmospheric ozone is the accumulation of polyamines,

generally observed as an increase in putrescine level, and in particular its bound form to thylakoid membranes. A study by Schraudner et al. (1990) also discovered a relationship between ethylene emission and PA biosynthesis was found in O_3-treated potato and tobacco plants, the leaves of which show early senescence in response to the pollutant. In the presence of O_3, all compounds of ethylene biosynthetic pathway in tobacco leaves were up-regulated. Put and Spd levels also increased, as did ornithine decarboxylase (ODC) activity (Bouchereau et al. 1999).

6 Polyamine Biosynthetic Genes and Stress Tolerance

The expression of genes responsible for the PA synthesis has benefited the plants to withstand environmental stresses. The over-expression or the down-regulation of the genes for PA metabolism in transgenic plants have been reported by many workers during environmental stress (Kumar et al. 1997; Walden et al. 1997; Malmberg et al. 1998; Capell et al. 1998; Rajam et al. 1998; Roy and Wu 2001; Bhatnagar et al. 2002). The genes which have been reported to be involved in the PA metabolism are *ODC*, *ADC* or *SAMDC*. Bhatnagar et al. (2002) have demonstrated that the cellular levels of Put increases by overexpressing *ODC* or *ADC* cDNA. Panicot et al. (2002a) have also reported that overexpression of *ODC* or *ADC* cDNA increases the Put levels in plants. Cheng et al. (2009) reported that transformation of yeast *SAMDC* in tomato increased Spm and Spd under high temperature stress. Overexpression of *SPDS* in Arabidopsis (Kasukabe et al. 2004); tobacco (Franceschetti et al. 2004) and sweet potato (Kasukabe et al. 2006) plants have conferred tolerance to multiple stresses. Polyamines have been proved to act as antioxidants and protect the plants from oxidative damage and maintain homeostasis in plant cells (Rodrıguez-Kessler et al. 2006). Accumulation of polyamines during environmental stresses in plants has been associated with increase in the levels of antioxidant enzyme activities like SOD, CAT, etc. Increase in MDA content has been observed during temperature stress in tomato, which leads to lipid peroxidation (Cheng et al. 2009). Overexpression of *ySAMDC* in transgenic tomato increases the Spm and Spd levels, which in turn decreases MDA content (Cheng et al. 2009). The overexpression of *SAMDC* gene in transgenic rice and tobacco showed increased levels of PA and confers tolerance to drought and salinity (Roy and Wu 2002; Waie and Rajam 2003). Table 19.1 provides further information about the PA transgenics.

7 Integration of Polyamines with Other Molecules During Stress Conditions

Polyamines affect several physiological processes in plants by activating the biosynthesis of signaling molecules like NO, H_2O_2; they affect ABA synthesis and signaling and are involved in Ca^{2+} homeostasis and ion channel signaling during the abiotic stress conditions. Figure 19.1 summarizes this information.

Table 19.1 Polyamine genes that can be expressed in plants for abiotic stress tolerance

Gene overexpressed	Plant	Response	Reference
ADC	*Oryza sativa*	Salt tolerance	Roy and Wu (2001)
ADC	*Brassica juncea*	Chilling and salt	Mo and Pua (2002)
ADC1, ADC2	*Oryza sativa*	Drought tolerance	Capell et al. (2004)
ADC1, ADC2	*Arabidopsis thaliana*	Freezing	Cuevas et al. (2008)
ADC	*Malus domestica*	Chilling, Salt and Dehydration	Hao et al. (2005)
ADC	*Oryza sativa*	Chilling	Akiyama and Jin (2007)
At ADC2	*Arabidopsis thaliana*	Salt tolerance	Urano et al. (2004)
ADC	*Solanum melongena*	Chilling, Salt and Dehydration	Prabhavathi and Rajam (2007)
ADC	*Zea maize*	Salt	Jimenez-Bremont et al. (2007)
MdADC	*Malus sylvestris* (L.) Mill. var. domestica	Salt tolerance	Liu et al. (2006)
PaADC2	*Pringlea antiscorbutica*	Chilling and salt	Hummel et al. (2004)
Mouse *ODC* cDNA	*Populous nigra* X maximowiczii cells	Stress tolerance	Bhatnagar et al. (2001)
Mouse *ODC* cDNA	tobacco	Salt stress	Kumria and Rajam (2002)
MdSAMDC2	*Malus sylvestris*	Cold and salt	Hao et al. (2005)
MdSAMDC2	*Pyrus communis*	salt	He et al. (2008)
SPDS cDNA from Cucurbita ficifolia	Arabidopsis	Chilling, salinity, drought	Kasukabe et al. (2004)
SPDS cDNA from Cucurbita ficifolia	Sweet potato	Increase in Spd	Kasukabe et al. (2006)
MdSPDS1	*Pyrus communis*	Salt, Heavy metal and osmotic stress	Wen et al. (2008)

Abscisic acid (ABA) is an anti-transpirant that reduces water loss through stomatal pores on the leaf surface in response to water deficit, resulting in the redistribution and accumulation of ABA in guard cells and finally closure of the stomata (Bray 1997). Many authors (Liu et al. 2000; An et al. 2008; Alcazar et al. 2010) have reported that Put, Spd and Spm also regulate stomatal responses by reducing their aperture and inducing closure, and Put modulates ABA biosynthesis in response to abiotic stress. Thus, polyamines are involved in the ABA mediated stress responses which affect the stomatal closure. Polyamines are also linked with reactive oxygen species (ROS) and NO signaling as amino oxidases during the catabolic process generate H_2O_2 which is a ROS (associated with plant defense and abiotic stress) and also there is evidence in which polyamines are reported to enhance the production of NO (Tun et al. 2006). NO is also known to enhance the salt stress tolerance in plants by regulating the content and proportions of the different types of free polyamines. According to Neill et al. (2008), both H_2O_2 and NO are involved in the

regulation of stomatal movements in response to ABA, in such a way that NO generation depends on H_2O_2 production. Thus, altogether, the available data indicate that polyamines, ROS (H_2O_2) and NO act synergistically in promoting ABA responses in guard cells (Alcazar et al. 2010).

Polyamines are positively charged compounds, which can interact electrostatically with negatively charged proteins, including ion channels. Indeed, polyamines at their physiological concentration block the fast-activating vacuolar (FV) cation channel in a charge-dependent manner ($Spm^{4+} > Spd^{3+} >> Put^{2+}$), at both whole-cell and single-channel levels, thus indicating a direct blockage of the channel by polyamines (Bruggemann et al. 1998). According to Alacazar et al. (2010), in response to different abiotic stresses, such as potassium deficiency, Put levels are increased drastically (reaching millimolar concentrations), whereas the levels of Spd and Spm are not significantly affected, and this increase of Put may significantly reduce FV channel activity. Bruggemann et al. (1998) have also reported that all PA levels increase in amount, and the enhanced Spm concentration probably blocks FV channel activity under salinity stress. These observations can be explained by the fact that polyamines in plants may thus modulate ion channel activities through direct binding to the channel proteins and/or their associated membrane components (Delavega and Delcour 1995; Johnson 1996; Alcazar et al. 2010). Phosphorylation and dephosphorylation of ion channel proteins are closely related to their activities. Thus, polyamines could also affect protein kinase and/or phosphatase activities to regulate ion channel functions (Bethke and Jones 1997; Michard et al. 2005; Alcazar et al. 2010). However, Zhao et al. (2007) points out that for elucidating the molecular mechanisms underlying polyamine action, identification of ion channel structural elements and/or receptor molecules regulated by polyamines would be of great importance.

Polyamines also tend to maintain Ca^{2+} homeostasis. Several examples have been reported by Alcazar et al. (2010). Yamaguchi et al. (2006, 2007) proposed that the protective role of Spm against high salt and drought stress is a consequence of altered control of Ca^{2+} allocation through regulating Ca^{2+}permeable channels. The increase in cytoplasmic Ca^{2+}results in prevention of Na^+/K^+ entry into the cytoplasm, enhancement of Na^+/K^+ influx to the vacuole or suppression of Na^+/K^+ release from the vacuole, which in turn increases salt tolerance (Yamaguchi et al. 2006; Kusano et al. 2007; Alcazar et al. 2010). Thus, polyamines have a definite role in calcium homeostasis during stress conditions.

8 Conclusions and Future Prospects

Considerable evidence shows that polyamines (PAs) are involved in a myriad of plant processes including DNA regulation, gene transcription, organ development, fruit ripening, leaf senescence and various environmental stresses. The use of the genetic approaches, proteomic approaches and various analytical techniques have made it possible to further understand their mechanisms of action, binding, interaction,

transport, signaling, homeostatic control of their metabolic pathways and their defensive role in biotic and abiotic stress conditions, although the exact reasoning is still difficult to interpret. Nevertheless, even this lack of information does not hamper further research into polyamines as they now constitute one of the widely distributed groups of organic molecules in nature with an important contribution towards maintaining plant growth and development, increasing crop production, defensive actions during stress conditions, combating various diseases and more recently acting as biomarkers for cancer detection. Thus, a spectral range of their applications in plants, animals and mammals offer a wide scope into their further research.

Polyamines have now been considered as secondary messengers in addition to being known as vital plant regulators (Liu et al. 2007). Although the exact mechanism of action of polyamines during the stressful conditions is not known, genetic tools have been found useful; traditional quantitative trait loci (QTL) mapping (Alonso-Blanco et al. 2009) and genome-wide association mapping (Nordborg and Weigel 2008) can be used for the identification of the genes underlying the mode of action and regulation of polyamines (Alcazar et al. 2010). Cloning of these genes would be another added advantage as these could be used in the same way as from chemicals to alleviate or mitigate stress derived injury for crop protection. Transfer of such technology to the other crops will help create germplasm which would be better adapted to the harsh stressful conditions and thus contributing to enhanced agricultural productivity.

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Part III
Crop Management

Chapter 20
Crop Diversification Practices in Saskatchewan, Canada

Ahmet Ruhi Mermut

Contents

Abstract Agriculture in the province of Saskatchewan is currently undergoing a significant structural change in response to changing economic, environmental, and political conditions. Diversification is becoming an agricultural strategy to reduce economic risk on the farm. Deciding on the type of diversification is not easy. The strategy is to integrate environmental concerns into the development process without fettering development itself. It has to improve environmental conditions and to increase productivity, especially of degraded lands. The Canadian Prairies pause to celebrate the centennial year of agriculture. The farmers and scientists look to a bright future as they continue to bring innovative ideas and technologies to crop producers. The farmers are increasingly adopting extended and diversified crop rotations together with conservation tillage practices. Many of these newer cropping systems are recognized as being more environmentally sustainable; however, often there is a conflict between achieving the long-term goal of resource sustainability

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and the short-term goal of economic viability. Adoption of technologies to improve nitrogen use efficiency will have the potential to significantly reduce energy use and Green House Gases (GHG). Application of all nitrogen at seeding or split within crop application will reduce the risk of loss of income due to under- or over-fertilization. Including oilseed and pulse crops in rotations that have traditionally been monoculture and cereal based, and reducing the frequency of summer fallow, contributes to higher net farm incomes in most regions, despite the higher production costs. In general, the profitability of cereal–oilseed–cereal pulse systems is>cereal–oilseed>monoculture cereal rotations in the more humid regions.

Keywords Crop diversification • Canadian agriculture and resources • Water management • Conservation tillage

1 Introduction

Centennial celebration of agriculture is now taking place in the Canadian Prairies. Farmers look to a bright future as they continue to bring innovative ideas and technologies to help the agricultural industry in the region to grow and prosper. Monocultures or cropping systems with a small number of crop components were the cropping systems and highly simplified in early stage of agriculture in western Canada.

Climatic conditions in North America are similar to those in Eurasian Steppes. Cold semi-arid regions of the Northern Great Plains (Prairies) receive about 350–400 mm rain per year. These numbers show that the Canadian Prairies are analogous to those in the Eurasian Steppes. Rainfall in Saskatchewan varies between 250 and 450 mm per year with a growing season of 100–120 days $year^{-1}$ (Cameron and Oram 1994). Great similarities in soils, landscapes, geology, hydrology, and ecology exist between Canadian Prairies and northern Kazak Plateau, including the cultivation history. Large areas were opened for agriculture after the World War II in both regions.

One of the fundamental arguments is that, about 50% of soil organic matter (SOM) is lost in the top soil, due to intensive agricultural practices in North America (Lal et al. 1999). Uncultivated soils were in equilibrium with the native vegetation and accumulated large SOC reserves and cultivation has disrupted the steady state equilibrium. The lost carbon primarily can be returned to the soil. With good management practices it may be possible to exceed the original native SOM content of many soils. Reduced grain price in the last 15 years has forced Canadian farmers to diversify agriculture and test new crops in the prairies. These include canola, chick peas, lentil, pigeon pea etc.

Research around the world has proven that soil fertility and organic matter is declining due to long-term use of conventional tillage techniques (Putterbaugh 1993; Acton and Gregorich 1995). The loss of soil fertility and organic matter are the two major factors that cause the reduction of soil quality. Commercial fertilisers can be applied to correct the soil fertility, but this will not directly influence the soil

organic matter. Loss of organic matter will adversely affect the soil erosion and increased erosion will cause a further decline of soil fertility and overall soil quality. Low fertility will produce less biomass, contributing faster decomposition of soil organic matter.

2 Land Resources

Farmland is concentrated in the south, reflecting severe limitations of climate and soils further north. Most productive land is located within 200 km of the U.S.A. border, in the agricultural heart lands of Ontario and Quebec, extending considerably farther north in the wheat land of Saskatchewan and adjacent parts of Alberta and Manitoba. About 7% of the land mass is used for agriculture, which is about 67.8 million ha. Of this land area:

1. Only 33 million ha (49%) were actually cultivated cropland.
2. The total number of farms is about 293 000 with an average size of 231 ha.
3. Today population employed directly in agriculture is a little over 3%.

About 47.6 million ha of land alone occur in the Canadian Prairie Provinces. The amount and distribution of land masses in different soil zones are shown in Table 20.1 and Figs. 20.1 and 20.2. Despite the long distance from markets and poor market structures, Canadian agriculture is so highly integrated within international trade through the *greater liberalization of international trade*. The results of this policy are yet to be seen.

3 Water Resources Management

1. Basic ideas of the water resource managements include:
2. Surface irrigation where it is possible
3. Groundwater management
4. Watershed development and management-participatory approach
5. Every drops counts: Ground water recharge/ rainwater harvesting a movement in the farmer's field- peoples' participation
6. Convergence approach

4 Crop Diversification Strategy

According to Agriculture Canada (1989), crop diversification strategy has four main pillars as shown in Fig. 20.3. These include: sustainability, diversification, market responsiveness, and self-reliance. This strategy includes the following principles:

1. More crops per drops
2. Productivity and yield gap syndrome- a human development setting

Table 20.1 Percent of land mass distribution in different soil zones of Prairie Provinces of Canada (Agriculture Canada 1989)

Soil zone	Manitoba	Saskat	Alberta	Total
Brown	–	25	17	19
Dark brown	–	35	19	25
Black	77	30	19	25
Dark grey	14	8	16	11
Grey luvisol	9	2	9	6
Total	11.1	45.1	24.2	80.4

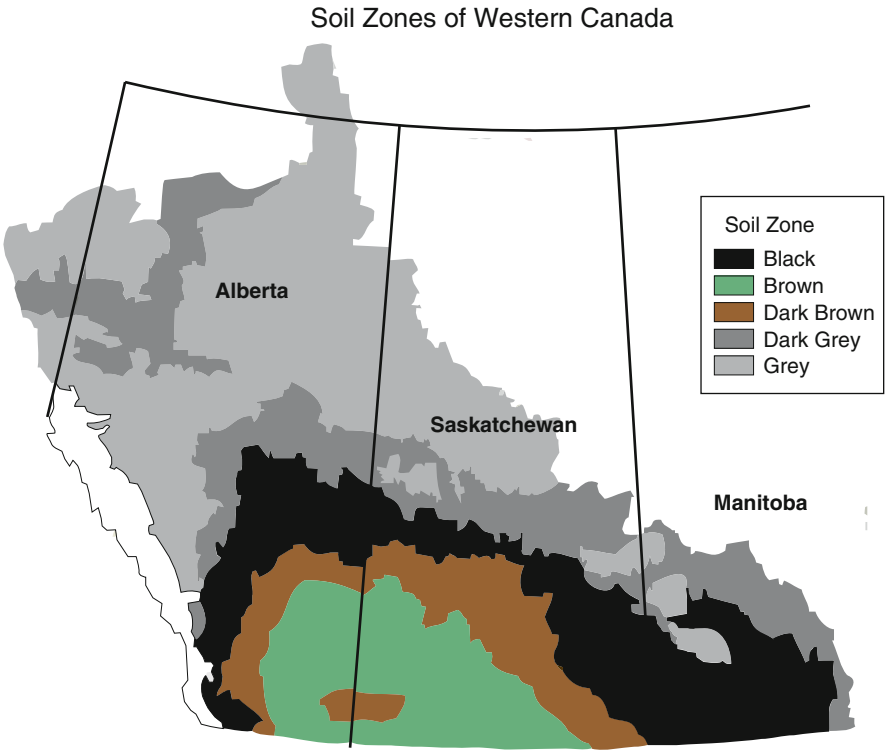

Fig. 20.1 Distribution of soil zones in three western provinces of Canada

3. Grey into green (Wasteland development)
4. Contract Farming
5. Reach the unreached in real time (Revamping of agricultural extension system)
6. Precision farming
7. Agricultural marketing – Post harvest management- in a global setting

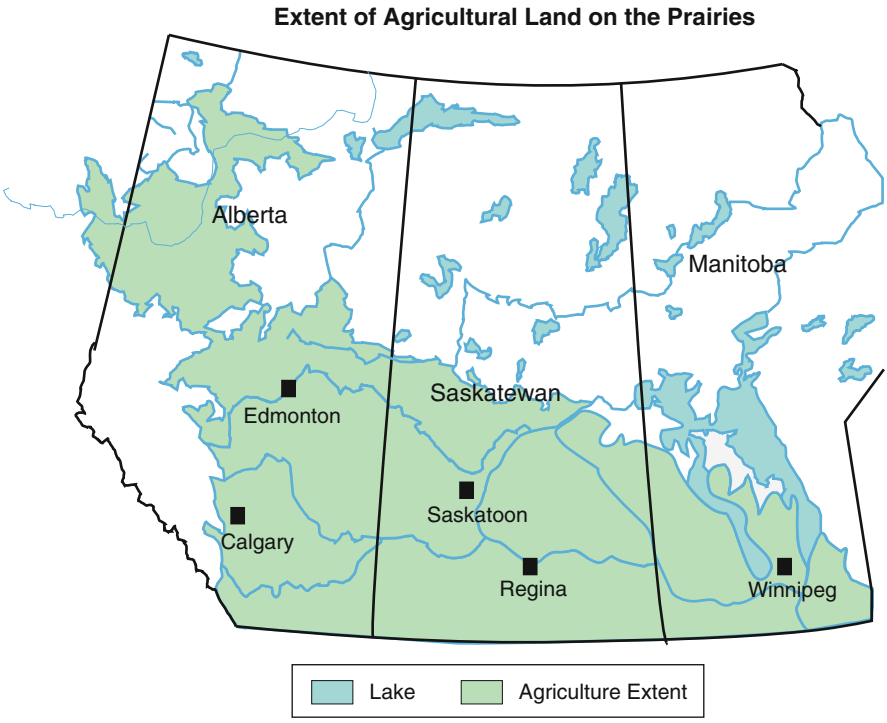

Fig. 20.2 Agricultural land area, in Manitoba, Saskatchewan, and Alberta (Agriculture Canada 1989)

5 Non-renewable Energy

This includes both as direct energy such as diesel, gasoline and indirect energy (fertilizer). Direct on-farm energy expenditure for mechanical power accounts for over $1 billion. This makes about 10% of total farm operating cost in the three Prairie Provinces. Gasoline, diesel and other fuels met over 70% of this requirement; electricity and natural gas constituting the remainder. Primary agricultural production also requires indirect energy embodied in machinery, fertilizer and pesticides. Nitrogen fertilizer can account for up to 70% of the energy used in crop production.

The types of crops produced and the amount of crop inputs will change in response to changes in climate and changes in the relative cost of inputs as they are affected by Green house gas (GHG) reduction policies and climate adaptation strategies in other sectors.

6 Project on Crop Diversification Scenarios

High fertilizer and energy efficiency of dry beans and chickpeas makes them significant crops in Western Canada. After a century of wheat farming Canadian farmers turn to tropical chickpeas, lentils etc., the increase in pulse and oilseed

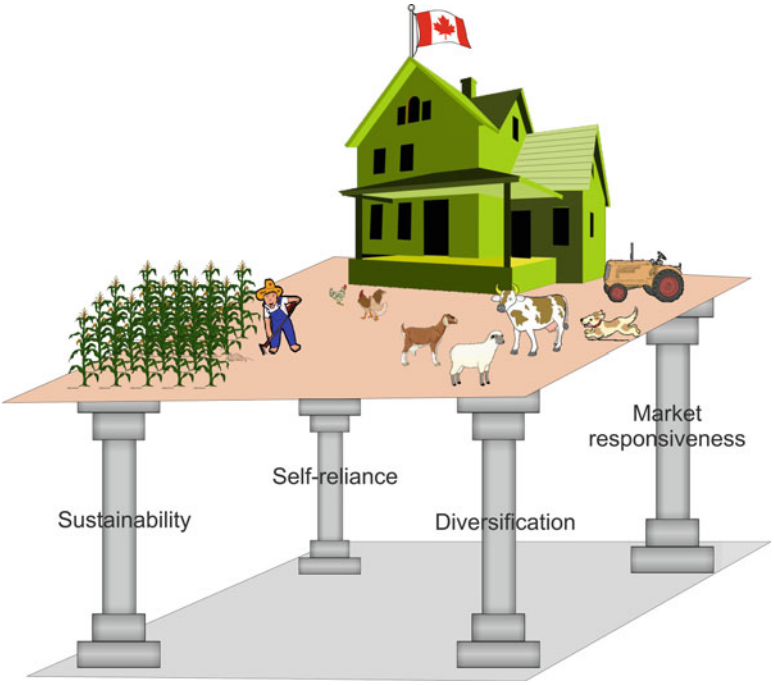

Fig. 20.3 A vision of agriculture in Canada, conceptualizing crop diversification as one of the four essential supporting pillars

Table 20.2 A comparison of pulse crops seeded in 2000 and 2001 (000 ha) (Statistic Canada 2002)

Crop	Year 2000	Year 2001	2000–2001 Change %
Dry peas	1,240	1,461	+18
Lentils	689	732	+5
Chickpeas	295	502	+70
Dry beans	169	163	–4

crops area for each province predicted to be in the year 2005 were 41%, 56%, and 51% for Alberta, Saskatchewan and Manitoba, respectively (Table 20.2).

7 Summer Fallow

In the past two decades, the area under summer fallow decreased in all soil zones, but the decline was small in the drier Brown soil zone (Statistic Canada 1988). The largest decline in fallow frequency occurred in the Black, Dark Gray, and Gray soil zones (subhumid region) where fallow area dropped 63% from 3.6 Mha in 1976 to 1.3 Mha in 1998. Over the same period, fallow area has decreased approximately

50% in the Dark Brown soil zone from 3.4 to 1.7 Mha. In the Brown soil zone, fallow remained constant at about 2.9 Mha until the period between 1996 and 1998 when fallow area dropped 16% to 2.4 Mha.

8 No Tillage or Conservation Tillage

No-till systems, do not use tillage for establishing a seedbed. Crops are simply planted into the previous year's crop residue. No-till planters are equipped with coulters that slice the soil, allowing a double disc opener to place the seed at a proper depth. The slot is closed with a spring press wheel. Herbicides are typically used as the sole means for weed control in no-till systems.

The term *conservation tillage* refers to a number of strategies and techniques for establishing crops in a previous crop's residues, which are purposely left on the soil surface. The principal benefits of conservation tillage are improved water conservation and the reduction of soil erosion. Additional benefits include: reduced fuel consumption, reduced compaction, planting and harvesting flexibility, reduced labor requirements, and improved soil tilth.

9 Conclusions

1. Most grain growers are well aware of the relationship between no-till and increased use of herbicides and the consequent exposure to herbicide resistance risk. This problem is causing a substantial number of growers to reduce their use of no-till. However, with the soil conservation benefits a key driver, no-till adoption is expected to increase over the next 5 years.
2. The adoption of minimum tillage, stubble retention, direct drill, and no-till sowing systems have allowed greater cropping intensity and been associated with reduced risk of soil degradation.
3. Including oilseed and pulse crops in rotations that have traditionally been monoculture and cereal based, and reducing the frequency of summer fallow, contributes to higher net farm incomes in most regions, despite the higher production costs.
4. In general, the profitability of cereal–oilseed–cereal pulse systems > cereal–oilseed > monoculture cereal rotations in the more humid regions.

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Chapter 21
Invasive Weed Species – A Threat to Sustainable Agriculture

Ghazala Nasim and Asad Shabbir

Contents

Abstract There are two main threats to biodiversity in the world. One of them is the direct inadvertent destruction of habitats by people which is done basically through inappropriate resource use or pollution the other highly serious but under

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estimated problem is the threat to natural and semi-natural habitats by invasion of alien organisms it is the lasting threat as compared to the first one because when exploitation stops the existing aliens do not disappear but continue to spread and consolidate. Our planet is quickly being taken over by hundreds of hardy aggressive species spread either knowingly or accidentally by human hands these are considered the second most significant threat to biodiversity after habitat destruction. These weeds are causing damages worth billions of dollars world wide and Pakistan being no exception. The countries rich repository of 6,000 of vesicular plants is highly threatened by a number of alien weeds among those some are worth mentioning such as Parthenium weed (*Parthenium hysterophorus*), Lantana (*Lantana camara*), Paper mulberry (*Brossonetia papyrifera*), Mesquet (*Prosopis juliflora*), Wild tamarind (*Leucaena leucocephala*), Khakiweed (*Alternanthera pungens*) etc. These weeds especially *P. hysterophorus* are highly noxious plant species and threatening the natural and agriculture ecosystem of the country. *Parthenium* is allergenic to both humans and animals and after invading Punjab is quickly moving towards NWFP. This is primarily because of unawareness about this plant and secondly that it is being constantly used in floral bouquets. Similarly there are many more in the list developed by IUCN in the year 2000 for Pakistan. Though it is difficult to manage these aggressive alien organisms but success stories have been documented regarding biological/chemical control and other integrated approaches. More over a careful revision of legislation regarding quarantine and enforcement of the same may prove useful to mitigate the threats of these weeds.

Keywords Invasive weeds • Biological pollution • Mycotrophy • Mycorrhiza • Phytosanitation • Management • Biological control

1 Introduction

A plant considered undesirable, unattractive, or troublesome, especially one growing where it is not wanted, as in a garden or any plant that is a hazard, nuisance, or causes injury to man, his animals, or his desired crops is called weed. Etymologically, "weed" derives from the Old English word for "grass" or "herb," but during the Middle Ages the meaning has changed to indicate an undesirable plant that grows where it is not wanted, especially among agricultural plots. This has historically been the primary meaning of the word, although in the nineteenth century, American writers grew increasingly aware that calling a plant a "weed" was an arbitrary human judgment, as there is no natural category of weeds. In the words of Ralph Waldo Emerson, a weed "is a plant whose virtues have not yet been discovered." Today, biologists tend to share that opinion, since many of the plants that are designated as weeds are, in fact, closely related to popular crops. Indeed, "weed" has fallen out of usage among biologists, although those who study agriculture still find the term useful in discussions of weed control and management (Radosevish et al. 2007).

Weeds are native or non-native plants that are unwanted in a particular area at a particular time. Change the area and the time and the plant might be desirable or even cultivated. When weeds become so wide-spread that they threaten crops, livestock, or native species, they may become more than just a "weed". They may then find themselves on a state list of plants to be attacked in a methodical manner with state support. They might then be termed "noxious weed", "invasive species", "exotic species", "alien species", or some similar term as set forth in law by each governing body. An invasive species id defined as a foreign species whose introduction does, or is likely to, cause economic or environmental harm or harm to human health. These noxious weeds find their way into new areas via boats, planes, soles of shoes, imported animals and agricultural products, etc. With increasing frequency, these most dangerous of weeds are causing the extermination of native flora and fauna from larger and larger areas. In the USA alone the invasive plants, animals and aquatic organisms have significantly reduced the economic productivity and ecological balance of the U.S. agriculture and natural resources. On land under cultivation, weeds compete with crops for water, light, and nutrients. On rangelands and in pastures, weeds are those plants that grazing animals dislike or that are poisonous. Many weeds are hosts of plant disease organisms or of insect pests. Some originally unwanted plants later were found to have virtues and came under cultivation, while some cultivated plants, when transplanted to new climates, escaped cultivation and became weeds in the new habitat. Density of single or many weed species can be changed depending on some factors during a long period. Because of globalization of economies and subsequent movement of people and materials, invasion by alien plant species is going to be the second important factor causing loss of biodiversity next to land use pattern. Changes in atmospheric composition and climate are regarded as long term factors influencing weed invasion, increasing in relative importance over time (Adkins 1995).

An invasive species is an introduced species that spreads out and often causes ecological effects on other species of ecosystems. These invasive exotic plants can cause significant displacement of native vegetation. But exactly what makes these exotic plants invasive? To begin with let's start with some basic definition: The vegetation historically found in a local area is termed native vegetation. These plants have traditionally been found in the area and are well-suited to maintain themselves in their environments. Exotic plants are those plants found in a particular area, but which originate from another continent or country. These plants can also be referred to as non-native. However non-native plants are not always exotic. Non-native plants may also be native elsewhere in the same country, but not found in the local area. For example, redwoods are native to California and would be non-native in Tennessee, but not exotic. Invasive plants are plants, native, non-native, exotic, that can cause significant ecological or economic damage. Invasive plants can displace more ecologically and economically valuable plants. These plants are typically characterized by a rampant rate of spread. This rate of dispersal is due to both human activity and vegetative characteristics. Humans assist in the dispersal of plants by moving plants materials to and from locations. Humans also cause significant disturbance to native

Table 21.1 A list of characters which enable weedy plants to become so competitive, persistent, and pernicious

1. **Number of seeds per plant**: Weeds are producers of large number of seeds. Example: Barnyard grass, 7,000 seeds; Common Purslane, 52,000 seeds; Common Lambs quarters, 72,000 seeds; Redroot Pigweed, 117,000 seeds; Russian Thistle, 200,000 seeds.
2. **Dormancy and longevity**: Dormancy is the ability of seeds to remain viable in the soil for extended periods of time. For Example: Johnson grass, 20 years; Field Bindweed, 20+ years; Common Lambs quarter, 40 years; Redroot Pigweed, 40 years.
3. **Special Adaptations or Appendages**: Plants have developed means to assist in their spread and distribution, these include: Hooks and spines – sandbur, puncture vine; Pappus (parachutes) – musk thistle, milkweeds; Good Looks – spotted knapweed, white top, Dalmatian toadflax.
4. **Vegetative Reproductive Capabilities**: Vegetative reproductive structures are those asexual portions of the plant which allow for new plants to arise without the fertilization of the flower. Examples include the following: Roots with adventitious buds Ex.: Leafy Spurge and Canada Thistle; Rhizome Ex. Johnson grass and Bermuda grass; Tubers Ex.: Yellow and Purple Nutsedge; Crowns Ex.: Dandelions and Plantains; Stolons Ex.: Bermuda grass; Special Characteristics: "Weeds have the greater will to live".
5. **Weapon of Mycotrophy**: Weeds owe their success towards the ease with which they establish symbiosis with the native soil inhabiting arbuscular mycorrhizal fungi however they do not entirely rely on the association for their survival.

plant communities that provide opportunities for the establishment of exotic and/or non-native invasives. The seeding and sprouting characteristics of the plants also significantly contribute to the rate of dispersal. Though a negative term, "invasive" plants can also be native plants that we usually do not consider to be a problem (Anderson 1996).

The introduction of many non-native or exotic plants has occurred both intentionally, as with ornamental plantings, and accidentally, as a by-product of commerce. For example, the movement of hey, carrying seeds of invasive plants, can introduce exotic and/or non-native invasive plants to new areas. Intentional plantings occur in both ornamentals, whore foliage, form and/or flowers make them attractive additions to landscaping, and other plants introduced to be used as erosion control or wildlife forage. Often, these plants exhibit reproduction traits that enable them to spread rapidly (Radosevish et al. 2007).

One reproductive tool of many invasive plants is the production of thousands of light, wind-blown seeds per plant that can rapidly disseminate to other areas (Table 21.1). Other types of invasive plants have seeds with very hard seed coatings. This seed coat protects the seed and allows it to remain viable for several years after being dropped by the parent plant. Invasive species with hard seed coats can lay dormant in the soil for years, waiting for the seed coat to wear away and for appropriate weather and site conditions to occur for germination. Conditions that facilitate seed sprouting can include several consecutive wet growing seasons, site disturbance that increase light availability and/or clears the soil surface of other vegetation, or droughts (Barrett and Richardson 1986; Barrett 1988).

Scarification of the seed coat, by animals or other means of abrasion, can also stimulate sprouting. Scarification occurs when the seed passes though an animal's

digestive track. The fruits and seeds produced by the invasive plants are usually very attractive to wildlife as a food source, which aids in subsequent seed dispersal. Birds, in particular, play a large role in disseminating seeds over broad areas. Another reproductive characteristic of invasive plants is the ability to sprout from the roots and root nodded along with the stems. If the above ground vegetation of an invasive plant is killed, the roots can rapidly send up a replacement sprout. Some plants reproduce by roots nodes located along the above aboveground stem. When one of the root nodded comes into contact with the soil surface, a new root system begins to develop and eventually forms a new plant (Baskin and Baskin 1988).

Invasive plants quickly colonize areas that are frequently disturbed, such as road embankments and construction areas. They can also be found along field edges, riparian areas and other managed places. With a few exceptions these types of plants are not found in shaded areas, such as the interior of forests. Most invasive plants are better suited for sunny sites and are considered intolerant of shade. However, species such as privet and Japanese grass can present major problems in forested and shaded areas of the United States. Forest disturbances, such as ice storms, fires, insect and disease infestations, harvests or canopy-removing event, can open forested areas to exotic plant invasions (Radosevish et al. 2007).

2 Weeds: Types and Classification

Weeds are really just one type of plant that we have decided shouldn't be growing in one particular place. It's just your point of view as to what makes a weed a weed. Some weed-type plants are invasive and fast growing. Their growth habit overtakes our cultivated turf plants, depriving them of food and water. Other weeds are extremely noxious and cause problems for humans if they get close to them. Weeds may be classified in various ways. The classification may be Based on economic importance, Lifestyle, leaf morphology, their likeness with grasses, herbs or shrubs, based on taxonomic position, based on place of occurrence (habitat), relationship with the host, nature of stem, and other such parameters (Radosevish et al. 2007).

3 Biological Pollution

Weeds are pollutants to our environment. Their characteristics are: they appear attractive, desirable, or harmless, weaken and may kill native vegetation, can be toxic, painful, or otherwise injurious to humans. can increase (or decrease) soil erosion and associated water quality problems, multiplies itself exponentially and can generate tons of itself in a matter of months, spreads naturally in water, wind, or soil, is also spread by wildlife, livestock, and recreationists. Effects are not usually apparent until the spread is already out of control because it can lay dormant and undetected underground for decades, then reappear and spread (Marwat et al. 2010).

Biological invasion by alien invasive species is now recognized as one of the major threats to native species and ecosystems. The unknown invader will cower in crates, mope in machines, and snuggle in ships. It can hide inside fruits, vegetables and meat. Sometimes it can even convince people to carry it across the border in their coat pockets. Parthenium weed (*Parthenium hysterophorus* L.), an alien invasive weed species, has spread in most parts of the world through one or a combination of above mentioned routes. It is now spreading rapidly throughout Pakistan. Worldwide, it has been designated as one of the most troublesome weed species. The adverse effects of this weed on human beings, livestock, crop production, and biodiversity are well-documented. As a result of a lack of information on its spread in Pakistan since its invasion, a an internal linkage project was designed in which a comprehensive phytosociological survey, with special reference to *Parthenium* weed management, was carried out in Punjab and Khyber Pkhtoonkhowa, Pakistan in later years of the past decade (Marwat et al. 2010).

4 What Makes Weeds Invasive

Biological invasion by alien species is now recognized as one of the major threats to native species and ecosystems, yet awareness of the problem is alarmingly low. The effects on biodiversity are immense and often irreversible. Invasive species occur outside their natural range. They are non-native plants and animals that harm or endanger native plants and animals or other aspects of biodiversity. Alien invasive species occur in all groups of plants and animals. They include competitors, predators, pathogens and parasites (McNeely et al. 2001). They have invaded almost every type of native ecosystem and caused hundreds of extinctions (Joshi 2001).

Through increased volume of trade and international transport over the past few centuries, natural barriers such as oceans and mountains that once prevented the movement of species have now become ineffective, ending millions of years of biological isolations. Introductions of alien species can happen deliberately or unintentionally, for example, by organism's transport in containers, ships, cars or soil. Tourists and homeowners often unwillingly introduce alien plants into wilderness areas, for example, by planting imported ornamental species in their gardens which then flourish and out-compete native species (McNeely et al. 2001).

Human exploration, colonization, and commercial trade have dramatically increased the diversity, scale, and impact of the invasion. Introduced species often find no natural enemies in their new habitat and therefore spread quickly and easily. Invasive species are a real threat to our environment and economy. Invasive species are a real threat to our environment and economy. Invasive species threaten biodiversity, habitat quality, and ecosystem function. They are the second greatest threat to native species, behind habitat destruction. Introduced species are also presenting an ever increasing threat to foot and fiber production. Most troublesome weeds in Pakistan are exotic in origin and they have arrived here sometimes in the past mainly through human activities (Tables 21.2, 21.3, 21.4, and 21.5). Once established in

Table 21.2 Common weed species of Pakistan

Scientific names	Family	Common name	Local name
Achyranthes asper	Amaranthaceae	Prickly chaff flower	Puth kanda, chirchita
Alhagi maurorum	Fabaceae	Prickly clover	Juvansa
Amaranthus spinosus	Amaranthaceae	Spiny pigweed	Cholai, Kandiali cholai
Amaranthus viridis	Amaranthaceae	Pig weed	Jangli cholai, tandulia
Anagalis arvensis	Primulaceae	Poison weed	Billi booti, chadder
Argemone maxicana	Papaveraceae	Prickly poppy	Sialkanta
Arundo donax	Poaceae	Thatch grass	Narra
Asphodelus tenuifolius	Lialiaceae	Jungle onion	Piazi, bhokat
Astragalus sp	Fabaceae	Milk vetch	Rotphullai
Avena fatua	Poaceae	Wild oats	Jangli jai
Atriplex crassifolia	Chenopodiaceae	Salt bush	Lani
Bidens biternata	Asteraceae	Black jack	Dipmal, Phutium
Boerhavia procumbens	Nyctaginaceae	Spreading hogweed	Biskhapra
Bracharia ramose	Poaceae	Signal grass	Bajra grass, bandri
Bracharia reptens	Poaceae	Bracharia	Chhota madhana
Brassica nigra	Brassicaceae	Black mustard	Jangli sarsoon
Bromus japonicus	Poaceae	Brome grass	Slai ghass
Calotropis procera	Asteraceae	Caltrope	Aak
Cannabis sativa	Cannabinaceae	Neck weed	Bhang
Carthamus oxycantha	Asteraceae	Wild safflower	Kandiari
Cenchrus ciliaris	Poaceae	Buffel grass	Dhaman
Cenchrus pennesetiformis	Poaceae	Burgrass	Kutta ghass
Centauria iberica	Asteraceae	Corn flower	Pohla otthkanda
Centaurium pulchellum	Asteraceae	Showy century	Rattan jot
Cerastium vulgatum	Caryophyllaceae	Field chickweed	Phullan booti
Chenopodium album	Chenopodiaceae	Common goose foot	Bathu
Chenopodium ambrosiodes	Chenopodiaceae	Alkali weed	Lani bathu
Chenopodium murale	Chenopodiaceae	Fathen	Talla bathu, krund
Cichorium intybus	Asteraceae	Blue daisy	Kasni
Cirsium arvense	Asteraceae	Creeping thistle	Leh, bhur bhur
Cleome viscose	Capparidaceae	Spider flower	Bogra, hul hul
Commmelina benghalensis	Commelinaceae	Asiatic dayflower	Kamlina
Convolvulus arvensis	Convolvulaceae	Common bindweed	Lehli
Convolvulus pluricaulis	Convolvulaceae	Summer bindweed	Hirankhuri
Corchorus antichorus	Tiliaceae	Jew's mallow	Baphali
Corchorus tridens	Tiliaceae	Wild jute	Jangli patsan
Conyza ambigua	Asteraceae	Tall fleabane	Loosen booti
Coronopus didymus	Brassicaceae	Swine cress	Naskari, gandi booti
Cuscuta campestris	Convolvulaceae	dodder	Amar bale, akash bale
Cynodon dactylon	Poaceae	Barmuda grass	Talla, khabbal
Cyprus difformis	Cypraceae	Umbrella plant	Ghoin
Cyprus iria	Cypraceae	Umbrella sedge	Buro-choocha
Cyprus rotundus	Cypraceae	Nut sedge	Deela, mork, motha

(continued)

Table 21.2 (continued)

Scientific names	Family	Common name	Local name
Dactyloctaenium aegypticum	Poaceae	Coast buttongrass	Madhana ghass
Datura fastuosa	Solanaceae	Jimson weed	Sufaid dhatura
Desmostachya bipinnat	Poaceae	Deep root grass	Dhabb
Dicanthium annulatum	Poaceae	Dicanthium	Bra jerga, phelwan
Digera muricata	Amaranthaceae	Digera	Tandla tendulia
Digitaria adscendens	Poaceae	Finger grass	Moti khabbal
Diplachne fusca	Poaceae	Beetle grass	Lumb ghass
Echinocloa colona	Poaceae	Millet rice	Kala swank
Echonocloa crusgali	Poaceae	Barnyard grass	Dhiddan
Eclipta prostrate	Asteraceae	False daisy	Daryai booti
Eichhnornia crassipis	Pontenderiaceae	Water orchid	Kalali
Eleusine indica	poaceae	Finger millet	Madhani
Eleusine flagellifera	Poaceae	Crowfoot grass	Chhimber ghaas
Eragrostis pilosa	poaceae	Soft love grass	Chiri ghaas
Euphorbia dracunculoides	Euphorbiaceae	Green spurge	Kanghi dodhak
Euphorbia helioscopia	Euphorbiaceae	Sun spurge	Chatter booti
Euphorbia granulata	Euphorbiaceae	Snake weed	Laal dodhak
Euphorbia hirta	Euphorbiaceae	Garden spurge	Hazardani
Festuca cristata	Poaceae	Winter grass	Domb ghaas
Fimbristylis dichotoma	Cypraceae	Hoora grass	Kalooro
Fumaria indica	Papaveraceae	Fumitory	Shahtra
Gallium aperine	Rubiaceae	Catch weed	Warri booti
Gnaphalium indicum	Asteraceae	Cud weed	Bairaksha
Heliotropium indicum	Boraginaceae	Indian heliotrope	Oont chara
Hydrilla verticillata	Hydrocharitaceae	Hydrilla	Jala
Ipomoea camea	Convolvulaceae	Railway creeper	Besharmi booti
Lactuca serriola	Asteraceae	Chinese lettuce	Jangli salad
Lantana camara	Verbenaceae	Lantana	Panj phulli
Lathyrus aphaca	Fabaceae	Crow pea	Jangli matri
Lathyrus sativus	Fabaceae	Grass pea	Kaseri
Launaea nudicaulis	Asteraceae	Yellow purge	Bathal
Lepedium sativum	Brassicaceae	Common cress	Halon
Laptochloa panicea	Poaceae	Henbit	Lamb ghaas
Lippia nudiflora	Verbinaceae	Prostrate vervane	Bukken booti
Lolium temulentum	Poaceae	Poison rye grass	Dhanak
Malva neglecta	Malvaceae	Dwarf mallo	Sonchal
Marsilia minuta	Marsiliaceae	Paper wort	Chopatti
Medicago polymorpha	Fabaceae	Bur clover	Maina
Melilotus alba	Fabaceae	White sweet clover	Sufaid senji
Melilotus	Fabaceae	Yellow sweet clover	Zard senji
Mentha longifolia	Lamiaceae	Wild mint	Jangli podina
Mukia maderaspatana	cucurbitceae	Wild cucurbit	Chibbher
Nelumbo nucifera	Nympheaceae	Water lily	Kanwal
Nicotiana plumbagifilia	Solanaceae	Wild tobacco	Jangli tambacco
Nonnea pulla	Boraginaceae		Lwien booti

(continued)

Table 21.2 (continued)

Scientific names	Family	Common name	Local name
Nymphaea nouchali	Nympheaceae	White water lily	Kutta kammi
Opuntia nigricans	Cactaceae	Cactus	Thohar
Orobanche aegyptica	orobanchaceae	Broom rape	Haddah
Oxalis corniculata	Oxalidaceae	Wood sorrel	Khatti booti
Oxalis corymbosa	Oxalidaceae	Wood sorrel	Khatkal
Oxalis pescarpae	Oxallidaceae	Bermuda	Khatti booti
Panicum antidotale	poaceae	Blue panic	Bansi ghaas
Panicum italicum	Poaceae	Foxtail millet	Kangni
Panicum ramosum	Poaceae	Brown top millet	Bandari
Panicum glaucum	Poaceae	Yellow foxtail	Lommar ghaas
Parthenium hysterophorus	Asteraceae	Parthenium weed	Gajar booti
Paspalidium flavidum	Poaceae		
Paspalum paspaloides	Poaceae	Knot grass	Naroo ghaas
Peganum hermala	Zygophyllaceae	Wild rue	harmal
Pennisetum purpureum	Poaceae	Elephant grass	Hathi ghaas
Phalris minor	Poaceae	Bird's seed grass	Dumbi sittee
Physalis minima	Solanaceae	Cape goose berry	Bhambool
Pistia stratiotes	Arucaceae	Duck salad	Aabi slad
Poa annua	Poaceae	Annual blue grass	Poa ghaas
Polygonum plebejum	polygonaceae	Smart weed	Dranak
Polypogon monspeliensis	Poaceae	Winter grass	Dumb ghaas
Portulaca oleracea	Portulacaceae	Parsely	Qulfa
Ranunculus muricatus	Ranunculaceae	Rough seeded	Chambal
Rhynchosia minima	Fabaceae	Rhyncosia	Maini
Rumex dentatus	Polygonaceae	Broad leaf dock	Jangli palak
Saccharum benghalensis	Poaceae	Tiger grass	Sarkanda
Saccharum spontaneum	Poaceae	Giant reed	Kahi
Sagittaria guayanensis	Alismataceae	Arrowhead	Chiri napay
Salsola baryosma	Chenopodiaceae	Soapwort	Kala takla
Saponaria vaccaria	Caryophyllaceae	Cow herb	
Schoenoplectus juncoides	Juncaceae	Softrush	Bhookal booti
Scirpus maritimus	Cypraceae	bulrush	Deela
Sesbania bispinosa	Fabaceae	Dunder fiber	Dhancha
Sesbania sesban	Fabaceae	Common giant	Jantar
Setaria pumila	Poaceae	Yellow foxtail	Zard loomer ghaas
Setaria italic	Poaceae	Ittalian millet	Kangni
Setaria viridis	Poaceae	Green foxtail	Bandri
Setaria verticillata	Poaceae	Bristly foxtail	Lahdra
Silene conoidea	Caryophyllaceae	Catchfly	Bhoora takla
Sylbium marianum	Asteraceae	Milk thistle	Kandiali
Sisymbrium irio	Brassicaceae	Hedge mustard	Jangli sarson
Solanum nigrum	Solanaceae	Black nightshade	Makko
Solanum xanthocarpum	Solanaceae	Spinay weed	Kandiari
Sonchus arvensis	Asteraceae	Perennial sowthistle	Peeli dodhak
Sonchus asper	Asteraceae	Spiny sowthistle	Kandiali dodhak
Sonchus oleraceous	Asteraceae	Annual sowthistle	Dodhak

(continued)

Table 21.2 (continued)

Scientific names	Family	Common name	Local name
Sorghum halepense	Poaceae	Arabian millet	Baru ghaas
Spergula arvensis	Caryophyllaceae	Corn spurry	Kalri booti
Sphenoclea zeylanica	Sphenocleaceae	Goose weed	Mirch booti
Sporobolus pallidus	Poaceae	Sprorobolous	Lamb ghaas
Stellaria media	Caryophyllaceae	Chick weed	Phullan booti
Striga lutea	Scrophulariaceae	Witch weed	Angari booti
Trianthema portulacastrum	Aizoaceae	Desert horse purslane	Itsit
Trianthema monogyna	Aizoaceae	Horse purslane	Itsit
Tribulus terrestris	Zygophyllaceae	Puncture vine	Bhakhra
Trigonella foenum-graecum	Fabaceae	Fenugreek	Methi
Trigonella monantha	Fabaceae	Trefoil	Maini
Typha domingensis	Typhaceae	Cattail	Dib
Veronica agrostis	Scrophulariaceae	Field speedwell	Veronica
Verbascum Thapsus	Scrophulariaceae	Common mullein	Gidar tambakoo
Vicia hirsuta	Fabaceae	Two-seeded vetch	Revari khurd
Vicia sativa	Fabaceae	Common vetch	Chhtri matri
Vicia tetrasperma	Fabaceae	Four-seeded vetch	Revari
Withania somnifera	Solanaceae	Winter cherry	Aksan
Xanthium strumarium	Asteraceae	Common cocklebur	Mohabbat booti

favourable habitats, these aliens spread like forest fire and threatened the environment and economy of the country. Invasive species threaten biodiversity, habitat quality, and ecosystem function. They are the second greatest threat to native species, behind habitat destruction. Introduced species also present an ever-increasing threat to food and fiber production (Marwat et al. 2010, 2011).

Most troublesome weeds in Pakistan are exotic in origin and they have arrived here sometimes in past mainly through human activities (Tables 21.2, 21.3, 21.4, and 21.5). Once established in favorable habitats, these aliens spread like forest fire and threatens the environment and economy. Pakistan has a rich repository of vascular plants and about 6,000 species have been reported. According to an estimate 8% of these species are endemic to Pakistan. According to Ali and Qaiser (1986) number of species per genus is much lower as compare to global average, reflecting a high diversity at generic level. Some of the world's worst alien invasive species have already been invaded country deteriorating the beautiful landscapes, agricultural lands plant biodiversity. Among those following are worth mentioning; Parthenium weed (*Parthenium hysterophorus*), Lantana (*Lantana camara*), Paper mulberry (*Brossunetia paperifera*), Mesquite (*Prosopis juliflora*) Wild tamarind (*Leucaena leucocephala*), Khakiweed (*Alternanthera pungens*) etc.

P. hysterophorus a noxious alien invasive weed worldwide is threatening the natural and agricultural ecosystems of the country. This weed is becoming a challenge for scientists as with in a short span of time it has invaded a significant portion of Punjab and now switching to NWFP. This noxious weed is allergenic to both humans and livestock but unfortunately florists are using this weed in bouquets showing lack of

Table 21.3 Grass land weeds at Quaid-e-Azam Campus, University of the Punjab, Lahore

Sr. #	Weed species	Sr. #	Weed species
1.	*Achyranthes asper*	27.	*Imperata cylindrical*
2.	*Ageratum conizoides*	28.	*Inula vestita*
3.	*Anagalis arvensis*	29.	*Lippia nodiflora*
4.	*Atriplex crassifolia*	30.	*Lamium sp.*
5.	*Abutilon indicum*	31.	*Launia nudicaulis*
6.	*Boerhavia diffusa*	32.	*Oligomeris glaucoscens*
7.	*Cynodon dactylon*	33.	*Mazus rugosus*
8.	*Calotropis procera*	34.	*Ncotiana plumbegiflora*
9.	*Convolvulus arvensis*	35.	*Oligomeris glaucoscens*
10.	*C. pleuricaulis*	36.	*Panicum antidotale*
11.	*Cenchrus pennisetiformis*	37.	*Polypogon monplensis*
12.	*C. setigerous*	38.	*Polygonum plebejum*
13.	*Cassis sp.*	39.	*Pullicaria crispa*
14.	*Croton sparsiflora*	40.	*Sporobolous pallidus*
15.	*Cyprus rotundus*	41.	*Rumax dentatus*
16.	*Dicanthium annulatum*	42.	*Solanum nigrum*
17.	*Dalbergia sisso*	43.	*Sonchus asper*
18.	*Dactyloctaenium aegypticum*	44.	*Suaeda fruticosa*
19.	*Desmostachya bipinnata*	45.	*Saccharum spontaneum*
20.	*D. lupinvola*	46.	*Setaria italica*
21.	*Eclipta alba*	47.	*Tribulus terrestris*
22.	*Euphorbia pilolifera*	48.	*Trigonella foenum-graecum*
23.	*E. prostrata*	49.	*Stellaria media*
24.	*Eleusine flagellifera*	50.	*Withania somnifera*
25.	*Eleusine flagellifera*	51.	*Xanthium strumarium*
26.	*Kochia indica*	52.	*Zizyphus numularia*

awareness at general public and municipal government level (Shabbir 2000). Its presence in metropolitan areas is threat to private and public property. Huge infestation in suburbs is also a potential threat to urban agriculture (Plates 21.1, 21.2, and 21.3a, b; Fig. 21.1).

Paper mulberry (*Broussonetia paperifera* Vent.) is an invasive alien weed tree became a menace in twin cities of Rawalpinia-Islamabad where thousands of residents are susceptible to its pollen allergy. Unfortunately, this weed is spreading to new localities like Lahore and Gujranwala; a huge population can be seen along the main drainage courses like of *Zafar Ali Road* in Lahore. In capital Islamabad where situation is worst, most of the citizens left the city and moved to pollen free areas. *Lantana cammra* a serious environmental weed and well known for its invasiveness globally, present in most parts of the country. Unfortunately awareness about this weed is little and people still using it as a hedge plant there by assisting its spread to newer localities. Mesquite (*Prosopis juliflora*) is another serious tree weed threatening the biodiversity of arid and semiarid environments of southern Punjab and Sind where it has reduced the primary productivity of the region. Mesquite was first introduced by British people in nineteenth century to green the Thar Desert but

Table 21.4 List of invasive weeds of Pakistan

Scientific name	Family	English name	Local name	Origin	Worst affected areas
Broussonecia papyrifera	Moraceae	Paper mulberry	Gul toot	South East Asia	From Lahore to Peshawar especially Islamabad, Rawalpindi and Northern Pakistan
Prosopis juiliflora	Mimosaceae	Mesquite	Kiker	West Indies & Mexico	Sindh
Eichhornia crassipes	Pontederiaceae	Water hyacinth	Gul-e-bakauli	Amazon Basin, South America	Water bodies of Sindh & Punjab
Salvinia molesta	Salvinaceae	Water fern	Not available	South America	Wetlands and irrigation channels of Thatta
Lantana camara	Verbinaceae	Lantana	Punch phuli	America	Islamabad
Parthenium hysterophorus	Compositae	White top	Booti	México, Central America	Islamabad & upper Punjab
Cannabis sativa	Cannabinaceae	Hemp	Bhang	Central and Western Asia	Waste areas of northern Punjab and NWFP
Pistia stratiotes	Araceae	Water cabbage	Jai kumbi	Old and New World Tropics	On the edges of old lakes
Ipomoea carnea	Convolvulaceae	Railway creeper	Railway creeper	Tropical America	In southern Sindh and Indus delta
Emex spinosa	polygonaceae	Prickly dock	Kafir kanda	Mediterranean region	In cooler parts of the country
Galium aprine	Rubiaceae	Catchweed bedstraw	Galium	Europe	Distributed in Pakistan from plains to 12,000 ft
Xanthuium stromarium	Compositae	cocklerbur	Puth kando	A New World Specie	Most of the rangelands of Pakistan
Leucanea leucocephala	Mimosaceae	Ipil ipil	As adopted local	Central America	Parts of Pakistan-in Punjab
Lolium temulentim	Graminae	Rye grass	Dhanak	Mediterranean region	Throughout Pakistan from plains to 2,000 ft

Modified from PARC (1999)

Table 21.5 Weeds of family Asteraceae from Pakistan (Khalid 1995)

Sr. #	Weed species	Sr. #	Weed species
1.	*Achillea millefolium*	28.	*Gnaphalium affine*
2.	*Achillea wilhelmsii*	29.	*Ifloga spicata*
3.	*Adenostemma lavenia*	30.	*Jurinea modesta*
4.	*Ageratum houstonianum*	31.	*Koelpinia linearis*
5.	*Artemesia scoparia*	32.	*Lactuca dissacta*
6.	*Bidens pilosa*	33.	*Lactuca serriola*
7.	*Caesulia axillaris*	34.	*Laggera aurita*
8.	*Calendula arvensis*	35.	*Launaea nudicaulis*
9.	*Carduus edelbergii*	36.	*Launea resedifolia*
10.	*Carthamus oxyacantha*	37.	*Matricaria aurea*
11.	*Carthamus lanatus*	38.	*Pentanema vestitum*
12.	*Centaurea iberica*	39.	*Picris hieraciodes*
13.	*Cichorium intybus*	40.	*Reichardia tingitana*
14.	*Cirsium arvense*	41.	*Saussurea heteromalla*
15.	*Cirsium wallichii*	42.	*Sigesbackia orientalis*
16.	*Conyza aegyptiaca*	43.	*Silybum marianum*
17.	*Conyza bonariensis*	44.	*Sonchus asper*
18.	*Conyza canadensis*	45.	*Sonchus lachnocephalus*
19.	*Conyza Sp.*	46.	*Sonchus oleraceus*
20.	*Conyzanthus squamatus*	47.	*Sphaeranthus indicus*
21.	*Cousinia minuta*	48.	*Taraxacum wallichii*
22.	*Echinops echinatus*	49.	*Tragopogon gracillis*
23.	*Eclipta alba*	50.	*Tridax procumbens*
24.	*Epilasia bungei*	51.	*Veronia anthelmintica*
25.	*Filago hurdwarica*	52.	*Xanthium strumarium*
26.	*Francoeuria undulata*	53.	*Youngia japonica*
27.	*Galinsoga parviflora*		

unfortunately this plant has become a weed and threat to unique desert flora of the country (Shabbir 2002).

A list of alien invasive species of Pakistan was developed by IUCN in 2000 but it is suspected that there would be many more to add as most of them are undocumented. Invasive species are very difficult to mange as these are equipped with weedy charters that native species are not. In developed countries like Australia millions of dollars are spent annually to eradicate invasive weeds. An exotic species is a species introduces into an area from somewhere else, often a different continent. Because the species is not native to the new area, it is frequently successful in establishing a viable population and quietly disappears. This is the fate of many pet birds, reptiles, and fish that escape or are deliberately released from their native habitats. Occasionally, however, an introduced species finds the new environment very much to its liking and can become an invasive species, thriving, spreading out, and perhaps eliminating native species native species by predation or competition for space or food. Exotic species are major agents in driving exotic species to extinction and are responsible for an estimated 39% of all animal extinctions since 1600 (Shabbir 2002).

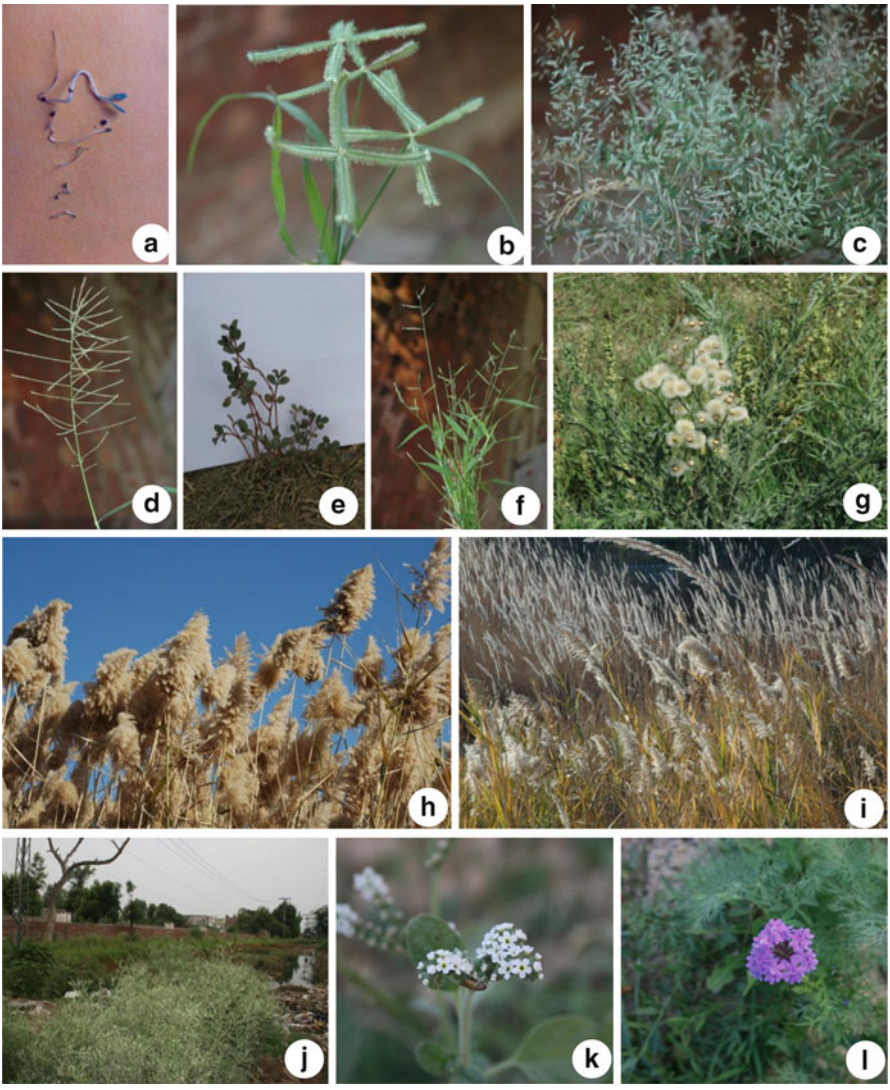

Plate 21.1 Some grass and non grass weeds of Pakistan; (a) Germinating weed seeds to evaluate soil seed bank of seeds; (b) *Dactyloctaenium aegypticum*; (c) *poa annua*; (d) *Sporobolus pallidus*; (e) *Euphorbia prostrate*; (f) *Cynodon dactylon*; (g) *Pullicaria crispa*; (h) *Saccharum spontaenium*; (i) *Imperata cylindrical*; (j) *Parthenium hysterophorus*; (k) *Heliotropium* sp; (l) *Verbena* sp

Other than this, global warming directly reflects on rising sea levels due to melting of ice caps and natural expansion of sea water as it becomes warmer. Consequently, areas adjoining the coast and wetlands could be frequently flooded and the distribution pattern of monsoon rains gets altered with more intense downpours, storms and hurricanes. Phytosociological survey of floristic composition of weeds reveals the recent invasion of rice fields by alien invasive weeds *Leptochloa chinensis* and

Plate 21.2 (a–d) Some invasive weeds of Pakistan; Invasion of *L. cammara* in Islamabad; (**A**) Mixed communities of invasive weeds in Islamabad; (**B**) Brousunetia paperifera; (**C**) Parthenium weed with Z. *bicolrata*

Marsilea quadrifolia. These two weed species dominated over the native weeds such as *Echinochloa* sp. and others by virtue of their amphibious adaptation to alternating flooded and residual soil moisture conditions prevalent during recent years in this region (Yaduraju and Kathiresan 2003; Kathiresan 2005).

5 Invasive Weeds of Pakistan

Beautiful flowers, interesting foliage, tough constitution, and fast growth are plant qualities that appeal to gardeners and landscapers. When these desirable horticultural characteristics occur in plants that are not native to a particular place, these exotics could escape from maintained landscapes, invade natural areas, and damage native plant communities of the area. Non-native plants that readily spread in natural areas, either vegetative or via seeds, pose a significant threat to the health and welfare of rich biodiversity of the area. These plants may also be considered as exotic invasive pests (Radosevish et al. 2007).

While in contrast to this category of exotic plants, Endemic plants are those which occur within a specified locality; not introduced and have a natural distribution confined to a particular geographic area. Endemic, in a broad sense, can mean "belonging" or

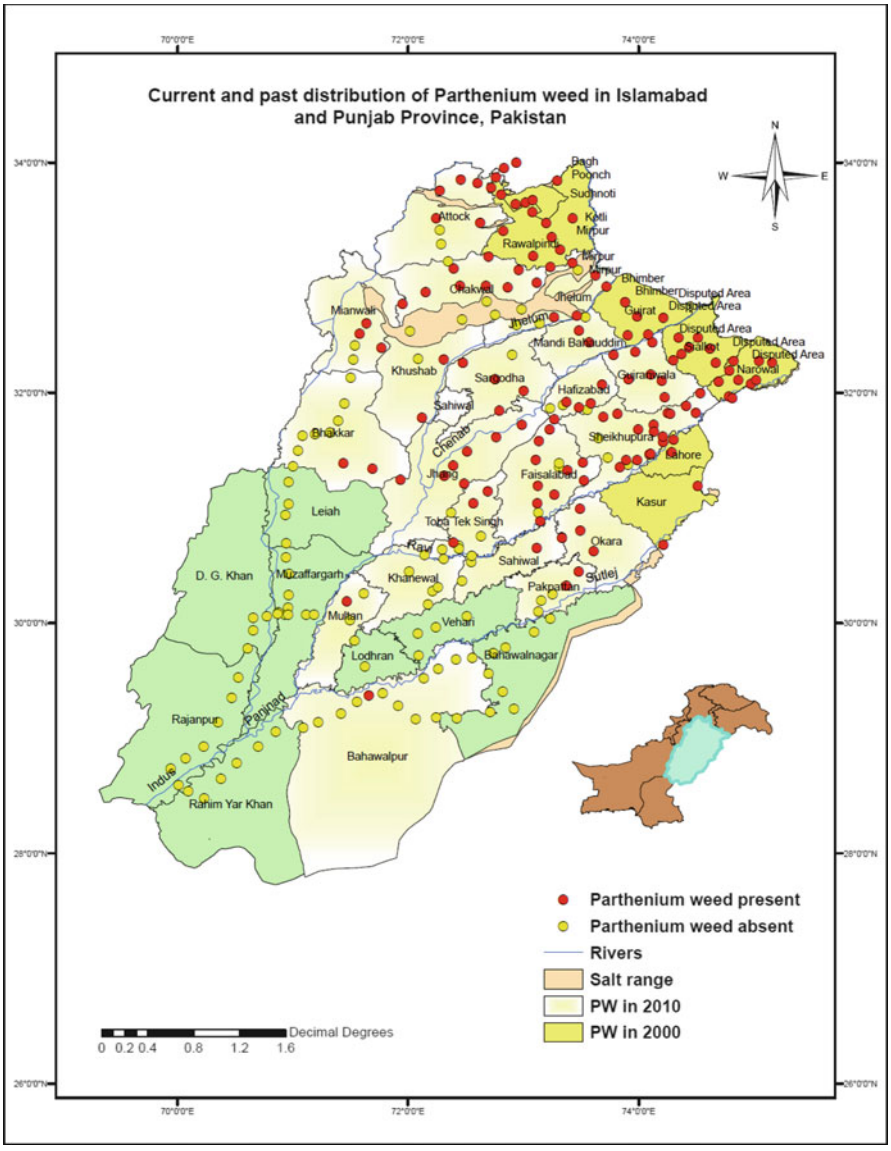

Plate 21.3 (**a**) Distribution of Parthenium weed in Punjab. (**b**) Distribution of Parthenium weed in Khyber Pukhtunkhwa

"native to", "characteristic of", or "prevalent in" a particular geography, race, field, area, or environment; native to an area or scope. The native and/or endemic plants have evolved through geological time in its native geographic location, developing a strong connection to the land, each other and the wildlife (Radosevish et al. 2007).

In contrast to it, some weeds find their way into new areas via boats, planes, soles of shows, imported animals and agricultural products, etc are called noxious or alien weeds.

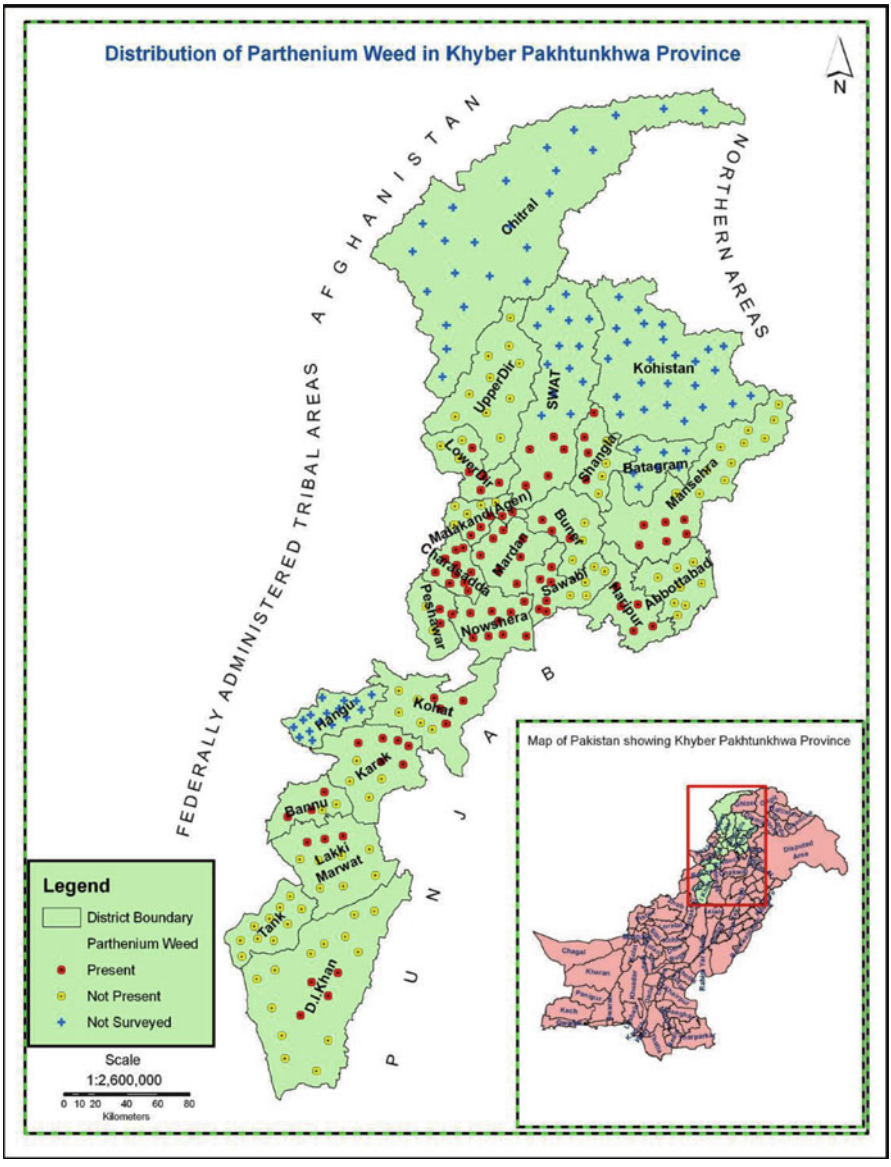

Plate 21.3 (continued)

Some of the plants introduced in Pakistan from other parts of the world are an important part of gardening and landscaping. Most of these plants are well behaved and rarely stray beyond the garden wall e.g. *Lantana camara*. Only about one percent of these non-natives readily escape into wild and become invasive in natural areas. Invasive plants exhibit certain traits (Marwat et al. 2011).

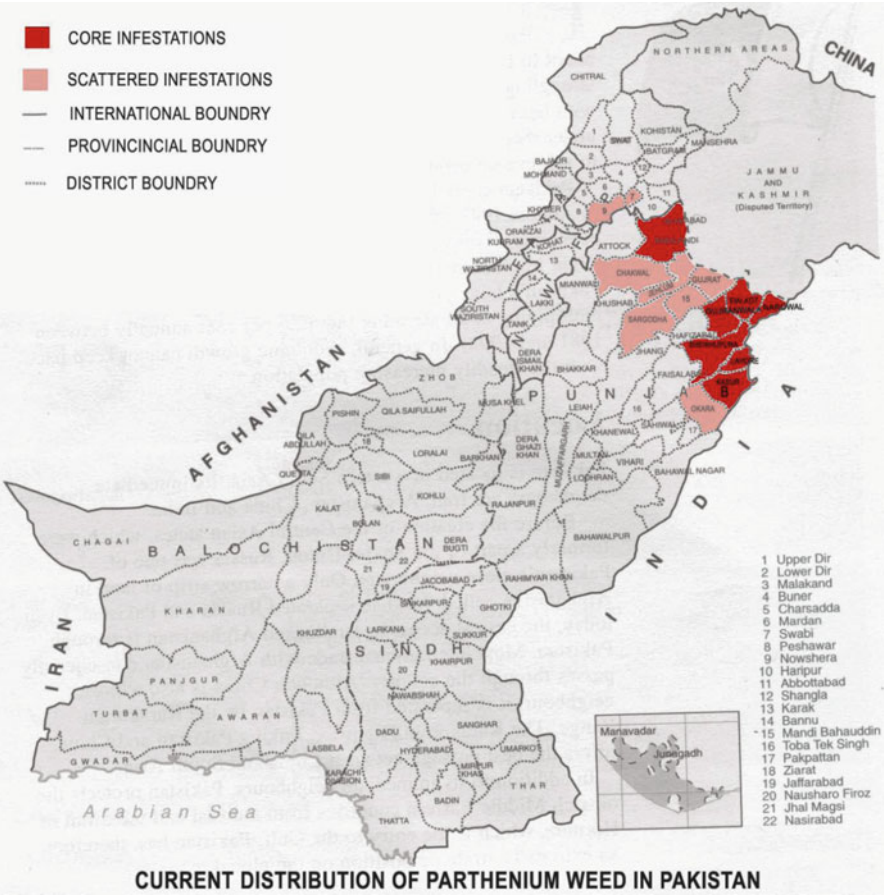

Fig. 21.1 Map of Pakistan showing Parthenium distribution

- Adaptation to local climate
- Rapid growth
- Mature quickly to flower and set seed
- Produce copious amounts of seeds
- Effective seed dispersal
- Rampant vegetative spread
- No major pests or disease problems

These traits can give exotic invasive plants undue advantage in wild habitats like forests, wetlands, marshes and grasslands. Exotic species can overwhelm native plants depriving them of nutrients, water, light and space and many totally displace native species, replacing a diverse ecosystem with a near sterile monoculture and resulting introduction of biodiversity, loss of endangered species and their habitats, loss of habitat and food sources for wildlife, and disruption of native plant-animal

associations. Exotic invasive plants threaten the health and stability of natural heritage of an area and needs to establish a handsome amount of funds for research to find proper control options (Pimental et al. 2004).

Some exotic invasives are agricultural pests-forage grasses and noxious weeds. Some were introduced to modify climate like paper mulberry while some hitched rides on boats or got dumped out of homes from floral bouquets or aquaria. Many are horticultural. Unfortunately some of the invasive traits listed above can increase a plant's horticultural desirability (Marwat et al. 2010).

The worldwide search for "new" and exciting landscape plants has played a role in the dramatic shift of plant species' distribution around the globe, and the introduction of potentially invasive species is a real danger. A research conducted by Reichard (1999) in the United States has shown that 85% of the non-native invasive woody plants in North America were originally brought in as ornamental or landscape plants. Gardner's, nursery owners, landscapers, and design professionals should be aware of this potential and understand the consequences.

6 Parthenium Weed: Spread Status and Management– A Case Study from Pakistan

Parthenium hysterophorus L. (Asteraceae), commonly known as parthenium weed, is an annual herb native to the Gulf of Mexico and central South America, and has become widespread in North America, South America, the Caribbean and many parts of Africa, Asia and Australia (Navie et al. 1996; Tamado 2002; Mahadevappa 1997). Parthenium weed is not native to Pakistan and it probably arrived in late 1990s and mainly occurs in central and upper Punjab, some parts of NWFP and Azad Jammu Kashmir (Shabbir 2002, 2006; Shabbir and Bajwa 2006; Adkins and Navie 2006) affecting the natural and agro ecosystems (Fig. 21.1; Plate 21.3a, b). Parthenium weed is potential threat to cultivated and grazing parts of country, In Ethiopia and India this weed has become a problem in some of major crops (Khosla and Sobti 1979; Nath 1981; Tomado and Milberg 2000) and range lands in Australia (Adkins et al. 1996, 2001; Navie et al. 1996). Where ever this weed has invaded, it resulted in severe economic, health and environmental problems (Chippendale and Panetta 1994; McFadyen 1995; Tamado 2002; Nadeem et al. 2005). Parthenium weed can be seen every where especially during summer in non-cropped areas of central and upper districts of Punjab. This weed is emerging as a major weed in wastelands, plotted housing colonies, degraded areas, rocky crevices, along water channels, roadsides and railway tracks (Shabbir and Bajwa 2006). It has recently also been reported in cultivated lands (Shabbir 2002, 2006; Shabbir and Bajwa 2006).

In addition to losses incurred on crop production, this noxious weed can severely affect animal and human health and biodiversity. Human health hazards of this weed are well documented by McFadyen (1995) from Australia. In Pakistan, this weed is growing concern in municipal areas where, unfortunately, florists are using excessively in bouquets in Pakistan. A multiphase collaboration study of Institute

of Agricultural Sciences, Punjab University, Lahore and Department of Dermatology King Edward Medical University Lahore revealed that an appreciable number of allergy patients in Lahore are sensitive to parthenium weed. It has caused a major outbreak of airborne contact dermatitis. The impact of *P. hysterophorus* on livestock production is also significant, both directly and indirectly, and it affects grazing lands, animal health, milk and meat quality (Tudor et al. 1982; Chippendale and Panetta 1994), and the marketing of pasture seed and grain. Very little, or sometimes no other, vegetation can be seen in *P. hysterophorus* dominated areas. Wherever it invades, it forms a territory of its own by replacing the indigenous natural flora including medicinal herbs utilised by man as a source of medicine (Shabbir 2002).

Since times immemorial a variety of natural herbs has become the basis of the traditional systems of medicine of treatment of many diseases in many parts of the globe. The traditional systems have been existed since remote past, in countries such as China, India and Pakistan. The traditional medicinal system continues to be of vital importance. It has been estimated by world health organization (WHO) that nearly 80% of human population depends upon traditional systems for primary health care. Amongst the world's ancient civilizations, Pakistan has been recognized as rich repository of natural herbs, which are being used in different indigenous systems of medicine i.e., Hikmat, Tib and Homeopathy. A rich heritage preserved the diversity of natural herbs widely distributed in different phyto-ecological zones of the country. The medicinal herbs viz, *Piccrorhiza* sp., *Aconitum* sp., *Artemisia* sp. etc., occur abundantly in western Himalayas, under arid and sub arid conditions. *Calotropis* sp. *Aloe barbidensis, Tribulus terristris and Withania sominefera* etc are very common. The medicinal herbs are adapted to wide range of agro-climatic regions and soil textures. Some of them also prefer to grow under degraded soils and wastelands. However, *P. hysterophorus* is quickly capturing these areas. They therefore, undergo continuous struggle/interference with *P. hysterophorus* in which this weed definitely becomes the winner, thus existence of economic herbs in these very areas in under threat (Shabbir 2002).

Parthenium hysterophorus grows well in almost all soil types although growth is more luxuriant in black as compared to latrite soil. It prevails all the years around due to its day neutral habit, temperature insensitivity, drought tolerance and absence of seed dormancy. Under arid conditions the weed has a very high survivability due to low photorespiration and emerges throughout the year, where other plants/herbs hardly withstand. Its seeds germinate in a wide range of soil pH that is 2.5–10. High regenerative and reproductive potential of the weed are the other favorable reasons for its wide spread. Root, stumps, petiole and even detached midribs have the ability to regenerate when in contact with soil. However, most of the natural herbs are not equipped with these versatile characters and as a result they cannot withstand in close competitions with it and their population is under gradual decline in Pakistan. Moreover, national parks and forest reserves are also a rich repository of many economic herbs, however, these areas were observed to be heavily infested with *P. hysterophorus* posing a serious threat to this useful flora (Shabbir 2002).

7 Management of Invasive Weeds

7.1 Prevention of Spread

Globalisation through increased trade, transport, travel and tourism will inevitably increase the intentional or accidental introduction of organisms to new environments, and it is widely predicted that climate change will further increase the threat posed by invasive species (Table 21.6). Enforcement of strict quarantine in developing countries is imperative to tackle the problem of invasive species. The establishment of wash-down sites for vehicles and implements, for thorough cleaning at international and national interstate borders will prevent much spread to newer localities (Radosevish et al. 2007).

Manual and mechanical methods: The control of invasive weed through manual and mechanical means is sometimes neither effective nor cost effective due to reasons that some weeds are allergic nature. Some of alien invasive weed species like parthenium weed is highly allergenic and prolonged exposures may result in serious contact dermatitis (McFadyen 1995) In Australia parthenium weed is a serious problem in perennial grasslands and some summer crops, where it has reduced beef production by an estimated Aus $16.5 million annually. Manual and mechanical control to such large property is economically not possible additionally such program also risks human health. If effective measures are not taken to manage the spread of this weed, a similar situation could emerge in Pakistan. In some cases mechanical control methods have shown success in controlling alien invasive weed. *Lantana cammra* an invasive weed in many parts of the world can be managed by hand pulling and bulldozing. Water hyacinth can also be managed mechanically with Hyacinth destruction boat in Florida. Manual and mechanical methods for control of invasive weed species are not effective in all situations. Some of other disadvantages of mechanical control of weeds are labour intensiveness, high cost and regrowth issues

Chemical control: Chemical control is not only partially effective and is expensive but also require repeated applications and likely to be environmentally toxic. Parthenium weed infestations along roadsides are the major sources of weed spread. One of the primary means of weed management for small, local populations is the use of Atrazine, or an Atrazine plus 2, 4-D mix, or Dicamba, or Hexazinone, or Metsulfuron or a Picloram plus 2, 4-D mix. Any one of these treatments could give good results depending upon the situation in which the weed was present (Adkins and Navie 2006). Glyphosate and Isoproturon were checked for their efficacy in controlling parthenium weed in wild situations. Glyphosate was found to be more effective in control of this noxious weed (Shabbir 2002). Chemical control of some of woody alien invasive species like paper mulberry is not possible due to its hardy nature of the plant to tolerate extreme condition

Competitive displacement ability: Several beneficial plants are known to behave highly competitive with many weeds. Some native plants can regain competitive

Table 21.6 Principles of weed management

Prevention of spread

Globalisation through increased trade, transport, travel and tourism will inevitably increase the intentional or accidental introduction of organisms to new environments, and it is widely predicted that climate change will further increase the threat posed by invasive species. Enforcement of strict quarantine in developing countries is imperative to tackle the problem of invasive species. The establishment of wash-down sites for vehicles and implements, for thorough cleaning at international and national interstate borders will prevent much spread to newer localities.

Field scouting

Field scouting is a key component of an Integrated Weed Management (IWM) system. It involves the systematic collection of weed and crop data from the field (weed distribution, growth stage, population, crop stage, etc.). The information is used, in the short term, to make immediate weed management decisions to reduce or avoid economic crop loss. In the long term, field scouting is important in evaluating the success or failure of weed management programs and for making sound decisions in the future.

Manual and Mechanical Control

The control of weeds through manual and mechanical means is sometimes neither effective nor cost effective due to reasons that some weeds are allergic nature. Manual and mechanical methods for control of invasive weed species are not effective in all situations. Some of other disadvantages of mechanical control of weeds are labour intensiveness, high cost and regrowth issues. The mechanical control of weeds involves:

- **Burial**: This method is most effective on annual weeds in which all the growing points can be buried. Burial is usually less effective on perennial weeds which have underground stems and roots and are capable of re-growth from these underground storage organs.
- **Cultivation**: The main objective in cultivation is to cut the root system of weeds and deep cultivation should usually be avoided due to damage to the crop roots. Deep cultivation may also bring more weed seed to the surface where they will germinate.
- **Mowing**: is another method of mechanical control. Mowing is usually the most effective on tall growing annuals, and not as effective on short growing plants or perennials.

Nitrogen fertility

Nitrogen fertilizer can affect the competition between crops and weeds, and in the subsequent crops. For example, nitrate promotes seed germination and seed production in some weed species. Nitrogen fertilization may increase weed growth instead of increasing crop yield. Selective placing nitrogen in a band can favour the crop over the weed. Using legume residues as opposed to chemical nitrogen fertilizer to supplement nitrogen needs of the crop can enhance weed suppression. Legume residues release nitrogen slowly with less stimulation of unwanted weed growth.

Mulches

Mulches may be of the following three types:

– Black plastic
– Clear plastic
– Infrared-transmitting plastic

Crop Competition

Crop competition is usually one of the cheapest and best methods of weed control; however, it is often one of the most overlooked methods. Weeds compete with crops for space, light, moisture, nutrients, and carbon dioxide. Usually the plant which starts first and is growing under ideal conditions will have the competitive advantage. Factors such as planting date, row spacing, seeding rate, planting depth, soil moisture, soil fertility, and soil pH have an influence on the competitive advantage of the crop or weed.

(continued)

Table 21.6 (continued)

Competitive Displacement Ability Several beneficial plants are known to behave highly competitive with many weeds. Some native plants can regain competitive edge if grazing pressure is managed.
Crop Rotation If the same crop is planted in the same field year after year, there usually will be some weed or weeds which are tolerant and favored by the cultural practices and herbicides used on that crop. By rotating to other crops, many of the cultural practices and herbicide programs are changed.
Biological Control Biological control by means of insects and pathogens has been used in several parts of the world to control the exotic weeds. This exploits coevolved natural enemies from the centre of the origin of the target pest species offering practical and sustainable solutions for the management of weeds.
Chemical Control Chemical control is not only partially effective and is expensive but also require repeated applications and likely to be environmentally toxic. Chemical control can be applied at three stages i.e., preplant, preemergence, and postemergence.
Prevention If effective weed control has been achieved using other methods, one further step should be considered. This is preventing weeds from re-infesting the area. Knowledge of how weeds enter the field is important. Weed seed may be distributed in crop seed, hay, straw, by wind, water, animals, machinery, and other ways. The use of certified, registered, and foundation seed, or clean planting material cannot be over emphasized in preventing weeds from infesting fields. It is also important to clean equipment before entering fields or when moving from one field to another.
Tillage Tillage and cultivation are the most traditional means of weed management in agriculture. Both expose bare ground, which is an invitation for weeds to grow. Bare ground also encourages soil erosion, speeds organic matter decomposition, disturbs soil biology, increases water runoff, decreases water infiltration, damages soil structure, and costs money to maintain (for fuel and machinery or for hand labor).
Watch the topsoil If establishing a landscape that requires the addition of topsoil, consider where that topsoil is coming from. It may be coming from an area that is heavily infested with perennial weeds, which means those vegetative structures may be getting a free ride into your yard.

edge if grazing pressure is managed. Parthenium weed is allelopathic (Adkins and Sowerby 1996) with root and shoot leachates capable of reducing growth and germination of numerous crops. The successful spread of the weed, in part, may be attributed to these allelopathic properties (Mersie and Singh 1987). Shabbir and Bajwa (2005) noticed in the field that *Senna occidentalis* is replacing *P. hysterophorus* gradually in patches in the capital city of Islamabad. Aqueous extract of *S. occidentalis* in different ratios were effective to check the germination and early growth of *P. hysterophorus*. *S. occidentalis* at different concentrations showed least germination of *P. hysterophorus* and a significant gradual depression in fresh biomass accumulation. *Senna occidentalis* and *P. hysterophorus* both are competitive weeds of wastelands. In view of health hazards and likely threats to biodiversity due

to *P. hysterophorus*, it is probably advisable to promote *S. occidentalis* growth, which is harmless medicinal plant in Parthenium infested areas in Pakistan. In a similar kind of study, Shafique et al. (2005) conducted aqueous extracts bioassays to evaluate the allelopathic potential of five tree species iz. *Azadirachata indica* L. *Mangifera indica* L., *Syzygium cumini* (L) Skeels., *Ficus benghalensis* L. and *Melia azdarach* L. for their use in *Parthenium hysterophorus* control. Aqueous extracts of 8 and 10% concentrations of all the test species invariably and significantly suppressed the germination and early growth of Parthenium weed. In few separate studies conducted by Javaid et al. (2005) revealed that some indigenous grasses like *Desmostachya bipinnata* and *Imprata cylinderica* had suppressive effect on distribution of parthenium weed, however, Shabbir (2002) observed an opposite trend when studied the distribution pattern of *D. bipinnata*. These results, obtained using a wide range of potentially useful pasture plants, create a foundation on which future field-based studies can now be undertaken to identify a useful plant or plants that can competitively displace parthenium weed (O'Donnell and Adkins 2005).

Fire and weed management: Fire has historically been an important part of the ecology of many of ecosystems. According to Keely (2001) burning may be effective at controlling noxious weeds but unless accompanied by revegetation with native species, is unlikely to diminish the alien dominance. A study carried out by Shabbir (2006) in Lahore revealed that when crop residues were burnt, the subsequent emergence of parthenium seedlings was significantly reduced in next coming crop. Wheat and rice fields, when watered after harvest showed that fire has significantly reduced the parthenium weed with no emergence in bare areas. On the other hand residues that escaped fire were stuffed with parthenium weed. This study suggests that parthenium weed may be a potential threat in those zero tillage rice – wheat cropping systems. In some other studies in Australia, smoke and heat did not significantly stimulate parthenium weed germination. Fire did, however, result in one-off increases in parthenium weed densities, which after subsequent fires rapidly declined (Vogler et al. 2002). In other studies smoke has been shown to stimulate parthenium weed seed germination and in some cases seedling emergence (Adkins et al. 2000, 2001).

Biological control: Biological control by means of insects and pathogens has been used in several parts of the world to control the exotic weeds. This exploits coevolved natural enemies from the centre of the origin of the target pest species offering practical and sustainable solutions for the management of alien invasive species (Plate 21.4). Biological control has been shown to be more cost effective than chemical means for parthenium weed. Encouraging results have been reported using biotrophic fungi (rusts) and several insect groups as agents for biological control of Parthenium weed (Dhileepan 2001, 2003a, b). *Zygogramma bicolorata* Pallister a beetle released as biocontrol agent against this weed in India and Australia has been reported by the author in Pakistan from Changa manga forest reserves (Shabbir

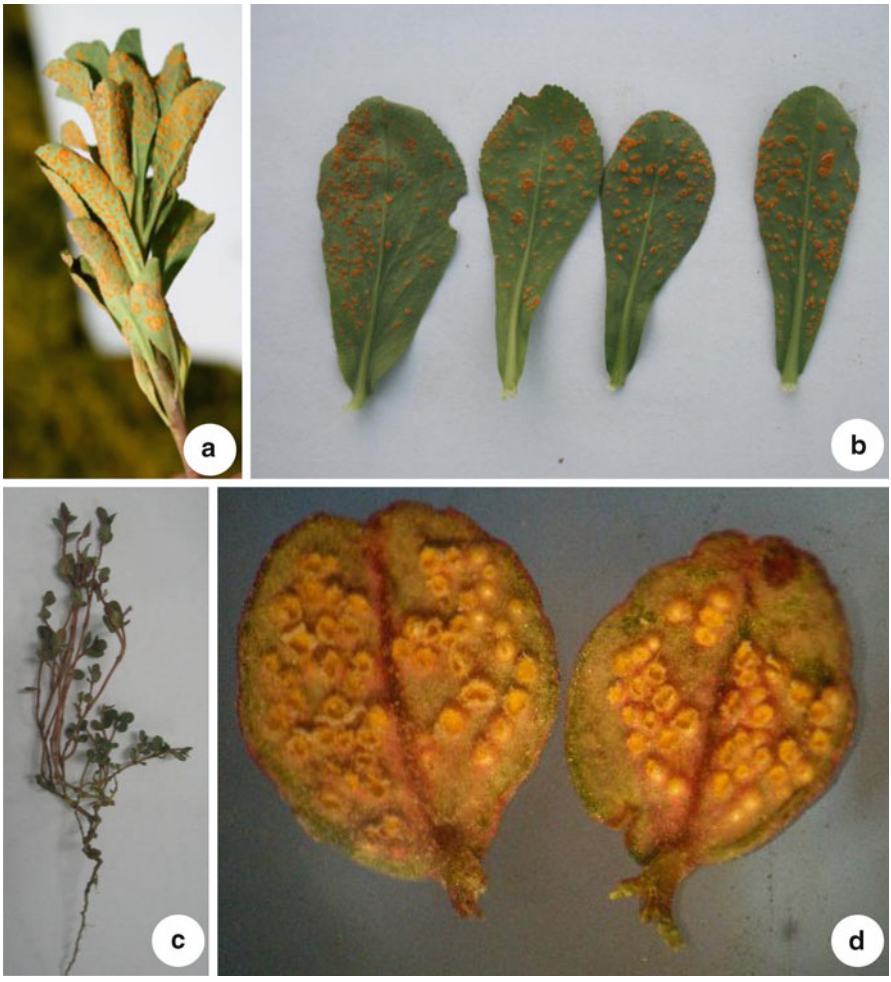

Plate 21.4 Two important weeds, *Euphorbia helioscopia* and *Euphorbia prostrate* and their biocontrol options: (**a** and **b**) *E. helioscopia* and its leaf rust; (**c** and **d**) *Euphorbia prostrate* and its leaf rust

2003; unpublished data) There is need to introduce some fungal pathogens along with some other insects as biocontrol agents in Pakistan to control its rapid spread. Unfortunately there exist no regulations regarding the release of fungal pathogens however; CABI-Pakistan has the prosvision to release the insect as biocontrol agents only. Moreover, a mealy bug has been reported on parthenium which seems to be very effective biocontrol agent but unfortunately not host specific and found attacking many wild and ornamental plants.

7.2 Mycotrophy in Relation to Ecological Success

Mycotrophy is the phenomenon of developing a mutualistic symbiotic relationship between plant roots/underground portions and fungi belonging to a specific group, Glomeromycota. The relationship facilitates a bilateral beneficent coordination. The fungus provides nutrients to plants ans in turn gets prepared food materials from plants. Two major types include ectomycorrhizae and arbuscular mycorrhizae. Of these two, later is the most ubiquitous and more than 50% of land plants are obligatory involves in this relationship. The relationship has existed millions of years ago when plants came to land. It is well established that plants which are ecologically predominant are able to establish strong mycotrophic relationship (Nasim 2010).

The invasion of alien plants can cause a serious threat to native ecosystems and economics (Pimentel 2002; Pimentel et al. 2004), and is the second cause of biodiversity losses after habitat destruction (Vitousek et al. 1997). Species invasion are favoured by the vulnerability of the invaded ecosystems (Sakai et al. 2001), the multiplication of introductions by human exchanges (Williamson 1996), or due to their own genetic, biological, physiological, and ecological attributed (Roy 1990; Prinzing et al. 2002). The investigation focusing on ecological attribute base invasion success of the alien plants on an enemy release hypothesis, where introduced species escape their natural enemies, thus giving them an advantage (Maron and Vila 2001; Kean and Crawley 2002). Other studies (Richardson et al. 2000; Klironomos 2002; Rudgers et al. 2005) have dealt with the potential role of the arbuscular mycorrhizal (AM) symbiosis to facilitate plant invasion in new areas. Most invasive plants are mycotrophic and thus preadapted to successfully establish in habitats containing AM fungi. Keeping in view the wide spread distribution of AM fungi and low host specificity, these mutualistic associations could favour invasion of many ecosystems by alien plants (Richardson et al. 2000; Fumanal et al. 2006). Mycorrhizal interactions could be a key point in invasive plant process, not only by facilitating local adaptation and/or reducing environmental stress, but also through their direct or indirect effects on interplant competition. Moreover, AM are now recognized as a major factor in structuring plant communities (Richardson et al. 2000).

Weeds also develop strong Mycorrhizal relationship to facilitate them for accelerated acquisition of the nutrients and water. *Parthenium hysterophorus* is an example of a notorious weed which is newly introduced in Pakistan. Besides exhibiting other "weedy" characteristics it is able to develop strong arbuscular mycorrhizal association. This relationship enables plants to out compete in ecologically diverse habitats. The year round active phase of the plant (except 1 ½ month in winter) enables AM fungi to actively absorb nutrients and save its energy from being concentrated to form spores and other perennating structures. Plant to plant colonization is also reported facilitating an easy spread of the weed (Nasim 2010).

7.3 Management Through Awareness Partnership and Participation

To address the issue of invasive weed species, public awareness and community participation and involvement are of the prime importance. Partnership between public and private sectors in activities to address IAS has shown great success in many countries. Promotion of awareness of invasive weeds issues by convening workshops and seminars, as well as conducting publicity events and media campaigns resulted in effective management program globally. Department of Mycology and Plant Pathology University of Punjab organized a national symposium on the awareness of Parthenium weed from 6 to 7th August 2004. Scientists, teachers and students from various parts of the country participated in this symposium and recommendations were formulated to create awareness about this dreaded weed in scientific and non-scientific community. PARTHENIUM NEWS: a biannual news letter is publishing form department of Mycology and Plant Pathology to create awareness about this weed (Plate 21.5). Ensure the sustainability of IAS prevention and management activities in the region by developing long-term programs of action.

Florists are excessively using parthenium weed, one of the most dangerous weeds that causes different diseases among humans, in the preparation of bouquets (Shabbir 2002). This misuse is not only threat to health of general public but also a source of spread to new localities. The rapid spread is probably due to its use in bouquets. An action group named Pakistan Parthenium Action Group (PPAG) was formed in 2002. This group ensures that the weed receives adequate publicity to create awareness that helps early identification when Parthenium weed germinate in newer locality. Another objective of this Action group is to establish protocols to reduce the movement and spread of parthenium weed particularly in municipal areas of metropolitan cities of Punjab and raise community's ability to recognize parthenium weed, know of its impacts and skills required for its management.

Plate 21.5 Face copies of publications of IMPP on awareness program of Invasive weeds of Pakistan

8 Recent Trends in Weed Management and Modeling

Weeds still represent an important constraint to crop production in the world. Agricultural practice has demonstrated that the same philosophy of integrated management used for insect control also needs to be adopted for weed control. Because of the vast number of serious weed problems in all regions of the world, just one method alone can no longer be relied on. The developments in weed management have taken place mainly through the application of new approaches and methods, rather than the introduction of new herbicides. Moreover, agriculture has promoted new methods, which also directly or indirectly affect weed control. These methods are original *per se* and demand a new approach for weed control. For example, organic agriculture does not permit the use of chemical herbicides, therefore cultural and biological control is the only possible means left to cope with weeds (Marwat et al. 2010).

Presently, the major problems in wheat fields are associated with herbicide-resistant weeds. In this context, one of the most serious incidences is the one associated with isoproturon-resistant grass *Phalaris* minor in North India. The entire rice-wheat area of 10 million ha is affected by the resistant biotype of *Phalaris* in the Indo-Gangetic plains. The problem is more serious in a 5 million ha area (Malik 2002, personal communication). To overcome this problem, Indian specialists have adopted an integrated approach for cropping wheat in rotation with direct-seeded rice. There are already more than 10,000 ha of wheat planted with the no zero till system in India, and it has brought about several benefits, such as improving water-use efficiency, preventing soil erosion and compaction, reducing the need for applying herbicides, and increasing wheat yields. The studies on long-term trials on zero till have shown that *Phalaris minor* stand decreased over the 3-year period because of the combined effect of the herbicide clodinafop and zero tillage (Rice-Wheat Consortium and CIMMYT 2003).

Herbicide-resistant crops offer the potential for simpler weed control, more effective management of problematic and resistant weeds, more timely weed control with potential to employ critical period, increased usage of minimum or zero tillage and avoidance of yield loss caused by current ‘selective’ herbicides (FAO 1998). Although the benefits are certain, herbicide-resistant crops (HRCs) may also pose direct risks to human health, and may cause indirect, ecological, and evolutionary risks, which have not received enough attention from regulatory agencies (Denison 2001).

9 Instruments of Quarantine, Environmental Law and Legislation

Some exotic plant pests leave immediate evidence of their presence. Signs of disease, crop damage or weed growth appear almost instantly. Other foreign pests however can go undetected for months or even years in the absence of proper surveillance.

Without early detection, these insidious organisms can become established in a new geographical range and permanently damage agriculture and natural resources of the area.

9.1 An Endangered Ecosystems Act

The endangered species act formally recognizes the importance of saving species that are in serious trouble. The act has enjoyed some success but is considered seriously flawed by many environmentalists because it does not directly address the major reasons species become endangered: the loss of crucial ecosystems that are their habitats, alien invasive species, pollution, climate change, disease etc. Thus, ecosystem diversity, a level of diversity higher than biodiversity, is also important to maintain (Wright 2005).

Ecosystem diversity is based on the idea that there should ne a variety of ecosystems in a given area, be it local national or global. By protecting many different types of ecosystems, many endangered species will be protected, and a bulk of other species will be prevented from becoming endangered. Thus protecting ecosystem diversity requires both the qualitative and quantitative preservation of ecosystem. In the United States only a number of ecosystems have been degraded from 85% to 98%. What has happened to these ecosystems? In large measures, they have been altered considerably by urban development, agriculture, exploitation of their resources, and pollution including the introduction of alien weeds. In response to such real and continuous losses, many scientists and environmentalists have called for a national Endangered Ecosystem Act (Wright 2005).

Implementation of endangered Ecosystem Act would involve steps similar to those taken to protect endangered species – that is. Taking an inventory of the status of ecosystem to identify those in trouble, protecting endangered ecosystems from further activities that would damage them, establishing recovery plans for many of the most critical ecosystems, and promoting research and monitoring to maintain surveillance and to gain knowledge for better management and protection (Wright 2005).

9.2 Plant Protection and Quarantine

The best and most cost-effective solution for mitigating agricultural damage is preventing the entry of exotic plant pests or invasive weeds into the country. Plant protection and quarantine (PPQ) is a regulatory program of USA, it is responsible for establishing effective regulations and policies to protect the country's agriculture through the exclusion of exotic species. PPQ determines what plants and plant products can be imported into this country and what products pose a high risk and, therefore, should be kept out. Based on varying levels of risk, PPQ's regulations

provide multiple layered of protection ranging from product bans to commodity treatments or other actions that lessen pest risk (e.g., restricting imports to certain geographical regions or certain seasons) (Shabbir 2002).

Plants and seeds requiring permits for propagation must enter the United States through specific ports-of-entry, where such items can be inspected and certified free of invasive pests and disease. At these plant inspection stations, PPQ also enforces the rules and regulations that apply to the import of plant species protected by the endangered species of Wild Fauna and Flora (CITES). PPQ currently operates 17 plant inspection stations nationwide and has been instrumental in surveying for invasive organisms, that do not exist in the United States but have been intercepted at U.S. port of entry. These pest potentially affect urban and natural areas and the Nations lumber and agricultural industry. The proactive work helps ensure that if these pests are found, they are found quickly before that can become established here. Early detection saves million of dollars in control ans eradication costs, protects natural resources, and presents the economic disruption of agricultural industry (Shabbir 2002).

9.3 Pakistan Environmental Protection Act (PEPA)

Pakistan Environmental Protection Act (PEPA), enacted in 1997, is an elaborate, comprehensive act to provide for the protection, conservation, rehabilitation and improvement of the environment, for the prevention and control of pollution and promotion of sustainable development. It rectifies CITES (Convention of International trade in endangered species of wild fauna and flora 1973); Ramsar (Conventional on wetlands of international importance 1971; 1975; Vienna convention for the protection of ozone layer, Montreal Protocol on Substance that deplete ozone, 1985; Stockholm Declaration on Human Environment, 1972; World's conservation strategy, 1980; World Charter for Nature, 1982; Man and Biosphere Program, 1970; Brundtland report, 1987). In all these conventions and declarations it indirectly addresses the issue of biodiversity conservation but no direct reference is made to the invasive species and not a single sub-section is focusing to tackle the problem. The new biosafety and quarantine rules have been laid down recently and are waiting for their enforcement and implementation. PEPA has badly failed due to illiteracy and unawareness prevailing in the masses, besides paucity of funds and corruption. An intensive revision of PEPA is therefore recommended keeping in view the recent development taking place in the developed world with special focus on the role of invasive species on economy and biodiversity of the region.

10 Conclusion

Weeds are a menace for agriculture in the world. These unwanted plants parasitize on the nutrients and other resources available for the crop plants. Billion of dollars are spent on weedicides to tackle with the problem. In developing countries like

Pakistan the damages caused by weeds are a nasty blow to the struggling economy of the country. In view of use of these weedicides and anthropogenic activities the problem become multifold with the invasion of alien weeds like *Parthenium hysterophorus*. Investigations are in progress to manage these weeds using biological methods or with competitor indigenous plants. It is also needed to strengthen our national biosafety regulations and bring them at par with the international criteria particularly for transportation of grains and other biological materials and soil. To avoid the problem, quarantine and phytosanitary measures should also strictly be reinforced.

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Chapter 22
An Overview of Plant Growth Promoting Rhizobacteria (PGPR) for Sustainable Agriculture

Rifat Hayat, Iftikhar Ahmed, and Rizwan Ali Sheirdil

Contents

Abstract Soil bacteria beneficial to plant growth usually referred to as plant growth promoting rhizobacteria (PGPR), are capable of promoting plant growth by colonizing the plant root. The mechanisms of PGPR-mediated enhancement of crop growth includes (i) a symbiotic and associative nitrogen fixation; (ii) solubilization and mineralization of other nutrients; (iii) production of hormones e.g. auxin i.e. indole acetic acid (IAA), abscisic acid (ABA), gibberellic acid and cytokinins; (iv) production of ACC-deaminase to reduce the level of ethylene in crop roots thus enhancing root length and density; (v) ability to produce antagonistic siderophores, *ß*-1-3-glucanase, chitinases, antibiotics, fluorescent pigment and cyanide

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against pathogens and (vi) enhanced resistance to drought and oxidative stresses by producing water soluble vitamins niacin, thiamine, riboflavin, biotin and pantothenic acid. Increased crop production through biocontrol is an indirect mechanism of PGPR that results in suppression of soil born deleterious microorganisms. Biocontrol mechanisms involved in pathogen suppression by PGPR include substrate competition, antibiotic production, and induced systemic resistance in the host. PGPR can play an essential role in helping plants to establish and grow in nutrient deficient conditions. Their use in agriculture can favour a reduction in agro-chemical use and support ecofriendly crop production. Trials with rhizosphere-associated plant growth-promoting P-solubilizing and N_2-fixing microorganisms indicated yield increase in rice, wheat, sugar cane, maize, sugar beet, legumes, canola, vegetables and conifer species. A range of beneficial bacteria including strains of *Herbaspirillum, Azospirillum* and *Burkholderia* are closely associated with rhizosphere of rice crops. Common bacteria found in the maize rhizosphere are *Azospirillum sp., Klebsiella sp., Enterobacter sp., Rahnella aquatilis, Herbaspirillum seropedicae, Paenibacillus azotofixans,* and *Bacillus circulans*. Similarly, strains of *Azotobacter, Azorhizobium, Azospirillum, Herbaspirillum, Bacillus* and *Klebsiella* can supplement the use of urea-N in wheat production either by BNF or growth promotion. The commonly present PGPR in sugarcane plants are *Azospirillum brasilense, Azospirillum lipoferum, Azospirillum amazonense, Acetobacter diazotrophicus*, *Bacillus tropicalis, Bacillus borstelensis, Herbaspirillum rubrisubalbicans* and *Herbaspirillum seropedicae*. Symbiotic N_2-fixing bacteria collectively known as Rhizobia are currently classified into six genera; Rhizobium, Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium and Sinorhizobium and 91 species. Their inoculation may increase nodulation and N_2-fixation in legumes. All these Rhizobiumn spp. can minimize chemical N fertilizers by BNF, but only if conditions for expression of N_2-fixing activity and subsequent transfer of N to plants are favourable. In this Chapter, PGPR role has been discussed in the process of crop growth promotion, their mechanisms of action and their importance in crop production on sustainable basis.

Keywords PGPR • BNF • P-solubilization • Phytohormones • Biocontrol • Cereals • Sugarcane and legumes crops

1 Introduction

Plant growth promoting rhizobacteria (PGPR) are capable of promoting plant growth by colonizing their roots and can play an essential role in helping plants to establish and grow in nutrient deficient conditions. Their use in crop production can reduce the agro-chemical use and support ecofriendly sustainable food production. Plant growth promotion by PGPR is due to root hair proliferation, root hair deformation and branching, increase in seedling emergence, early nodulation,

nodule functioning, enhanced leaf surface area, vigor, biomass, increasing indigenous plant hormones levels, mineral and water uptake, promoted accumulation of carbohydrates and yield in various plant species (Podile and Kishore 2006). The demand of PGPR biofertilizers has been increasing day by day with increase in the importance of organic agriculture with minimum inputs of chemicals. The population of PGPR in rhizospheric soil varies and depends largely on crop species and soil health (Tilak et al. 2005). Numerous studies clearly indicated the positive effect of PGPR on growth of different crops at different agroecological boundaries. Trials with soil rhizospheric N_2-fixing and P-solubilizing *Bacillus* species showed yield increase in wheat (de Freitas 2000), rice (Sudha et al. 1999), maize (Pal 1998), sugar beet (Çakmakçi et al. 2006), canola (de Freitas et al. 1997), and conifer species (Bent et al. 2002). One of the most important and oftenly reported PGPR is *Bacillus polymyxa,* also known as *Paenibacillus polymyxa* (Timmusk et al. 1999) with a range of properties, including P-solubilization (de Freitas et al. 1997), nitrogen fixation (Coelho et al. 2003); production of cytokinin (Timmusk et al. 1999), antibiotic (Rosado and Seldin 1993), hydrolytic enzymes (Nielson and Sorenson 1997), colonization hair and cortical cells (Shishido et al. 1999), and increased root and shoot growth of crops (Sudha et al. 1999). Some strains of *Rhodobacter* are known to fix N_2 (Drepper et al. 2002), but extensive studies are needed to validate its ability to fix N_2. *Pseudomonas* sp. effectively adapt to new environments and colonize winter wheat roots (Misko and Germida 2002) thus significantly increasing root dry weight in spring wheat (Walley and Germida 1997), yield in sugar beet (Çakmakçi et al. 2001), and promote the growth of spinach (Urashima and Hori 2003).

Sustainable agriculture production can be achieved by emphasizing the use of PGPR as biofertilizer inoculants (Schippers et al. 1995). Generally, bacteria promote plant growth in three different ways (Glick 1995, 2001): synthesizing growth promoting harmones for the plants (Dobbelaere et al. 2003), facilitating the uptake of nutrients from the soil (Çakmakçi et al. 2006), and lessening or preventing the plants from diseases (Saravanakumar et al. 2008). The indepth mechanisms involved in plant growth by PGPR are yet to be investigated (Dey et al. 2004). However, the possible explanations include (i) solubilization of mineral phosphates and mineralization of other nutrients (Richardson 2001; Banerjee and Yasmin 2002); (ii) a biological nitrogen fixation (Kennedy et al. 2004a, b); (iii) ability to produce hormones like auxin i.e. indole acetic acid (IAA) (Patten and Glick 2002), abscisic acid (ABA) (Dobbelaere et al. 2003), gibberellic acid and cytokinins (Dey et al. 2004); (iv) ability to produce ACC-deaminase to reduce the level of ethylene in root of developing plants thereby increasing the root length and growth (Li et al. 2000; Penrose and Glick 2001); (v) antagonism against phytopathogenic bacteria by producing siderophores, *ß*-1,3-glucanase, antibiotics, chitinases, fluorescent pigment and cyanide (Glick and Pasternak 2003); and (vi) mediated resistance to drought (Alvarez et al. 1996) and oxidative stresses (Stajner et al. 1995, 1997) and production of water soluble vitamins thiamine, niacin, pantothenic acid, biotin and riboflavin (Revillas et al. 2000).

2 Biological Nitrogen Fixation (BNF)

Nitrogen is the most important nutrient and its deficiency severely affects crop yields. Most of the soils around globe are deficient in nitrogen and applications of nitrogenous fertilizer are essential for achieving maximum crop yield. Nitrogen is required for all living organisms. Although 78% of the atmosphere consists of dinitrogen, but it cannot be used by most organisms and consequently the availability of nitrogen in a form suitable for assimilation is often a major limiting factor for crop growth. The production of chemical nitrogen fertilizers not only depletes non-renewable energy resources but also poses human and environmental hazards, besides being very expensive. Urea is the cheapest and readily available N source, but unfortunately less than 50% of the applied urea is used by plants. This low efficiency of use is mainly caused by NH_3 volatilisation and denitrification, and losses from leaching. Leaching of NO3-N causes ground water toxicity. Volatilisation and denitrification pollute the atmosphere through the evolution of greenhouse gases like N2O, NO and NH3. BNF not only complements and substitutes the mineral fertilizers but can be an economically beneficial and ecologically sound alternative (Glick et al. 1999).

BNF is a major source of nitrogen for plants as a part of environment friendly agricultural practices. It contributes for 65% of the total nitrogen currently utilized in crop production and will be important contributor for future agriculture (Matiru and Dakora 2004). This fixation is an important biochemical reaction next to plant photosynthesis for life on earth and occurs through symbiotic N_2-fixing bacteria possessing the nitrogenase enzyme and in association with legumes (and some woody species) that converts atmospheric elemental dinitrogen (N_2) into ammonia (Shiferaw et al. 2004). The potential of nitrogen fixation for most legumes species is in the range of 200–300 kg N ha^{-1} $crop^{-1}$ (Peoples et al. 1995).

In the recent years, use of biological inoculants for sustainable crop production is attaining popularity in various parts of the world and biological nitrogen fixation represents the major source of N input in agricultural soils including those in arid regions. Symbiotic nitrogen-fixing bacteria include the cyanobacteria, the genera *Rhizobium*, *Azorhizobium, Bradyrhizobium*, *Sinorhizobium, Allorhizobium, Mesorhizobium* and *Frankia* (Paul and Clark 1996; Brock et al. 2000). The mechanisms of symbiotic N_2-fixation between *Rhizobium* and legumes has been studied intensively. The symbiosis between *Frankia* and non-leguminous actinorhizal plants, is also been investigated now a days. Symbiotic system is the major N_2-fixation system, playing a significant role in improving the fertility and maximizing productivity of low-N soils. Biological N_2-fixed through rhizobium-legume symbiosis can also be beneficial to cereals growing in intercrops or to subsequent crops rotated with legumes. In many natural grassland systems, the grasses use N_2 fixed by their legume counterparts for their nitrogen requirement and the protein available through this association enhances the forage quality for livestock production (Paynel et al. 2001). In addition to symbiotic N_2-fixation in legumes, rhizobia as PGPR are also capable of contributing to growth promotion in non-legume species (Hoflich 2000). To act as PGPR, rhizobia naturally produce molecules (auxins, abscisic

acids, cytokinins, riboflavin, lumichrome, lipo-chitooligosaccharides and vitamins) that promote crop growth, and their colonization and infection of cereal root would be expected to increase vigor and grain yield (Matiru and Dakora 2004). Other PGPR role of *Rhizobium* includes their ability to produce phytohormones (Arshad and Frankenberger 1998), solubilization of inorganic phosphorus (Chabot et al. 1996), siderophore release (Plessner et al. 1993; Jadhav et al. 1994) and antagonism against plant pathogenic microorganisms (Ehteshamul-Haque and Ghaffar 1993). Application of rhizobium inoculants under rainfed conditions on legumes like guar (*Cyamopsis tetragonoloba* L. Taub), moth (*vigna acontifolia*), mung (*vigna radiate*), and mash (*vigna mungo*) give up to 10–25% yield benefits (Rao 2004; Hayat et al. 2008a, b).

Free living bacteria as well as rhizobial strains can promote the growth of cereals by contributing to N-economy through their ability to fix N_2 (Zahir et al. 2004). It is reported that biological N_2-fixed by the diazotrophic PGPR may be a contributing factor of rice growth promotion in addition to other mechanisms (Biswas et al. 2000). Kennedy and Islam (2001) also explained the possible role and mechanisms of non-symbiotic bacteria to crop growth from BNF view point. Providing the plant with essential nutrients, e.g. NH_4-N through atmospheric nitrogen fixation or aiding the plant in nutrient uptake is also considered as direct plant growth promotion. The important free living and associative nitrogen fixing genera are; *Azospirillum, Azotobacter, Acetobacter* (also known as *Gluconacetobacter*) *Azoarcus, Achromobacter, Bacillus, Burkholderia*, *Clostridia, Citrobacter*, *Enterobacter, Herbaspirillum*, *Kelbsiella*, *Mycobacterium, Pseudomonas, Rhodobacter* and *Serratia*. This list is increasing day by day.

3 Mineral-Solubilization and Uptake

PGPR directly contribute to the plant growth by facilitating plant nutritions through solubilization of inorganic phosphates and production of iron chelating siderophores thus increasing phosphorous and iron uptake.

3.1 Phosphorus Solubilization

Phosphate solubilizing bacteria (PSB) could play an important role in supplying phosphate to plants in a more environment friendly and sustainable manner. The naturally abundant PSB solubilize Calcium-bound phosphatic compounds in an alkaline soil environment and convert the insoluble phosphatic compounds into soluble forms and make them available to crop plants. PSB are widely applied in agronomic practices in order to increase the productivity of crops while maintaining the health of soils. The beneficial effects of PSB on plant growth vary significantly depending on environmental conditions, bacterial strains, plant and soil conditions (Şahin et al. 2004; Çakmakçi

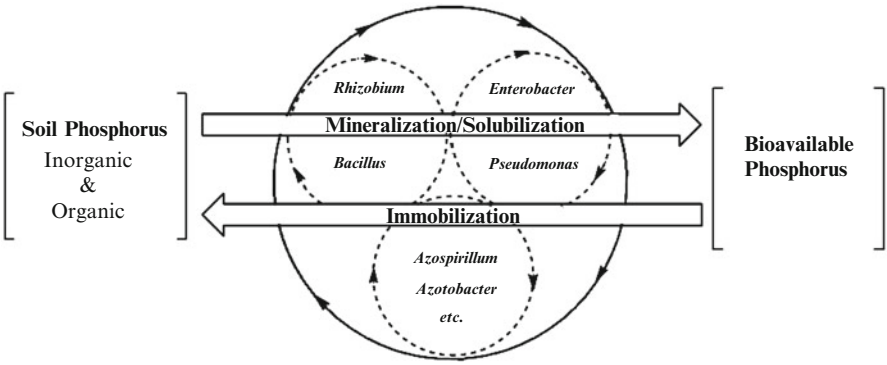

Fig. 22.1 Schematic diagram of soil phosphorus mobilization and immobilization by bacteria (Khan et al. 2009)

et al. 2006). Various bacterial species in the genera *Bacillus, Rhizobium* and *Pseudomonas,* have proven to be the most powerful phosphate solubilizing bacteria (Banerjee et al. 2006). There are also reports of phosphate solubilization by non-symbiotic nitrogen fixer, *Azotobacter* (Kumar et al. 2001). The phosphate-solubilizing activity of *Rhizobium* (e.g., *Rhizobium*/*Bradyrhizobium*) is associated with the production of 2-ketogluconic acid, indicating that phosphate-solubilizing activity of the organism is entirely due to its ability to reduce pH of the medium (Halder and Chakrabarty 1993). The phosphate-solubilizing ability also depends on nature of nitrogen source used in the media, with greater solubilization in presence of ammonium salts than when nitrate is used as nitrogen source. This has been attributed to extrusion of protons to compensate for ammonium uptake, leading to a decreased extra-cellular pH (Roos 1984). In some cases, however, ammonium can lead to decrease in phosphorus solubilization (Reyes et al. 1999). Several strains of P-solubilizers have been identified in vitro as *Bacillus brevis*, *B. megaterium, B. polymyxa, B. sphaericus, B. thuringiensis* and *Xanthomonas maltophilia* (de Freitas et al. 1997). P-solubilizing bacteria of *Bacillus* and *Paenibacillus* (formerly *Bacillus*) sp. have been applied to soils to successfully enhance the phosphorus status of plants (van Veen et al. 1997).

Since 1950s it is reported that P-solubilizing bacteria release phosphorus from organic and inorganic soil phosphorus pools through mineralization and solubilization (Fig. 22.1; Khan et al. 2009). Lowering of soil pH by microbial production of organic acids such as acid phosphatases, lactate, citrate, and succinate gluconic and keto gluconic acids etc. (Goldstein 1995; Deubel et al. 2000) and proton extrusion is the main principal mechanism of mineralization of organic form of phosphorus. Release of phosphorus from insoluble and adsorbed forms is also an important aspect of phosphorus solubilizing bacteria regarding phosphorus availability in soils. Phosphorus solubilizing bacteria transform soil phosphorus to forms that can easily be taken up by crops. Bacteria assimilate soluble phosphorus, and make it available by preventing it from adsorption (Khan and Joergensen 2009). Bacteria also enhance phosphorus availability to crops by solubilizing precipitated forms of phosphorus (Chen et al. 2006).

Fe - and Al - P and Ca - P are the main precipitated forms in acidic and alkaline soils respectively and can be solubilized involving organic anions and associated protons. Complex form of adsorbed phosphorus my be released by chelating metal ions through ligand exchange reactions. Soil bacteria use carbon and serve as a sink for phosphorus by immobilizing it from soil (Bünemann et al. 2004). Phosphorus solubilizing bacteria mainly depend on soil phosphorus and organic matter contents and release phosphorus from their cells for crops (Kim et al. 1998a). Agricultural and range land soils are main habitat of phosphorus solubilizing bacteria (Yahya and Azawi 1998) and their use in crop production can reduce chemical P by 50% without any significant reduction of crop yield (Yazdani et al. 2009). PSB biofertilizers have great potential for sustaining crop yield along with optimized phosphorus fertilization. Examples of P-solubilizing PGPR include *Azotobacter chroococcum* (Kumar and Narula 1999) and *Bacillus circulans* in wheat (Singh and Kapoor 1998), *Enterobacter agglomerans* in tomato (Kim et al. 1998b), *Pseudomonas chlororaphis* or *Pseudomonas putida* in soybean (Catellan et al. 1999) and other prominent P-solubilizing genera are *Klebsiella*, *Balkhurdaria* and *Azospirillum* etc. The availability of nutrients i.e. C, N and metals ions are the determining factors of P-solubilizing ability of PGPR. The activity of P-solubilizing rhizobacteria varies with nitrogen source and increases in the presence of low levels of Ca^{2+} and EDTA (Nautiyal et al. 2000). Soil bacteria could increase the P nutrition of plants through increased solubility of Ca-phosphates. Phosphate solubilization is the result of combined effect of pH decrease and production of organic acids (Fankem et al. 2006). Bacteria through secretion of different types of organic acids e.g. carboxylic acid and rhizospheric pH lowering mechanisms (He and Zhu 1988) dissociate the bound forms of phosphate like $Ca_3(PO_4)_2$. Nevertheless, buffering capacity of the medium reduce the effectiveness of PSB in releasing P from tricalcium phosphates (Stephen and Jisha 2009). Acidification due to proton substitution/excretion of H^+ (accompanying greater absorption of cations than anions) or release of Ca^{2+} by microbial population releases P from apatite (Goldstein 1994; Villegas and Fortin 2002). The reverse phenomenon become operative when uptake of anions increases than that of cations, with excretion of OH^-/HCO_3^- (Tang and Rengel 2003). PSB produce carboxylic anions, which have high affinity to calcium. This phenomenon results in more solubilization of phosphorus than acidification alone (Staunton and Leprince 1996). Complexation favoured by organic acids results in cations complexing structures in P solubilization, which is an important mechanism. Decrease in pH as well as synthesis of carboxylic acids results in the release of calcium phosphate (Ca-P); however, release of protons cannot be the single mechanism (Deubel et al. 2000). Crop yields improve with increased solubilization of the fixed soil P as well as the applied phosphates by PSB (Gull et al. 2004).

Most annual crops often do not get benefitted by direct application of phosphate rock in a short time. Phosphatic rocks can be solubilized by acid producing microorganisms (Gyaneshwar et al. 2002) to release more P for plant uptake. The inorganic P-solubilizing activities by PSB ranges between 25 and 42 μg P mL^{-1}, whereas the organic P mineralization may occur between 8 and 18 μg P mL^{-1} (Tao et al. 2008). The application of P fertilizers can be reduced by 25% and 50%, by using PSB inocula along with single super phosphate and rock phosphate, respectively

(Sundara et al. 2002). It is reported that 29–62% P can be released by *Pseudomonas putida*, *P. fluorescens* Chao and *P. fluorescens* Tabriz; along with highest value of 0.74 mg P/50 mL from Fe2O3 (Ghaderi et al. 2008). *Pseudomonas fluorescens* is very effective and can solubilize 100 mg P L^{-1} containing $Ca_3(PO_4)_2$ or 92 and 51 mg P L^{-1} containing $AlPO_4$ and $FePO_4$, respectively (Henri et al. 2008). Rock phosphatic minerals are often insoluble to provide sufficient P for plant uptake; however, using PSBs can release P from the fixed insoluble minerals and thus help to increase crop yields (Zaidi 1999). PGPR not only improves BNF but also contribute in increasing the availability of soluble P and thus, enhance plant growth (Ponmurugan and Gopi 2006). It is reported that PSB enhanced seedling length of *Cicer arietinum* (Sharma et al. 2007) and increase sugarcane yield (Sundara et al. 2002). Co-inoculation of PSB and PGPR can reduce application of P fertilizers by 50% without affecting corn yield (Yazdani et al. 2009). Grain yield of wheat increased 20–40% by the application of inoculation along with P fertilizers against sole P fertilization (Afzal and Bano 2008). The indigenous PSB populations often are not effective in releasing P from bound phosphates mainly due to high buffering capacity of soil; however, inoculants of PSB biofertilizers may contribute considerably in more plant P uptake. Plant available P increased by the activity of PSB especially belonging to the genera: *Bacillus, Pseudomonas* and *Enterobacter*. So, PSB has enormous potential in biofertilizer formulations to be exploited in increasing crop yields by increasing fixed P in the soil, as well as by making good use of natural reserves of phosphate rocks.

3.2 Iron Uptake

Iron being essential micronutrient of plants plays a key role in several enzymes with redox activity. Its role is important in photosynthesis, NO_2 and SO_4 reduction and N_2 assimilation, and is therefore indispensable for chlorophyll biosynthesis (Rashid 1996). Iron is essential component of different steps involved in biosynthetic pathways and formation of chlorophyll required physiologically available iron to the plant (Lopez-Millan et al. 2001). Catalase and peroxidase are considered as important protoheme enzymes which can be used as biological markers in iron acquisition studies. Around 30% of the agronomic crops growing around the globe are facing the problem of chlorosis due to iron deficiency (Imsande 1998). Total soil Fe is always in excess of crop requirements, which is present in highly insoluble form of ferric hydroxide. In nature, various forms of iron are present in abundance but remain unavailable to plants due to their different solubility and bioavailability behaviour. The amount of soluble Fe in soil is much less than the total Fe contents thus iron remains unavailable for crops even in iron rich soils and contributes little in crop production (Podile and Kishore 2006). When iron availability to crops is inadequate for growth, leaves become pale green, yellow or white and eventually brown (Brittenham 1994). The availability of iron in soil solution is 10–18 M, a concentration even not sufficient for sustaining microbial growth. A large number

of soil bacteria produce siderophores that bind Fe^{3+} and help in iron uptake (Podile and Kishore 2006). These siderophores can be used by rhizosphere bacteria and crops can absorb bacterial Fe^{3+} siderophore complexes. This mechanism is involved in iron absorption by crops especially in calcareous soils. Different kind of PGPR produce different kinds of siderophores. Pseudomonads are leading siderophore producers among PGPR and siderophore producing strains of *fluorescent psuedomonas* may be inoculated in calcareous soils for solubilization of non-available forms of iron (Sharma and Johri 2003).

Siderophores are iron chelating compounds having low molecular weight which are released under iron limited rhizospheric soils. These siderophores possess high binding affinity and specificity for iron (III) and help to facilitate its bioavailability into the biological cell (Schalk et al. 2001). The transportation of ferric siderophore complexes is a heterologous uptake and mediated by specific receptor proteins (Meyer et al. 2000). PGPR with the ability to produce siderophore play a vital role for iron acquisition in rhizo spheric soil. It is reported that under non-sterile soil system, crops show no iron-deficiency symptoms and have more iron uptake in roots as compared to crops grown in sterile soil (Masalha et al. 2000). This supports the possible role of PGPR in iron acquisition. Iron is present in different complexes each having different solubilities in natural system; therefore, the bioavailability of iron depends upon the potential of siderophores to chelate the iron from its complexes (Hersman et al. 2001). A cold-tolerant mutant of *Pseudomonas fluorescens* with 17-fold increase in siderophore production enhances colonization and PGP effect on mungbean (Katiyar and Goel 2004). Similarly, seed inoculation of maize with siderophore-producing *Pseudomonas Pseudomonas chlororaphis* enhances seed germination and root shoot biomass (Sharma and Johri 2003). Siderophores producing PGPR suppressed root pathogens by consuming available soil iron (Kloepper et al. 1980) and with the addition/availability of iron even in acidic soils ($pH < 6.0$), these siderophores becomes less effective (Neilands and Nakamura 1991). Different bacterial proteins are involved in iron uptake and transport. This uptake by different bacterial species depends on the available concentration of soil iron.

4 Plant Growth Regulators

PGPR synthesize and export phytohormones also called as plant growth regulators (PGRs), that may be synthesized in defined organs of plant and can be translocated to other sites, where these trigger specific biochemical, physiological, and morphological role in plant growth and development (Hayat et al. 2011). PGRs are organic in nature that promote physiological processes of plants even at low concentrations and also take part in the development of tissues where they are produced. Among PGRs, gibberellins, cytokinins, auxins, abscisic acid and ethylene are well documented. The most common and active auxin in plants is indole-3-acetic acid (IAA), that regulates many aspects of plant growth and development throughout the plant

cell cycle, from cell division, cell elongation and differentiation to root initiation, apical dominance, tropistic responses, flowering, fruit ripening, senescence and stimulation of plant growth. Regulation of these processes by auxin is believed to involve auxin-induced changes in gene expression (Guilfoyle et al. 1998). In addition to IAA, bacteria such as *Paenibacillus polymyxa* and *Azospirillum* also release other compounds in rhizosphere that could indirectly contribute to plant growth promotion like indole-3-butyric acid (IBA), Trp and tryptophol or indole-3-ethanol (TOL).

Cytokinins too are important phytohormones usually present in small amounts in biological samples (Vessey 2003). These enhance cell division, root development and root hair formation (Frankenberger and Arshad 1995), and are also involved in the processes such as photosynthesis or chloroplast differentiation. They are also known to induce opening of stomata, suppress auxin-induced apical dominance, and inhibit senescence of plant organs, especially in leaves (Crozier et al. 2001). More than 30 growth promoting compounds of cytokinin group are reported that are produced by plant associated PGPR. Cytokinin producing bacteria *Azotobacter chroococcum* and cytokinin precursor's isopentyl alcohol (IA) and adenine (ADE) have been tested on maize crop under controlled and field conditions by Nieto and Frankenberger (1991). They observed improvement in crop growth. PGPR also produce widely recognized gibberellic acid (GA) and gibberellins (GAs).Over 89 GAs are known to date, most of which are primarily responsible for stem elongation (Dobbelaere et al. 2003). GAs also affect reproductive processes in a wide range of plants (Crozier et al. 2001). PGPR like *Azospirillum* and *Pseudomonas* sp. produce cytokinins and gibberellins (gibberellic acid). In addition to IAA, abscisic acid (ABA) has also been detected by radio-immunoassay or TLC in supernatants of *Azospirillium* sp. and *Rhizobium* sp. cultures (Dobbelaere et al. 2003).

Ethylene is a plant growth regulator synthesized by almost all species of bacteria (Primrose 1979). It acts as a ripening hormone, promotes adventitious root and root hair formation, stimulates germination, breaks dormancy of seeds; promote plant growth, development, and senescence. However, if ethylene concentration remains high after germination, root elongation, as well as symbiotic N2 fixation in leguminous plants is inhibited. PGPR produces enzyme like 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyzes ACC and lowers the level of ethylene in crop rhizosphere. The product of this hydrolysis, ammonia and α-ketobutyrate, can be used by the bacterium as a source of nitrogen and carbon for growth. In this way the bacterium acts as a sink for ACC and thus lowers the ethylene level in plants, preventing some of the potentially deleterious consequences of high ethylene concentrations (Glick et al. 1998). PGPR with ACC-deaminase characteristics improve crop growth and yield and may be included bio-fertilizer biotechnology (Shaharoona et al. 2006). Role of PGPR in the production of phosphatase, β-glucanase, dehydrogenase and antibiotics has also been recognized. Another recently identified mechanism for plant growth promotion is due to production of volatiles by PGPR. Ryu et al. (2004) discussed in detail the role of bacterial volatiles in plant growth promotion *in vitro*. PGPR release different volatile blends and the differences in these volatile blends stimulate the plant growth. Volatile compounds like 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol, produced by *Bacillus subtilis*

and *B. amyloliquefaciens* stimulated the growth of *Arabidopsis thaliana* in *in-vitro* experiments. The volatile-mediated growth promotion by PGPR is by activation of cytokinin-signaling pathways.

5 Role of PGPR in Biocontrol Aspects

PGPR indirectly help in plant growth by suppression of deleterious microorganisms that inhibit plant growth or root pathogens through antibiosis, parasitism, competition for nutrients and space within the vicinity of plant roots, and/or activation of host defense responses (Podile and Kishore 2006). The strains of *Bacillus subtilis* are the most widely used PGPR due to their disease reducing and antibiotic producing capabilities (Kokalis-Burelle et al. 2006). Fluorescent pseudomonads are also known to suppress soil born fungal pathogens by producing antifungal metabolites and by sequestering iron in rhizosphere through the release of iron-chelating siderophores, rendering it unavailable to other organisms (Dwivedi and Johri 2003).

Suppression of deleterious microorganisms by PGPR is mainly by parasitism, by competing for available nutrients, production of enzymes or toxins and inducing resistance by activating plant defense response against pathogens (Podile and Kishore 2006). Fluorescent pseudomonads establish themselves on plant roots and sink the available nutrients, thus limiting the available nutrients required for the growth of pathogen (Walsh et al. 2001). PGPR also compete for nutrients with native rhizosphere microbes for elimination of pathogens. Siderophore production by PGPR, sequester most of available Fe^{3+} in the rhizosphere and force the pathogens for iron starvation, thus is a major contributor for pathogen suppression (O'Sullivan and O'Gara 1992). Suppression of *Fusarium wilt* of radish by *Pseudomonas* strain WCS358 through siderophore-mediated competition for iron (Costa and Loper 1994) is another example. Some PGPR's degrade the cell wall of pathogenic fungi through production of hydrolytic enzymes (chitinase, β-1,3-glucanase). Purified chitinases of *Bacillus subtilis* AF 1 (Manjula et al. 2004), *Serratia marcescens* (Kishore et al. 2005b; Ordentlich et al. 1988) and *S. plymuthica* (Frankowski et al. 2001) are highly antifungal. β-1,3-glucanase producing strain of *Pseudomonas cepacia* inhibits the rhizosphere proliferation of various phytopathogenic fungi including *Rhizoctonia solani*, *Sclerotium rolfsii* and *Pythium ultimum* (Fridlender et al. 1993) and synergistic action of the two hydrolytic enzymes chitinases and β-1,3-glucanases was more effective in the inhibition of fungal pathogens than either enzyme alone (Tanaka and Watanabe 1995). Hydrolytic enzyme produced by pathogenic fungi includes pectolytic enzymes (polygalacturonases, pectin, lysis), cellulases and cutinase. PGPR's inhibit (Podile and Kishore 2006) these pathogenic fungi and thus activity of these enzymes can be reduced with the reduction in virulence (Beraha et al. 1983). *Bacillus megaterium* B 153-2-2 inhibits the activities of extracellular enzymes, like cellulase, pectin lyase and pectinase produced by *Rhizoctonia solani,* by producing an extracellular endoproteinase (Bertagnolli et al. 1996). A groundnut seed endophytic bacterium *Pseudomonas aeruginosa* GSE 18,

effective in control of groundnut stem rot disease, when applied as seed treatment or soil amendment, inhibits the production of cell wall-degrading enzymes (CWDE) polygalacturonase and cellulase by the pathogen *Sclerotium rolfsii* tested in vitro (Kishore et al. 2005c).A few strains of rhizobacteria activate plant defense responses against a broad spectrum of plant pathogens, termed as induced systemic resistance (ISR). Rhizobacteria-mediated ISR has been demonstrated in many plant-pathogen systems wherein the bacterium and the challenging pathogen remained spatially separated, and these observations indicate that ISR is genetically determined (Pieterse et al. 2001).

Inoculation of different strains of *Pseudomonas fluorescens* strains reduce the seedling mortality caused by *Aspergillus niger* (Dey et al. 2004) and show strong inhibition to *Sclerotium rolfsii* by reducing the incidence of stem rot severity. They also produce siderophores and antifungal metabolites. Production of antifungal metabolites by fluorescent *Pseudomonads* has been found to suppress soil-borne fungal pathogens on many occasions (Dey et al. 2004; Catellan et al. 1999). Similarly, *Pseudomonas putida* produces siderophores which convert a fusarium-conducive soil into a fusarium-suppressive soil for growth of different crops. Improvement in plant growth and disease resistance to a broad array of plant pests can be accomplished using PGPR (Kloepper et al. 2004). It is well recognized that PGPR can influence plant growth through creating resistance against pathogens. *Bacillus subtilis* is one such commercialized PGPR and it acts against a wide variety of pathogenic fungi (Banerjee et al. 2006).

6 Role of PGPR in Crop Production

Rice, wheat and maize are the three major staple food crops for world's population. A variety of PGPR's participate in interaction with C3 and C4 plants and can significantly increase their yield (Kennedy et al. 2004a, b). Rice crop removes around 16–17 kg N to produce 1 t dry weight of rice including straw. Wheat crop requires about 26–28 kg N to produce 1 t of grain including straw (Angus 2001). Maize plants require 9–11 kg N to produce 1 t biomass (Kennedy et al. 2004a, b). The N requirement of cereals is normally met by fertilization at a rate depending on soil fertility with chemical urea (Scharf 2001). PGPR inoculant biofertilizers can, in principle, be used to supplement or reduce the use of urea-N (Kennedy et al. 2004a, b). Those closely associated with rice rhizosphere are *Azospirillum*, *Burkholderia* and *Herbaspirillum*. A free living heterotrophic diazotroph like *Azotobacter (A. vinelandii* and *A. chroococcum)* uses C from sugar as energy source (Kennedy and Tchan 1992). There are obligatory anaerobic heterotrophs like *Clostridia* which are only capable of fixing N_2 in the complete absence of oxygen (Kennedy and Tchan 1992; Kennedy et al. 2004a, b) and are usually isolated from rice fields (Elbadry et al. 1999). Their activity in rice may be enhanced with the addition of organic source like straw (Kanungo et al. 1997), presumably as a result of microbial breakdown of cellulose into cellobiose and glucose. Yield of rice can be increased with the application of *Azotobacter, Azospirillum lipoferum* and *Azospirillum brasilense,*

*Azospirillum (*Reis et al. 2000). Similarly, *Burkholderia vietnamiensis* increases rice grain yields significantly up to 8 t ha^{-1} (Tran Vân et al. 2000) by supplementing 25–30 kg N ha^{-1} as synthetic fertilizer. Family *Enterobacteriaceae* has several diazotrophs like *Klebsiella* (*K. pneumonia*), *Enterobacter* (*E. cloacae), Citrobacter* (*C. freundii*), and *Pseudomonas* (*Ps. putida* or *Ps. fluorescens*) isolated from rice rhizosphere with plant growth promoting traits (Kennedy et al. 2004a, b). Another rice endophyte *Herbaspirillum seropedicae* (James et al. 2000) can fix upto 45% of total plant N in rice from the atmosphere (Baldani et al. 2000). The N_2- fixation range by *Herbaspirillum* was assessed upto 58 mg $tube^{-1}$ under aseptic conditions (Reis et al. 2000). *Azoarcus* sp. is also endophytic Gram-negative N_2-fixing bacterium firstly isolated from Kallar grass *(Leptochloa fusa* Kunth*)* growing in saline-sodic soils of Pakistan (Reinhold-Hurek et al. 1993). *Azoarcus* colonise rice under both laboratory and field condition (Hurek et al. 1994; Reinhold-Hurek et al. 2002). Multi-strain biofertilizer (BioGro) containing three different PGPR like *Pseudomonas* (*Ps. fluorescens*, *Ps. putida*), *Klebsiella* (*K. pneumonia*) and *Citrobacter* (*C. freundii*) isolated from rice rhizospheric soil of Hanoi, Vietnam (Nguyen et al. 2003) significantly increased rice grain yield in Vietnam and Australia (Williams and Kennedy 2002). National Institute for Biotechnology and Genetic Engineering (NIBGE) of Pakistan also prepared a multi-strain rice biofertilizer rice with the commercial name "BioPower" (Malik et al. 2002) and similar product is also available in Egypt (Hegazi et al. 1998). These inoculants are claimed to give similar yield increases on rice farms upto 20% (Kennedy et al. 2004a, b).

Similarly, strains of *Azospirillum, Azotobacter, Azorhizobium, Bacillus, Herbaspirillum* and *Klebsiella* can supplement the use of urea-N in wheat production either by BNF or growth promotion (Kennedy and Islam 2001). The N requirement of wheat is higher than that for rice, because of its higher grain protein content. Wheat yields vary widely from 1 to 7 t ha^{-1} dependent on inherent soil fertility, amount of applied fertilizer, wheat variety, diseases, other management practices and environmental conditions (Angus 2001).Thus, estimated amount of N removed by wheat crop varies between 26 and 200 kg N ha^{-1}, depending on the yield (Reeves et al. 2002). To maximize wheat yields in soils that are not capable of supplying enough N, chemical fertilizers such as urea are used to enhance N supply. Biofertilizers are also being used to supplement the use of urea worldwide. The estimated amount of BNF by such wheat-bacterial associations was between 10 and 30 kg N ha^{-1} (Kennedy and Islam 2001) or about 10% of their total-N requirement. Wheat transformed about 30% of carbon assimilates into the process of rhizodeposition and part of this below ground translocated C is incorporated by rhizosphere micro-organisms (Kuzyakov and Domanski 2000). Studies indicate that PGPR may act as natural elicitor for improving the growth and yield of wheat. Important PGPR responsible for increased wheat yield in different parts of world are *Pseudomonas* (*ps. cepacia, ps. aeruginosa*, *ps. fluorescens* and *ps. putida, ps. chlororaphis*), *Bacillus (B. cereus), Azospirillum (A. brasilense, A. lipoferum) and Herbaspirillum (H. seropedicae).* Common PGPR species found in rhizosphere of maize are *Enterobacter sp., Rahnella aquatilis, Paenibacillus azotofixans, Herbaspirillum seropedicae, Bacillus circulans, Klebsiella sp*. and *Azospirillum sp.* (Chelius and Triplett 2000). The positive effects of *Azospirillum* on maize growth are mainly

derived from physiological changes of the inoculated plant roots, which enhance water and mineral nutrient uptake (Okan and Kapulnik 1986). Both *Azospirillum brasilense* and *Azospirillum irakense* are used as inoculant biofertilizer for maize. Other species of *Azospirillum* capable of increasing the yield of maize are *A. lipoferum,* and *A. indigens*. *Azorhizobium caulinodans* is also capable of giving such beneficial effects (Riggs et al. 2001). *Herbaspirillium seropedicae* can improve the ability of maize plant to use fertilizer N more efficiently and the yield increase due to *H. seropedicae* was up to 19.5% (Riggs et al. 2001). PGPR strains *Burkholderia* (*B. cepacia), Pseudomonas (P. fluorescens), Serratia (S. proteamaculans,* and *S. liquefaciens), Rhizobium (R. etli* bv. *Phaseoli, R. leguminosarum* bv. *Trifolii) and Sinorhizobium* sp. increase corn growth, plant height and grain yield of maize in different agro-ecological zones. The diazotrophs commonly present in sugarcane plants are *Acetobactor* also known as *Gluconacetobacter (A. diazotrophicus)*, *Azospirillum (A. brasilense, A. lipoferum, A. amazonense), Burkholderia (B. brasilensis, B. tropicalis),* and *Herbaspirillum (H. seropedicae, H. rubrisubalbicans)* (Kennedy and Islam 2001). Sugarcane requires approximately 1.45 kg N ha^{-1} to produce 1 t moist biomass (Bhuiyan 1995) or about 7 kg N ha^{-1} for 1 t of dry cane. Generally 150–250 kg urea-N ha^{-1} is applied for sugarcane cultivation depending on soil fertility, genotype and the targeted yield. Evidence from Brazil indicates fertilizer-N of sugarcane can be reduced to half by exploiting BNF systems, claimed to be based on diazotrophic PGPR such as A*cetobacter* (*Gluconacetobacter*) and *Herbaspirillum* (Döbereiner 1997; Döbereiner and Baldani 1998). More than 70% of sugarcane N (200 kg N ha^{-1} y^{-1}) was derived from biological fixed N2 by *Azospirillum* (*A. diazotrophicus*) (Boddey et al. 1991). Similarly, *Acetobacter* (with *nif*H^+) -sugarcane system has also been well established (Lee et al. 2002). *Azotobacter sp.* and *Klebsiella mobilis* are reported for improving potato yield. Similarly *Pseudomonas fluorescent* and *Achromobacter piechaudii* increase tomato yield. Positive effect of PGPR (*Bacillus cereus; Pseudomonas putida; Pseudomonas fluorescens; Serratia liquefaciens; Arthrobacteer cetreus; Escherichia coli, Mesorhizobium loti* and *Delftia acidovorans*) inoculation on the growth and yield of rapeseed have been reported by many researchers. *Burkholderia cepacia* alone or in combination with *Enterobacter* spp. and *Pseudomonas fluoroscens* have also been tested for its ability to promote growth of sorghum (*Sorghum bicolar*) (Chiarini et al. 1998). Inoculation with effective bacterial strains (*Pseudomonas alcaligenes, Pseudomonas denitrificans, Bacillus polymyxa, Azospirillum brasilense* and *Mycobacterium phlei)* increases the root and shoot growth of cotton (Anjum et al. 2007). Çakmakçi et al. (2007) also conducted a study on barley under greenhouse conditions in order to investigate seed inoculation with five different N_2-fixing (*Bacillus licheniformis*, *Rhodobacter capsulatus*, *Paenibacillus polymyxa*, *Pseudomonas putida*, and *Bacillus* spp. OSU-142) and two different phosphate solubilising (*Bacillus megaterium* and Bacillus spp. M-13) bacteria in comparison to control and mineral fertiliser (N and P) application. Co-inoculation of legumes with PGPR and *Rhizobium* has received increasing attention in recent years. Co-inoculation with symbiotic bacteria and rhizosphere bacteria may increase nodulation through a variety of mechanisms. PGPR strains, from a range of genera, enhance legume growth, nodulation and nitrogen fixation when co-inoculated with

their effective rhizobia. Examples of these are *Azospirillum* (*A. lipoferum, A. brasilense*), *Azotobacter (A. chroococcum)*, *Bacillus* (*B. cereus, B. endophyticus*, *B. pumilus*, *B. subtilis, B. firmis, B. megaterium*), *Paenibacillus (P. lautus*, *P. macerans*, *P. polymyxa,), Pseudomonos* (*Ps. fluorescens, Ps. putida, Ps. aeruginosa)* Serratia (*S. lequefacians, S. proteamaculans), Aeromonas hydrophia* and, *Streptomyces* (Pal et al. 2004; Kishore et al. 2005a; Figueiredo et al. 2007) . All these bacteria including cyanobacteria can supplement urea-N by BNF, but only if conditions for expression of N_2-fixing activity and subsequent transfer of N to plants are favourable. In general, it is believed that PGPR are more effective in promoting plant growth under limited supply of nutrients; however, present scenario does not allow to compromise on actual potential of crop productivity by reducing use of chemical fertilizers. Hence, it is of prime importance to isolate such PGPR strains that could be effective even under optimum nutrition. The biosynthesis of ethylene in plant roots is significantly affected by concentration of NO_3-N present around the roots (Ligero et al. 1999). Higher levels of NO_3^- in rooting medium stimulate ACC-oxidase activity leading to increased ethylene production, which is generally believed to be inhibitory to root growth (Glick et al. 1998). The nitrogenous fertilizer applied in ammonical form is readily oxidized to NO_3-N under aerobic conditions. It is very likely that NO_3-N present in the vicinity of roots reduces the efficiency of PGPR by inducing ethylene synthesis. However, PGPR containing ACC-deaminase minimizes the NO_3^- induced ethylene synthesis.

7 Conclusion

PGPR are able to enhance significantly the yield of cereals, legumes and sugarcane crops etc. Soil and plant health can be enhanced through PGPR-crops rhizospheric interactions. From the agricultural viewpoint, the aim will be to increase the yield on sustainable basis, while maintaining soil health. To achieve this, knowledge about indigenous PGPR for different crop rhizospheric environment must be improved. It is also necessary to adopt and maintain minimal standards for production of PGPR inoculants/biofertilizers. PGPR biofertilizers not only promote crop growth but also enhance the resistance against pathogens. Integrated use of PGPR along with inorganic and organic nutrient sources provides a sustainable agricultural ecosystem.

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Chapter 23
Arbuscular Mycorrhizae for Sustainable Agriculture

Ghazala Nasim

Contents

Abstract Arbuscular mycorrhizal (AM) associations have now been recognized having a cosmopolitan and ubiquitous occurrence forming with the roots and underground portions of plants. AM associations have been reported to enhance plant vigor by enabling them to absorb more nutrients. The thread like hyphae of the fungi work as conduits for pumping essential nutrients into the plant body, thereby imparting plant ample amount of resistance to combat with the soil born and other diseases. Due to these promising features of AM, they are being widely used as

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biological fertilizer at commercial level in many countries of the world and it has considerable practical significance in sustainable agricultural practices.

Keywords Arbuscular Mycorrhiza • Plant nutrition • Biocontrol agent • Metal toxicity • Micropropagated plantlets • Transgenic plants • Responsiveness • Sustainable agriculture

1 Introduction

The term mycorrhiza, which literally means fungal root, was first coined by AB Frank in 1985 during his study of endophytic fungi (Frank 1985). Since then, plethora of literature has mounted on various aspects of mycorrhizae. This is a unique example of symbiosis between two eukaryotes, soil fungi and plants. This association induces important morphological and/or physiological changes in each partner that lead to reciprocal benefits, mainly in terms of nutrient supply (Balestrini and Lanfranco 2006). Mycorrhizae comprise several distinctive groups that are recognized based on the type of host, fungus and the structures formed by the fungus. Seven such groups of mycorrhizae have been recognized out of which arbuscular mycorrhizae and ecto-mycorrhizae are the most common and widely distributed ones (Allen et al. 2003). Arbuscular mycorhizal (AM) fungi are the obligate mycotrophs and are the most ubiquitous and cosmopolitan in nature. More than 80% of land plants form arbuscular mycorrhizae (Brundrett 2002). These not only include plants belonging to angiosperms but algae, gymnosperms, pteridophytes, bryophytes and mosses are also beneficiaries of this relationship (Fonseca et al. 2006; Zhang et al. 2004; Nasim 2010). Recent studies have shown that AM fungi are much more widely distributed than had been considered before (Nasim and Iqbal 1991a, b; Iqbal and Nasim 1991). There are now reports on the occurrence of AM fungi in portions other than roots (Nasim 1990a, b). The submerged leaves of vascular plants and algae (Bagyaraj et al. 1979), senescent leaves of mosses, rhizoids, thalli of bryophytes and pteridophytes (Nasim et al. 1987; Nasim 1990a, b; Nasim 2010), decaying peanut leaves (Taber and Trappe 1982), pegs and nodules of the peanut plant (Graw and Rehm 1977; Nasim and Shaheen 1992), decaying leaf litter and leaves of *Dalbergia sisso* and *Termanalia arjuna* (Nasim and Iqbal 1989; Nasim et al. 1996a, b.; Nasim 2010) and scale leaves and rhizomes of a number of plants have been reported to harbor AM structures through several investigations.

Ectomycorrhizae are distinguished from arbuscular mycorrhizae on the basis of the presence of a Hartig net (Brundrett 2004). Hartig net consists of labyrinthine hyphae between root cells and is meant for the exchange of nutrients between the two partners. The thickness of the Hartig net may be variable depending upon the host-fungus combination (Burgess et al. 1994). The presence of an outer hyphal sheath called mantle confers protection from the pathogens by creating a physical barrier. While the hyphae protruding from the sheath enhance host nutrient supply by tremendously increasing the surface area. The fungi forming ectomycorrhizae

belong to Ascomycota and Basidiomycota. On the other hand, AM fungi which belong to Glomeromycota (Schüβler et al. 2001), form branched tree-like structures as the primary sites of nutrient exchange. Arbuscules are ephemeral structures which are often absent or hard to observe due to poor staining or host age and pigmentation. Vesicles are the storage structures formed at later stages of the host growth. The vesicles may serve as propagules for vegetative reproduction besides azygospores which are formed extramtrically by the fungus (Brundrett 2004).

Mycorrhiza is mutually beneficial relationship and the organisms involved in linking plant and soil, transporting mineral nutrients to the plants and C compounds to the soil and its biota (Bago et al. 2003). Mycorrhizal associations involve three-way interactions between host plants, mutualistic fungi and soil factors. They are therefore both agents of plant nutrition and soil nutrition (Hock 2001; van der Heijden and Sanders 2002; Allen et al. 2003; Querejeta et al. 2003a, b.). The hyphae of the arbuscular mycorrhizal fungi transport resources such as HPO_4, NH_4, Ca, S, K, Zn, Cu, H_2O to the host (Treseder and Allen 2002; Chen et al. 2004; Goicoechean et al. 2004). Reports on the effect of the arbuscular mycorrhizae on uptake of cesium and some other cations are also available (Berreck and Haselwandter 2001; George et al. 1994). Arbuscular mycorrhizal (AM) fungi contribute to the formation of relatively stable aggregate structures. Fibrous roots and AM fungal hyphae can be viewed as a "sticky-string bag" that contributes to the entanglement and enmeshment of soil particles to form macro-aggregates (Sylvia et al. 1997), a basic building block of soil structure. Furthermore, glomalin a glycoprotein, produced by AM fungi is deposited on their outer hyphal walls and on adjacent soil particles. Glomalin appears to be a rather stable hydrophobic glue that might reduce macro-aggregate disruption during wetting and drying events by retarding water movement into the pores within the aggregate structure (Miller and Jastrow 2000). In a nutrient poor environment such as sand dunes or rugged mountain slopes, AMF contributes not only to plant nutrition, but also to the process of dune stabilization by binding sand grains into wind-resistant aggregates (Forster and Nicolson 1981; Nasim and Bajwa 2001). They do so by binding the soil particles together (Miller and Jastrow 1992) and may serve as a substrate for other polysaccharide-producing microorganisms. The bacterial polysaccharides in fact cement the soil particles together (Tisdall and Oades 1979).

Land degradation due to salinity, waterlogging, erosion, etc., are serious and growing problems all over the world (Bell 1988; Scott 1992). These fungi also play a vital role in alleviating the effects of salinity (Al-Karaki et al. 2001). By improved nutrient acquisition, AM fungi compensate for the nutritional imbalances imposed by salinization (Sylvia and Williams 1992). Some other environmental stresses such as drought (Neumann and George 2004; Auge et al. 2003, 2004a, b.), micronutrient imbalances and industrial effluents (Oliveira et al. 2001), heavy metal toxicity (Chaudhry et al. 1999; Andrade et al. 2004), biocide treatment (Heggo et al. 1990), slurry application (Chistie and Kilpatric 1992), sulfur dioxide fumigation (Clappert and Reid 1990), and wild fire recovery (Puppi and Tartnlini 1991), involve the use of AM fungi (Barea et al. 1993). Some AM fungi are adapted to adverse conditions so they can benefit plants under a variety of environmental stresses (Mosse et al.

1981). AM can also reduce the toxicity of certain metals for plants, while at non-toxic or such optimal level, their acquisition is enhanced by symbioses (Bethlenfalvay 1992; Khan et al. 2000; Kochian et al. 2004). AM also plays a positive role in protecting plants from pH extremes (Sylvia and Williams 1992).

2 AM in Relation to Plant Nutrition

Arbuscular mycorrhizal associations are mutually beneficial relationships in which both the partners achieve advantage. The fungal partners which are the obligate symbionts rely on carbon provided by their plant hosts to complete their life cycle. In return, the fungus provides essential nutrients like phosphorus and nitrogen to the plant host. This nutrient exchange takes place in between root cortical cells and arbuscules. Arbuscules never penetrate the host plasmalemma and cytoplasm rather are enveloped by the newly synthesized host membrane. They remain in this interfacial space where the exchange process occurs (Balestrini and Bonfante 2005).

Mycorrhizal development initiates with the germination of AM fungal spores present in the soil. Hyphae that enter the vicinity of the suitable host start to ramify more vigorously (Akiyama et al. 2005). When it comes in contact with the host root surface, a hyphopodium is formed and the host epidermis is penetrated by the fungus (Genre et al. 2005). After getting into the host root cortex, the hyphae start forming highly branched tree-like arbuscules (Smith and Read 1997). Arbuscules are short-lived strictures and begin to degenerate after 4–10 days of activity (Strack et al. 2003). After the complete senescenece of arbuscules, the host cell may be ready for recolonization. AM colonization is a continuous process and a mature plant root may have cells at various stages of colonization. The extent to which the plant root is colonized depends upon various host, fungal and environmental factors. Even the genetic makeup of the host plays an important role in its responsiveness towards mycotrophy (Maeda et al. 2006; Javot et al. 2007a).

The whole process of nutrient exchange takes place in the interfacial apoplast (Balestrini and Bonfante 2005). During arbuscule development the peri-arbuscular membrane continues to produce cell wall materials, including pectins, xyloglucans, polygalactrunans and hydrxyproline rich glycoproteins. The components are secreted into the interfacial apoplast without being assembled into a wall (Balestrini and Bonfante 2005). Van Buurnan et al. (1999) and Balestrini et al. (1997) identified transcripts encoding proline rich proteins in cells of certain plants colonized with arbuscules. The permeability and nutrient transfer properties of the arbscular membranes are further enhanced by the presence of aquaporin proteins (Uehlein et al. 2007). These proteins are also known to regulate water relations in plants (Javot and Maurel 2002). The expression of aquaporin genes has been reported to be variable in drought and salt stressed conditions (Porcel et al. 2006; Ouzaid et al. 2005).

Phosphate is an essential nutrient and is limiting for plant growth in many environments (Bucher 2007). Phosphate is present in the soil in the form of inorganic orthophosphate and is readily sequestered by cations like iron, aluminum and calcium

resulting into reduced mobility of this phosphate form. This ultimately results into the establishment of a localized depletion zone (Bucher 2007). To combat deficiency in the soil tons of phsopahte fertilizers are added globally as soil supplement. This results into to an increasing cost of extraction of this component and eutrophication of surface and subsurface water bodies.

The mycorrhizal associated pathway of P-uptake is operated through specific phosphate transporter genes encoding members of the phosphate transporter 1 (Pht 1) family (Bucher 2007; Javot et al. 2007b). Pht 1 proteins are proton:phosphate symporters belonging to the major facilitator superfamily and play a key role in the transport of phosphate across the peri-arbuscular membrane (Karandashove and Bucher 2005; Javot et al. 2007b)

Arbuscular mycorrhizal fungi are heterotrophic organisms and obligate symbionts totally relying on host for the supply of carbon rich compounds. Studies have shown that the form of carbon transferred from host plant to fungus is hexose sugar (Ravnskov et al. 2003.). However, it still needs to be verified that whether arbuscules are the point of hexose delivery or it takes place from elsewhere. The most prevalent form of sugar translocated in the plants is sucrose. It needs to be broken down into hexoses under the action of invertase and sucrose synthase. AM fungi have not been reported to release such enzymes rather mycorrhiza-stimulated production of synthase and invertase genes have been identified in various plant hosts including maize (Ravnskov et al. 2003) and tomato (Schaarschmidt et al. 2006). Once in the intraradical hyphae, much of the carbon is converted into storage lipids predominantly trigylycerides. Lipids not only act to store carbon but are also the main form of carbon translocated from intra- to extra-radical hyphae, where they provide the major respiratory substrate (Pfeffer et al. 1999).

AM fungi extend a number of benefits to their host plants. In addition to enhanced mineral nutrition, they can increase tolerance to water and salt stress, induce greater resistance to pathogens, and reduce sensitivity to toxic substances or pollutants present in the soil (Smith and Read 1997). The host plant, however, has to pay a fairly high price for all these services, with up to 20% of the host fixed carbon being delivered to the microbial symbionts.

3 AM as a Biocontrol Agent

Arbuscular Mycorrhizal associations between roots of higher plants and fungi have been reported to exist for long ago. Since most of the land plants are able to form mycorrhizae, it is logical to attempt to relate the mycorrhizae to the incidence and severity of plant diseases. Such efforts were also needed to find alternative means of controlling diseases without the use of expensive and potentially harmful chemicals in order to achieve the goal of sustainable agriculture (Bethlenfalvay and Linderman 1992). Rhizosphere microbial changes occur when mycorrhizae are formed. When these associations are formed, the fungi live both within root tissue and external to those tissues. They could have direct interactions with other soil organisms or they could

influence those organisms indirectly by changing host plant physiology, especially root physiology and, in turn, pattern of exudation into the '*mycorrhizosphere'*. Similarly, the behavior of AM fungi is also indirectly affected by other soil microbes capable of influencing the host physiology.

A number of investigations have been carried out to draw conclusions regarding the role of mycorrhizae in plant-pathogen interactions (Azcón-Aguilar and Barea 1996; Bagyaraj 1984; Caron 1989; Dehne 1982; Hooker et al. 1994; Ingham 1988; Jalali and Jalali 1991; Linderman 1988; Linderman and Paulitz 1990; Schenck 1983, 1989; Schoenbeck 1979; Zak 1964; Akhtar and Siddiqui 2008). Various authors seem to suggest different mechanisms regarding the role of mycorrhizae in plants disease control. The common agreement is on the enhanced nutrition, competition for host photosynthates and infection sites, morphological changes in roots and root tissues, changes in chemical constituents of plant tissues, reduction of abiotic stresses and microbial changes in the mycorhizosphere (Linderman 2000).

It has been reported that where arbuscular mycorrhizae reduce disease, the same effects could be reproduced by correcting the nutrient deficiency particularly for phosphorus. However, the enhanced plant vigor provides better substrate for obligate pathogens and pathogens causing foliar diseases to infect and multiply (Meyer and Dehne 1986). While some other investigations report that P-induced changes in root exudation could affect spore germination by the pathogens (Graham and Menge 1982). On the other hand, in some of the reports it has been shown that P-nutrition is not involved in disease reduction (Caron et al. 1985, 1986a, b, c.).

It is now well established that AM formation enhances the rate of photosynthesis in the host plant, thus, compensating the C withdrawn by the fungus for its use (Dauds et al. 2000). Competition may exist between the AM fungus and the pathogen for the same nutrients or the infection site. It has been indicated by Dehne (1982) that the pathogen infects plant root tissue adjacent to those colonized by the AM fungus. This further testifies that there is no competition for nutrients or space. For nematode pathogens it has been documented that host nutrients are required for reproduction and development and a direct competition between nematodes and AM fungi may result in reduced reproduction of nematodes (Hussey and Roncadori 1982).

There are very few reports available in the literature suggesting that localized morphological changes occur in mycorrhizal roots (Becker 1976; Dehne and Schoenbeck 1979; Nasim et al. 2012). These authors have suggested that the changes may include increased lignifications of the root cells of the endodermis or may cause hypertrophy and swelling in plant root tissue. Other workers (Wick and Moore 1984) showed an increased wound-barrier formation that inhibited the black root rot pathogen of holly. Such studies indicate that AM colonization in some plants may create physical barrier for the entry of the pathogen (Linderman 2000).

Changes in specific chemical components in plants that could deter pathogens have been reported, such as increased levels of arginine in tissues suppressing sporulation of *Thielaviopsis* (Baltruschat and Schoenbeck 1975), increase in chitinase in mycorrhizal plants that could be detrimental to pathogens with chitinous walls (Dumas-Gaudot et al. 1992), or accumulation of phenolics in mycorrhizal plants

(Grandmaison et al. 1993). There is also considerable research concerning the mycorrhiza-induced defense mechanisms to explain reduced responses of AM plants to infection by specific pathogens (Cordier et al. 1999; Vierheilig et al. 1994; Dumas-Gaudot et al. 2000). These studies have examined AM and non-AM plants for changes in defense reactions and have shown that arbuscular mycorrhizae may induce slight, often transient, but potentially significant activation of metabolic and morphological changes related to disease resistance mechanisms. These' changes are thought to be elicited, and are often reversed unless challenged by a pathogen. However, the magnitude of the plant reaction is generally low compared to pathogen-induced reactions, and there is still no proof or demonstration that the mycorrhiza-induced reactions are directly involved in reducing plant disease (Dassi et al. 1998; Smith and Read 1997). Even the reported increase in phytoalexin-like isoflavanoid compounds, such as reported by Harrison and Dixon (1994), and Sundaresan et al. (1993) have not been implicated directly in the suppression of a specific disease. Benhamou et al. .(1994) showed the activation of plant defense mechanisms, but their study was conducted on transformed carrot roots in an *in vitro* root organ culture system, far removed from a real agrosystem or natural ecosystem where plants are growing in soil and mycorrhizae are associated with a myriad of other soil microbes.

There are plant diseases that definitely are influenced by environmental stresses such as drought or nutrient deficiency or excess. The literature is extensive regarding the improved nutrition and tolerance to soil drought by arbuscular mycorrhizae (George 2000; Auge 2000). These effects could be very real in natural and agro-ecosystems, and could play a major part in preventing, suppressing, or reducing the severity of diseases. If nutrient stress weakens the plant, making it more susceptible to pathogen ingress or spread, then indirectly arbuscular mycorrhizae have influenced the disease by compensating for the nutrient deficiency. Form of nitrogen has been shown to influence root disease incidence by providing a kind that plants can use but pathogens cannot. Different forms of nitrogen have been shown to alter the bacterial populations that can suppress the pathogen. However, mycorrhizae were not evaluated in such studies, so their role in inducing the microbial shift cannot be assessed.

There is now substantial indication in the literature that mycorrhizae induce changes in the microbial populations in the mycorrhizosphere, some of which could directly or indirectly influence the incidence and/or severity of plant diseases. Recent research, however, has focused on other mechanisms of interaction that largely relate to the host response to AM fungus colonization that could limit development of the pathogen and disease. Many researchers believe that host defense responses are not of sufficient magnitude or timely enough to have a significant impact on disease (Filion et al. 1999). This underscores the need to search for more universal and acceptable explanations for why plants with mycorrhizae usually tolerate or resist diseases better than plants without mycorrhizae. Furthermore, since plants in undisturbed ecosystems seem to tolerate diseases, or seem fully resistant or immune to them, one might surmise that they benefit from the full complement of mechanisms that function in tandem to suppress pathogens, even when they are

introduced. An underlying feature of these mycorrhizal plants that could contribute to their success in resisting disease, as well as to many other mycorrhiza-induced *traits,* is that they have a balanced and fully-stocked mycorrhizosphere microbial composition.

The literature suggests that arbuscular mycorrhizae alter the composition of functional groups of microbes in the mycorrhizosphere, including the numbers and/or activity of pathogen antagonists (Secilia and Bagyaraj 1987). Yet a number of other papers provide results, showing of potential increased antagonistic activity against the pathogen, even though that potential was not acknowledged. Results of Caron et al. (1986a), and St-Arnaud et al. (1994) suggest that microbes in the mycorrhizosphere might have influenced the disease or pathogen level in studies on *Phytophthora* root rot, *Fusarium* tomato root rot, and marigold *Pythium* root rot, respectively. The natural tendency is to consider other mechanisms to explain the results. Usually, this is because one expects control treatments consisting of a microbial filtrate from the mycorrhizal fungus inoculum (excluding AM fungal propagules), which would compensate for any effects associated with microbes contributing to the measured response. While those microbes might have been added to all the experimental units equally, the further population enhancement of certain components only occurs when mycorrhizae are present due to changes in root exudation and by hyphal exudation. Thus, the level of antagonism might not be the same for the treatments with or without mycorrhizae.

A number of studies have been carried out in Pakistan to monitor the effect of pre-inoculation of arbuscular mycorrhizae on the incidence of plant diseases (Table 23.1). In these investigations it has been observed that presence of AM in plant roots confers resistance against root born plant pathogens. The host and AM-pathogen combinations had been variable. Some of the studies were survey works that established that the mycorrhizal plants were more resilient to the incidence of diseases.

4 AM Ameliorating the Adverse Effects of Nutritional Stresses and Metal Toxicity

The modern agriculture demands a reduction of chemical biocides to control plant diseases and insect pests. It actually lies within the premise of sustainable agriculture that the production inputs should be reduced without significant losses to productivity. Although the mainstay of Pakistan's economy is agriculture, the crop production in the country is confronted with various constraints, among these, low soil fertility status being the most common. On an average, the agricultural soils of province Punjab contain less than 1% organic matter (Farooq-e-Azam 1988). It necessitates the application of chemical fertilizers. Although chemical fertilizers are effective in increasing the yield, these may cause some problems such as soil structure deterioration, ground water pollution and high nitrates in vegetables.

Table 23.1 An overview of studies conducted in Pakistan

Host/AM fungi	Pathogenic fungus	Effect	References
Brassica napus	*Rhizoctonia solani*	VA mycorrhiza as a deterrent to pathogenic infection	Iqbal et al. (1977)
Brassica napus	*Rhizoctonia solani*	VA mycorrhiza as a deterrent to pathogenic infection	Iqbal and Mehmood (1982)
Brassica napus	*Rhizoctonia solani*	VA mycorrhiza as a deterrent to pathogenic infection	Iqbal and Mehmood (1982)
Brassica napus	*Rhizoctonia solani*	VA mycorrhiza as a deterrent to pathogenic infection	Iqbal and Mehmood (1986)
Brassica oleraceae/Glomus mosseae	*Rhizoctonia solani* + *Fusarium moliniforme*	VA mycorrhiza as a deterrent to mixed pathogenic infection	Iqbal et al. (1987)
Brassica oleraceae/Glomus mosseae	*Fusarium moliniforme*	VA mycorrhiza as a deterrent to pathogenic infection	Iqbal et al. (1987-I)
Brassica oleraceae/Glomus mosseae	*Rhizoctonia solani*	VA mycorrhiza as a deterrent to pathogenic infection	Iqbal et al. (1987-II)
Brassica oleraceae/Glomus mosseae	*Rhizoctonia solani*	VA mycorrhiza as a deterrent to pathogenic infection	Iqbal and Nasim (1988-IV)
Chlorophytum commosum	*Thermomyces stellatus*	Behaviour of vesicular arbuscular mycorrhizal fungi against a pathogen in the scales of *Chlorophytum commosum* Baker	Nasim (1994)
Solanum tuberosum/glomus mossease + *Glomus fasciculatum*	*Rhizoctonia solani*	VA mycorrhiza as a deterrent to mixed pathogenic infection	Nasim et al. (1996a, b)
Lycopersicon esculentum, Brassica oleracea/Glomus mossease + *Glomus fasciculatum*	*Rhizoctonia solani* + *Fusarium moniliforme* + *F. oxysporum* + *Drechselera sorokiniana*	VA mycorrhiza as a deterrent to mixed pathogenic infection	Nasim (2005)
Capsicum annum/Mixed AM inoculum	*Macrophomina phaseolina*	Occurrence of vesicular arbuscular mycorrhizal association in chillies infected with root borne pathogens.	Iqbal et al. (1990)
Cicer areitinum/mixed inoculum	*Ascochyta rabiei*	Effect of foliar application of Ascochyta rabiei on growth and vesicular arbuscular mycorrhizal status of eight chick pae varieties	Nasim et al. (2003)
Lycopersicon esculentum/ mixed inoculum	*Fusarium* spp	Effect of variable soil texture and VAM preinoculation on the occurrence of damping off caused by *Fusarium* spp. on tomato.	Sheikh (1991)
Lycopersicon esculentum/ mixed inoculum	*Fusarium* spp	Effect of preinoculation of tomato seedlings with VAM, *Trichoderma viride* and *Aspergillus flavus* on the pathogenicity of *Fusarium* spp.	Naz (1991)

Similarly, severe insect/pest attack has led to the over-use of pesticides. The residues from these pesticides persist both in food and the environment, posing menace to human and animal health on one side, while they can interrupt natural processes and ecosystems on the other. There is a strong conviction among researchers to probe into alternatives of chemical based agriculture.

It is now generally recognized that AM enhances the uptake of nitrogen and of relatively less mobile nutrients such as phosphorus, sulfur, copper, zinc and boron (Giasson et al. 2008). AM fungi increase the plant contact area with the soil for up to 47-folds (Smith and Read 1997). Nutrients are taken up via the fungal hyphae by specific uptake systems and can be mobilized and transported to the plant via extra-metrical and intramatrical hyphae and arbuscules.

Heavy metals occur naturally on the soil. This term is often used for metals and metalloids that have been associated with contamination and potential toxicity (Duffus 2002). The term "heavy metal" or "trace metals" refers to the metal ions like arsenic, cadmium, chromium, copper, manganese, mercury, molybdenum, nickel, lead, selenium and zinc. In the agroecosystems, the main sources of metal contamination are increased use of chemical fertilizers and biocides, from the application of metal containing wastes such as sewage sludge, animal manure, coal and wood ashes to soil, and from atmospheric deposition (Mhatre and Pankhurst 1997; Kabata-Pendias 2001; Kabata-Pendias and Mukherjee 2007). Although some of these metals are essential nutrients for plants and are needed for growth and development of plants, but some are toxic pollutants like Cd, Pb and As. The presence of high concentration of these heavy metals in the agricultural soils is a threat to the quality of soils and surface waters (Kabata-Pendias and Mukherjee 2007). There are certain factors which greatly influence the uptake of metal ions by plants or microbes (Kloke et al. 1984). These include: (1) nature of soil types; (2) nature of the metal ion and its interaction with soil colloids and other soil components; (3) concentration and chemical form of the metal entering the soil; (4) minerological composition; (5) sorptive properties of soils and their binding; (6) physical, chemical and biological soil properties; (7) biological activity of the rhizosphere; (8) duration of contact with the surface binding of these metals; (9) chemical composition of the soil solution; (10) plant type and plant exudates.

Metal tolerance of arbusculer mycorrhizal fungi has been determined using several methods including AM spore number, root colonization and extent of root colonization (Weissenhorn et al. 1993). Unfortunately, such methods did not provide sufficient information on conditions, limitations, and threshold values ensuring the survival and growth of AM fungi, or about the genetic basis for multi-metal resistance and tolerance. However, AM fungi co-exist with other microbial communities and plant roots that can tolerate and accumulate metals, and this could confound the real interactions between AM fungi and metals in the medium (Giasson et al. 2008). Several reports have shown that AM fungi from metal contaminated soils have developed tolerance against metal toxicity and are well adapted (Weissenhorn et al. 1993, 1994; Toler et al. 2005; Sudova et al. 2007). At present, potential interaction mechanism between AM fungi and metals, and the cellular and molecular mechanism of heavy metal tolerance in AM fungi is poorly understood (Leyval and Joner 2001;

Martin et al. 2007). Metal transporters and plant encoded transporters are involved in the tolerance and uptake of heavy metals (Gohre and Paszkowski 2006; Hildebrandt et al. 2007) from the extracellular media, or in their mobilization from intracellular stores (Gaither and Eide 2001). In general, mobilization of metals in the soil by soil microbes can be achieved by protonation, chelation and chemical transformation (Gadd 2005). The exudates such as citric acid and other organic compounds, released from both plants and microbes are very effective in solubilizing and releasing metals from soil components. AM fungi have been reported to sequester and accumulate metals in their biomass as well as in the roots of host plants (Burke et al. 2000; Gadd 2005). Arbuscular mycorrhizal fungi have great potential in the remediation of disturbed land and low fertility soils through heavy metal mobilization, uptake, metal hyperaccumulation, phytoextraction, metal stabilization and phytovolatilization.

5 Soil Aggregation and Role of AM

Soil is the natural covering on most of the earth's land surface. It is a natural medium for the growth of plants. Soils support plants by holding down their root masses, although strong winds or saturated soils cause withdrawal of some trees. Soils hold enough water to supply plant requirements for several days, sometimes even weeks between rains or irrigation (Kapulnik and Douds 2000).

Soils on the basis of their particle size and composition may be classified as sand, gravel, loam, silt and clay loam (Miller and Donahue 1995). According to this classification, loams are soils with both fine (silt and clay) and coarse size classes well represented. The effects of soil texture on its moisture relations are rather complicated. However, fine textured soils have a great advantage in holding much water. The excess of moisture is held in the upper layers, which are highly vulnerable to drying. The water is not admitted in readily and is mostly lost as run off. Root penetration is also discouraged in such soils so that seedlings may not be able to approach the deeper moisture before surface soil dries. Moreover, these soils tend to be poorly aerated especially in deeper layers. Such conditions of anoxia lead to shallow rooting which results in drought susceptible plants (Doubenmire 1979).

In moderately coarse textured or in heavy well aggregated soil, there exists large interstitial spaces which facilitate the diffusion of gases. As a result, CO_2 produced by respiring organisms and roots, is able to escape rather more easily and oxygen used up in the process also moves into the soil with corresponding ease. In heavy soils, especially those that are not well aggregated or poorly aggregated, the deficiency of O_2 and toxicity of excess of CO_2 become limiting factors for many plants. Moreover, in these coarse soil water readily percolates down into deeper layers and becomes unavailable in upper soils layers. Nelson (1987) in a careful review concluded that most effects of arbuscular mycorrhizae on water relations of their host are probably due to improved phosphorus nutrition.

Higher moisture levels and congested plant growth are the conducive conditions for the development of damping off (Strange 2003). The moisture level of a soil is

very much dependent upon its water holding capacity. This water holding capacity depends strongly upon a variety of soil characteristics such as its texture, size of constituent mineral particles, the manner in which these particles are arranged and the amount of organic matter (Linderman 2001).

Soil fertility largely depends upon soil humus, which is the end product of degradative and metabolic activities in soil. Soil humus, the complex array of substances left after extensive chemical and biological breakdown of fresh plant and animal residues make up 60–70% of the total organic carbon in soils and are quite resistant to further microbial decomposition. Because of its complexity, humus is often divided by solubility separations into *humic acid* (HA), *fulvic acid* (FA) and *humin.* The three compounds resemble structurally but differ in molecular weight and functional group content (Schnitzer and Khan 1972). Both fulvic and humic acids are soluble in dilute sodium hydroxide solution, but humic acid is larger and will precipitate out (be insoluble) when the solution is made acidic. Humin is the portion of humus that is insoluble in dilute sodium hydroxide (Miller and Donahue 1995). These compounds have beneficial effects both on soil structure and plant productivity. They enhance the uptake of ions by chelate formation and hence an increase in growth is caused by establishing positive effect on root enzymes and soil O'Donell (1973) regarded soil humus as a plant food. It enhances the water holding capacity and nutrient level of the soil. The high-molecular-weight humic substances, humic acids and humates, alter the physical characteristics of the soil, while the low-molecular-weight humic substances, fulvic acids and fulvates, are involved in chemical reactions in the soil that influence plant's metabolic processes. Fulvic acids can form through the enzymatic and/or chemical oxidation of humic acids. Both fulvic acids and humic acids found in soil result from the chemical and biological degradation of dead organisms. The formation of these substances may come about by the oxidative changes of organic fragments, microbial synthesis, or chemical condensation after biological breakdown or self-digestion of humic biomass (Miller and Donahue 1995).

Soil aggregation is of prime importance for the growth of plants and determines the quality and fertility of field soils. This soil characteristic is based on the spatial and temporal actions of various organomineral binding agents which may be classified into three classes (Miller and Jastrow 2000). The first category of these binding agents composed of microbial and plant-derived polysaccharides and mucigels undergo decomposition quite rapidly and are referred to as transient binding agents. The second category referred to as temporary agents is composed mainly of living or dead fibrous roots and fungal hyphae and can normally persist through a growing season or even longer in perennial systems. The third category considered to be persistent binding agents is composed of decayed or more rendered materials having humic acid moieties in association with clays, amorphous mineral complexes, or both. Thus, among these categories, AM plays an important role as temporary stabilizing agents of macroaggregates. It can be conceptualized by viewing three dimensional network of fibrous roots and AM fungal hyphae as a "sticky string bag" that physically entangles or enmeshes smaller aggregates and particles creating rather stable macroaggregates (Miller and Jastrow 2000). In fect,

recent studies have shown that a hydrophobic glycoprotein occurs on the surface of AM fungi. This glycoprotein is named as glomalin and is actually exuded from both intra- and extra-radical hyphae and deposited on soil surface. It has also been established that it is produced by majority of AM strains, though they may differ considerably in the amount they produce (Miller and Jastrow 2000). Hyphae of AM fungi also help create the conditions that are conducive to the formation of microaggregates. This is achieved through the exudation of organic substances from the hyphae which favour the establishment of microbial communities. In the presence of clays and silts, these substrates along with hyphal fragments can act as nucleating points for the formation of microaggregates.

6 AM and Micropropagated and Transgenic Plants

The group of fungi forming arbuscular mycorrhizae is now being placed in Glomeromycota. This is a small group comprising seven genera and 160 species. The genus *Glomus* comprising 110 species is the most important genus of order Glomerales (Schüβler et al. 2001.). However, the fungi are ubiquitous in nature and are world-wide in distribution forming the mutualistic symbiotic association with over 95% of the plant species (Krishna 2005; Johnson et al. 2006). During the last 20 years, these fungi are reported to be of much wider occurrence than were thought 3–4 decades ago. The literature is ample confirming their presence in portions other than roots (Nasim and Iqbal 1991a, b; Iqbal and Nasim 1991). Colonization of roots by mycorrhizal fungi has been shown to improve growth and productivity of several field crops (Javaid et al. 1993; Cavagnaro et al. 2006) by increasing nutrient element uptake (Al-Karaki 2002). Mycorrhizal symbiosis is frequently associated with increased photosynthetic rates of mycorrhizal plants (Martins et al. 1997, 1999; Wu and Xia 2006). Mycorrhizae also impart other benefits to plants including production of certain secondary metabolites (Schliemann et al. 2008), enhancement of nitrogen fixation by symbiotic or associative N_2-fixing bacteria (Javaid et al. 1994), osmotic adjustment under drought stress (Augé et al. 2008; Ruiz-Lozano 2003), increased resistance to pests (Khaosaad et al. 2007), tolerance to various abiotic stress factors (Takeda et al. 2007), and improving soil aggregation (Wu et al. 2008). Thus they improve soil physical properties and stability. Studies have shown that AM fungi alleviate allelopathic stress and improve crop growth and yield (Bajwa et al. 1999, 2003; Javaid 2007).

6.1 AM and Micropropagated Plants

The significance of AM fungi for a tissue culture raised plantlets is of special concern. The plantlet mass multiplied through the aseptic technique has to face vicissitudes of the environment when are transplanted in the unsterilized field soil. Root born plant

diseases are one of them. Over 50% plantlet mortality has been reported at this stage. This hampers the utility of this technology for human well-being. The plant tissue culture experts have been using chemicals for acclimatization of these fragile plantlets (Short 1990, 1991). In view of the escalating cost of chemicals like antitranspirents and synthetic waxes and their unfriendliness to the environment, AM fungal preinoculation may serve as a vital tool to reduce the mortality percentage. However, to fulfill the objective, selection of the right organism or consortia of microbes is needed to be done carefully as it has been evidenced by Bougher et al. (1990) that the response of plants to mycorrhization does not only depend upon nutrient availability, but also on the fungus species or even strains of the same species.

The abilities of mycorrhizal species and strains to promote plant growth has opened new perspectives for the use of these fungal inoculums in nurseries and forestries. Inoculations of forestry species were performed with different fungal-host combinitions and inoculums types under different conditions. The results of these studies have shown that the plants grow better, have more extended root systems and both roots and shoots have increased dry weights, although the ratios between the dry weight of roots and shoots were lower for mycorrhizal plants (Smith and Read 1997). The total number of short roots of mycorrhizal plants has been higher than for non-mycorrhizal plants. A study conducted by Nasim et al. (1992) and Nasim and Bajwa (2008) the wheat plants responded differently to various types of fungal inocula. The inoculum types included spore concentrates of AM fungi, AM root fragments, scale-like leaves of underground plant portions, earthworm fecal pellets, and cycas rhizosphere soil. Root colonization by mycorrhizal plants can result in lower plant growth rates if fungal compatibility, nutrient availability, light intensity or temperature is not suitable for plant development (Table 23.2; Plate 23.1; and Fig. 23.1) (Conjeaud et al. 1996).

In another study, the fungi forming AM with four tissue culture raised potato varieties have been listed and their dynamics has been studied in the field/tunnel system (Nasim 2010). The spore number in the rhizosphere of four potato varieties cultivated in Pakistan has been recorded so that the suitable associative fungi may be selected carefully. The major objective of the present study was to indicate and characterize AM fungus or a group of fungi potentially beneficial for the crop of tissue culture raised plantlets in potato growing area (Nasim 2010). Micropropagated plants are adversely affected by water stress, either due to low absorption capacity of roots or due to stomata, which are deficient in regulation of water loss (Bonga 1977; Flick et al. 1983). Acclimatization of micropropagated plants corresponds to transition period when roots become adapted to a substrate with less available nutrients, and to an autotrophic condition. At this stage, the presence of mycorrhizae could increase the availability of limiting nutrients such as phosphorus and nitrogen facilitating the absorption. Water stress can be responsible for the high mortality of many micropropagated plants (Martins 2008).

Tissue culture raised plants develop under high moisture and low light intensity, often with low lignifications levels and decreased functionality of the root system have low survival rates during hardening. Mycorrhizal inoculation of such tissue

Table 23.2 List of Glomaromycota spores isolated from soils of potato growing fields, Sahiwal district, Pakistan

Species name	Percentage of spores (100 g^{-1} soil)
Acaulospora bireticulata Rothwell & Trappe	+++
Acaulospora sp. (Unidentified)	++
Gigaspora sp. (Unidentified)	++
Glomus albidum Walker & Rhodes	+
G. claroideum Schenck & Smith	+
G. dimorphicum Boyetchko & Tewari	+++
G. deserticola Trappe, Bloss & Menge	+
G. etunicatum Becker & Gerd.	++
G. fasciculatum (Thaxter Sensu Gerd.) Gerd. & Trappe	++++[a]
G. halonatum Rose & Trappe	++
G. heterosporum Smith, Schenck & Tewari	++
G. macrocarpum Tul & Tul	+++
G. microaggregatum Koske, Gemma & Olexia	++++
G. microcarpum Tul & Tul	+++
G. monosporum Gerd & Trappe	++
G. mosseae (Nicol. & Gerd.) Gerd. & Trappe	++++[a]
G. multicaule Gerd & Bakshi	+
G. pallidum Hall	++
Glomus sp. I (Unidentified)	+
Glomus sp. II (Unidentified)	+
Glomus sp. III (Unidentified)	++
Glomus sp. IV (Unidentified)	+
Sclerocystis sp. (Unidentified)	+++
Scutellospora sp. I (Unidentified)	++
Scutellospora sp. II (Unidentified)	+
Spore Type I (Unidentified)	+
Spore Type II (Unidentified)	++
Spore Type III (Unidentified)	++
Spore Type IV (Unidentified)	+

Key: + = 0–25%, ++ = 50–75% and +++ = 75–100%
[a]Highly abundant

culture raised plantlets before acclimatization increases survival thereby enhancing the functionality of the root system and the plant mineral nutrition (Martins 2004, 2008; Martins et al. 1996).

6.2 AM and Transgenic Plants

Most plant species except those belonging to certain plant families, are involved in a symbiotic association with mycorrhizal fungi (Trappe 1987). Most crop species form arbuscular type of mycorrhizae which are important in uptake of diffusion

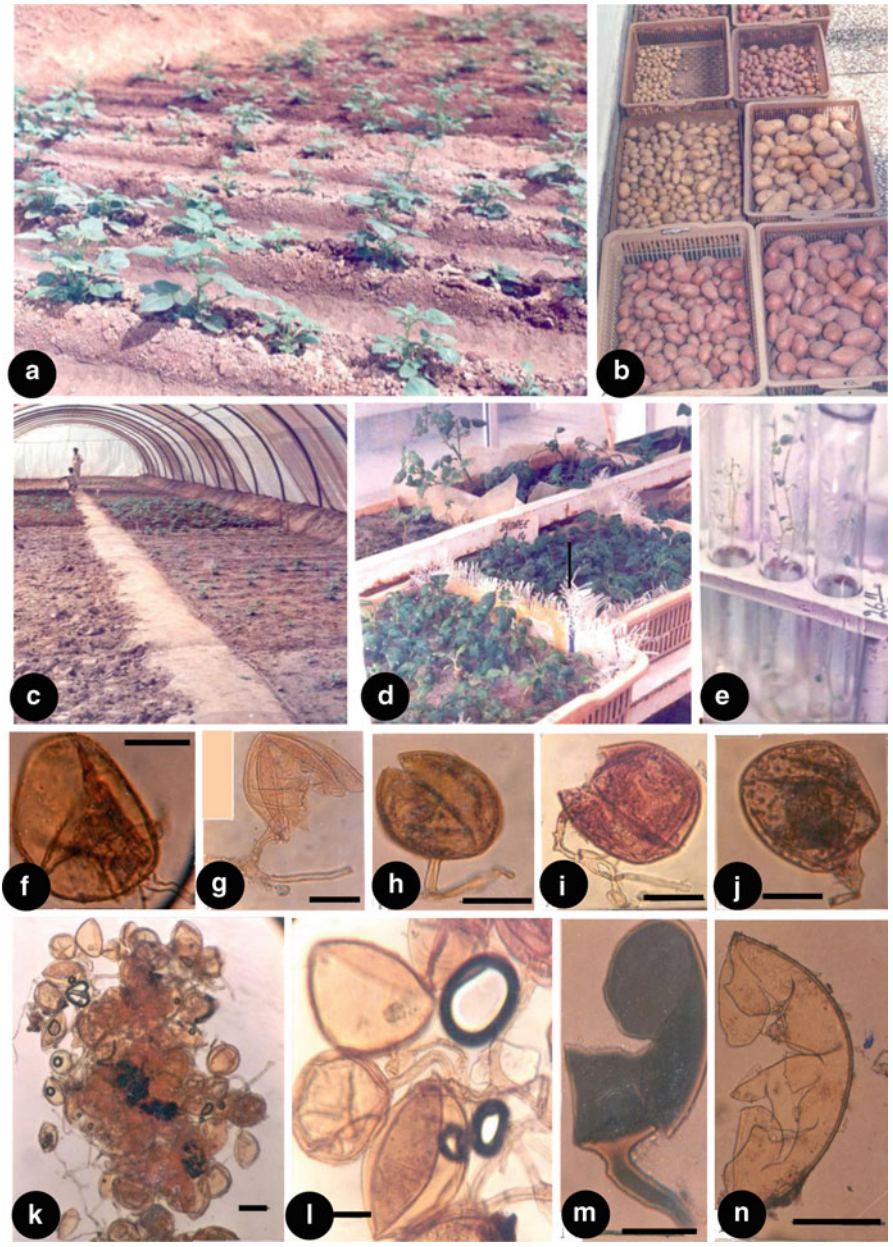

Plate 23.1 (**a**–**e**) Tissue culture raised potato plantlets in growth tunnels, test trays and tubes in Sahiwal area of Pakistan. (**f**) *Glomus albidum*. (**g** and **h**) *Glomus claroideum*; (**i**) *Glomus microcarpum*; (**j**) *Glomus multicaule*; (**k** and **l**) *Glomus mosseae*; (**m**) *Glomus monosporum*; (**n**) *Glomus* sp. (bar = 30 μm)

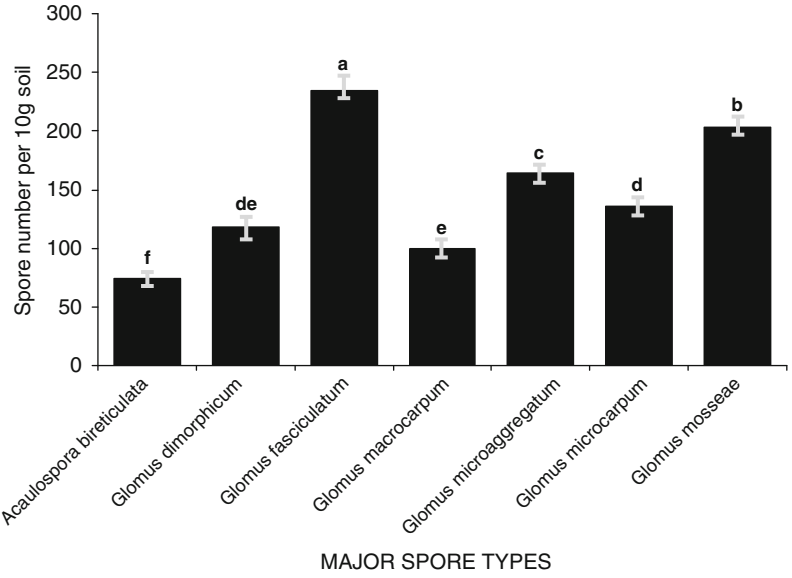

Fig. 23.1 Spore counts for seven major AM species in the rhizosphere of tissue cultured potato plants. Means sharing same lowercase letters are non-significant at 5% level

limited soil nutrients (Smith and Read 1997). They increase the host resistance to pathogens (Linderman 2000), contribute to soil aggregate stability (Miller and Jastrow 2000), and influence plant water relations (Auge 2000). Arbuscular Mycorrhizae (AM) are host non-specific that means one fungal species can form association with more than one plant species and one plant species may be host to many AM fungus species. Never-the-less variation exists in the extent of colonization and host growth benefits among plant species and even among cultivars within a species, and preferential associations between mycorrhizal fungi and particular hosts are frequently observed. Colonization of roots by AM fungi and the formation of structures that characterize the AM symbiotic association, i.e., appresoria, extraradical and intraradical hyphae, arbuscules and vesicles, involve a complex series of events. Scientists have raised plant mutants that show a block at some stage of the colonization process and are useful in determining the factors involved in the interaction between hyphae and root cells at each step in that process.

6.2.1 Differences Among Plant Species and Mycorrhizal Species

Preferential association of mycorrhizal fungus species and plant species are indicated by differences in spore densities in different cropping systems. Johnson et al. (1992), found that spore density of *Glomus aggregatum* and *Glomus occultum* was higher in fields with maize history and *Glomus microcarpum* higher in field with soybean history. These differences were found in fields which had been planted to the same

crop for 5 consecutive years. Dodd et al. (1990) also found that spore densities are dependent upon the dry or wet weather, and also upon the cropping rotation of sorghum, cassava, cowpea and kudzu. *Glomus occultum* spore density increased marledly with the sorghum crop. *Acaulospora mellea, A. marrowae* and *Entrophospora colombiana* spore density was greatest with the cow pea crop. The fact that *G. occultum* spore density increased under the maize (Johnson et al. 1992) and sorghum crops (Dodd et al. 1990) indicates that mycorrhizal species preferences for a specific host may extend across related plant species. Bethlenfalvay et al. (1982) found differential interaction of mycorrhizal species when used to infect soybean and sorghum and further showed that soil type was important in determining the extent of the response. The results of these studies indicate that plant and mycorrhizal fungus species interactions are important in the ecology of agricultural system. A study conducted by Miller et al. (1985) on assessing the comparative response of seven *Glomus* species on apple showed a twofold difference. Interestingly, the AM fungus treatment resulted in the greatest response and the least response was with two different isolates of *Glomus occultum*. Hetrick et al. (1992), found substantial differences among AM fungus species for their ability to colonize and promote the growth of wheat and wheat relatives. Different isolates of *Glomus etunicatum* provided different growth benefits. These studies further support the observation that variation in plant response to an isolate or a mycorrhizal fungal species can be as great as variation in response to various species of the fungi. Little is understood about the genetic differences between effective vs. ineffective isolates, because it is not possible to manipulate them genetically (Park and Kaeppler 2000).

It is likely that isolates and species differ with regard to optimal temperature (Baon et al. 1993) and soil conditions for mycorrhiza formation. Although AM fungi are remarkable for their ability to colonize a wide host range, the combined results of both retrospective spore density analysis and controlled experimental studies clearly show that some level of host preference or functional capability (Gianinazzi-Pearson 1984; Giovannetti and Mosse 1980) may occur between mycorrhizal genotypes and certain plant species.

6.2.2 Differences Among Cultivars and Isolates

There are numerous examples of cultivars within plant species that differ in the extent of colonization by mycorrhizal fungi and their responsiveness to them. These include *Arachis hypogaea* (Kesava-Rao et al. 1990.), *Hordeum vulgare* (Baon et al. 1993), *Medicago sativa* (Lackie et al. 1988), *Oryza sativa* (Dhillion 1992), *Pennisetum americanum* (Krishna et al. 1985), *Triticum aestivum* (Hetrick et al. 1996), *Vigna unguiculata* (Mercy et al. 1990), and *Zea mays* (Toth et al. 1990).

Mycrorrhizal fungi are especially important in absorption and P uptake of plants under P-limiting conditions, but colonization is generally suppressed by P sufficiency of the host (Smith and Read 1997). Baon et al. (1993) compared eight cultivars of barley that differed in P efficiency for their responsiveness to inoculation with

Glomus etunicatum. The results showed that the cultivars least able to grow at low P were most responsive to added P or mycorrhizae and the cultivars which attained the greatest growth at low P were the least responsive to P amendment or mycorrhizae. Their results provided evidence that cultivars best able to grow at low P are not likely to be the most efficient under high P conditions. Mycorrhizal responsiveness of ten wheat cultivars was compared at three P regimes (Hetrick et al. 1996). At the lowest P level, six of the cultivars responded positively to colonization with AM fungi, and four cultivars had no response or responded negatively to inoculation. Mycorrhizal responsiveness declined with increasing P for the six responsive cultivars, but was unaffected for the four non-responsive cultivars. Previous investigations on wheat have shown that landraces and cultivars released before 1950s, before the era of chemical fertilization, were more responsive to mycorrhizal fungi than more recently released cultivars (Hetrick et al. 1992, 1993, 1995). From this observation it was possible to conclude that selection under high fertilization may result into the loss of alleles which allow colonization and control responsiveness to mycorrhizal fungi.

Toth et al. (1984) have shown that in the case of maize breeding and selection for efficiency of P uptake may have resulted in a change in the genetic potential for colonization. High P inbreds were colonized to a greater extent than low P inbreds ones. In this case, selection of maize plants for P efficiency may have resulted in the inadvertent selection of maize susceptible to mycorrhizal fungi, but other selection criteria may have resulted in an opposite trend, namely of selecting plants "resistance" to infection by mycorrhizal fungi (Toth et al. 1990).

In another study with 28 inbred lines of maize in low P soil, the mycorrhizal responsiveness varied from 66% to 653% in the greenhouse (Kaeppler et al. 1998). There was also a twofold difference in root colonization in root colonization among these lines which was not related to mycorrhizal responsiveness. These workers have also found a significant difference among mycorrhizal fungal species in enhancing maize growth; *G. etunicatum* providing a consistent positive response across three inbred lines; *G. intraradices* showing differential response across lines and several species; and *Acaulospora morrowae* which caused no significant response in any of the lines studies.

The above cited studies on barley, wheat and maize exemplify the range of variation present among genotypes within a plant species and within species of mycorrhzal fungi for the ability to form a mutually beneficial interaction. These genetic interactions, coupled with environmental factors such as soil fertility, light intensity, variation in the density of mycorrhizal populations in soil and microbial competition within the rhizosphere have made it challenging to harness the mycorrhizal interaction in agronomic applications.

6.2.3 Genetic Basis in Host Determination of Colonization and Responsiveness

The genetic basis of the plant-micorrhizal fungus interaction has not yet been fully characterized. Genetic control of the interactions between AM fungi and host-plant

exists both within host as well as symbiont species. Genetic analysis of variations among AM fungal isolates has been nearly impossible since these fungi propagate sexually and have multinucleate spores and cells (Park and Kaeppler 2000). Information on host genes controlling the interactions is now becoming available both through the analysis of mutants (Duc et al. 1989; Peterson and Guinel 2000) and through the studies of quantitative variation in populations. The genetic relationship between AM symbiosis phenotypes (e.g. percent of root colonized) and agronomic traits such as yield, biomass accumulation, and disease resistance is of particular interest from a plant improvement perspective.

Toth et al. (1990) have observed a positive correlation between disease susceptibility and colonization in 13 inbred lines of maize. Their results have suggested that selection for resistance to fungal pathogens may also result in decreased AM fungus colonization. Hetrick et al. (1995) provided the first data on the chromosomal location of genes controlling mycorrhizal responsiveness in wheat. Kaeppler et al. (1998) mapped quantitative trait loci (QTL) controlling growth at low P in the absence of mycorrhiza and mycorrhizal responsiveness in an inbred population of maize.

These studies futher define genetic factors affecting the host symbiont interaction and indicate that the inbred lines developed in the era of chemical fertilization contain alleles that allow a positive interaction with AM fungi.

The debate that current cultivars contain alleles for mycorrhizal responsiveness has important implications for future agricultural production systems. Cultivars for sustainable systems will likely need to maximize microbial interactions to replace chemical inputs. If current elite cultivars retain alleles for this function, then it is reasonable to expect high yielding genotypes for reduced input systems to be bred in a limited amount of time. However if breeders reach back to less developed germplasm to develop these cultivars, years of selection for disease resistance, quality, and other agronomic charecteristics present in current hybrids will be sacrificed.

7 Culturing of AM Fungi for Commercial Purposes

AM fungi have not been grown *in vitro* and therefore it is difficult to produce large quantities of fungal biomass for biochemical and genetical studies. Numerous attempts have been made to culture these fungi, but these have met with little or no success (Hepper 1984; Burggraff and Beringer 1989) because of the obligate nature of these fungi. The most common method employed for culturing AM fungi is trap culture, where a bait plant is grown in diluted soil from the field. This usually results into a mixer of species. Mosse (1962) was the first to establish that surface sterilized spores could infect plant roots under axenic conditions. Subsequently, various methods were developed to successfully infect a host plant (Menge and Timmer 1982) for starting a pure line of an AM fungus under sterilized conditions (Hepper 1981). AM fungi can be maintained pure and viable for long-term periods on a suitable synthetic growth medium or substrate in association with excised roots.

The current practice of inoculating plants involves the use of soil/turface-based pot-culture inoculum, produced on a suitable host plant grown under sterilized growth conditions for a period of about 2–3 months (Augé et al. 2008.). To break the spore dormancy, the inoculum is incubated in refrigerator or at room temperature for another month prior to application (Mehrotra 2005). To produce standard AM inoculum, the plant species used for raising AM inoculum are *Zea mays, Pennisetum typhoides, Allium porrum, Sorghum bicolor, Glycine max, Paspalum notatum, Cenchrus ciliaris, Chloris guyana* and *Panicum maximum*. The commercial inoculum is available in USA, India, Canada, Japan, and UK and contains several kinds of propagules, including spores, mycorrhizal root fragments and living mycelium (Mehrotra 2005). Time constraints are, however, there and it takes almost 1 year for the inoculum to get ready.

Although AM fungi have been reported to exhibit host non-specificity, however, a number of studies have shown that plant genetic makeup and AM responsiveness are closely related (Krishna 2005). The variations exist between species and genera of the same family, cultivars of the same crop species and different transgenic lines of the same plant.

Genetic control of the interactions between AM fungi and host plant exists both within host as well as symbiont species. However, the genetic basis of the plant-micorrhizal fungus interaction has not yet been fully characterized. The reason that genetic analysis of variations among AM fungal isolates has been nearly impossible as these fungi propagate asexually and have multinucleate spores and cells (Park and Kaeppler 2000).

In a study conducted by Nasim et al. (2010) it has been indicated that some of the transgenic lines of tobacco responded remarkably well to the inoculation by *Glomus intraradices*. It addresses the hypothesis that the transgenic events in tobacco has enhanced the responsiveness of *G. intraradices*. Moreover it also attempts to compare the rate of multiplication of fungual inoculum in transgenic tobacco roots with that of wild type Xanthi.

The data on fresh and dry biomass of tobacco lines at three different harvests have shown a significant difference of some of the genetic lines as compared to wild types (Nasim, G. Unpublished data). However, the determination of mycorrhizal parameters has indicated a multifold increase in colonization and sporulation by *G. intraradices*. The first harvest of experiment I was conducted after three and a half months considering for the provision of enough time to allow the fungal hyphae to invade the plant root system and initiate sporulation. The monstrous sporocarps were first observed at that stage. The second experiment was then planned keeping in view the summary of the results of the first experiment. Eight genetic lines were then short-listed including seven synthetics and one wild type. The modified seed lines were picked on the basis of their interaction with the test fungus. In this experiment, first harvest was taken after a period of 6 weeks allowing the minimum time for colonization by the AM fungus. The results were very exciting and even at this stage of host growth, the colonization and sporulation by *G. intraradices* was remarkably high (Plate 23.2). The spore aggregates/sporocarps were much bigger (Plate 23.3). The maximum size reported was 2.1×2.8 cm. These were not only

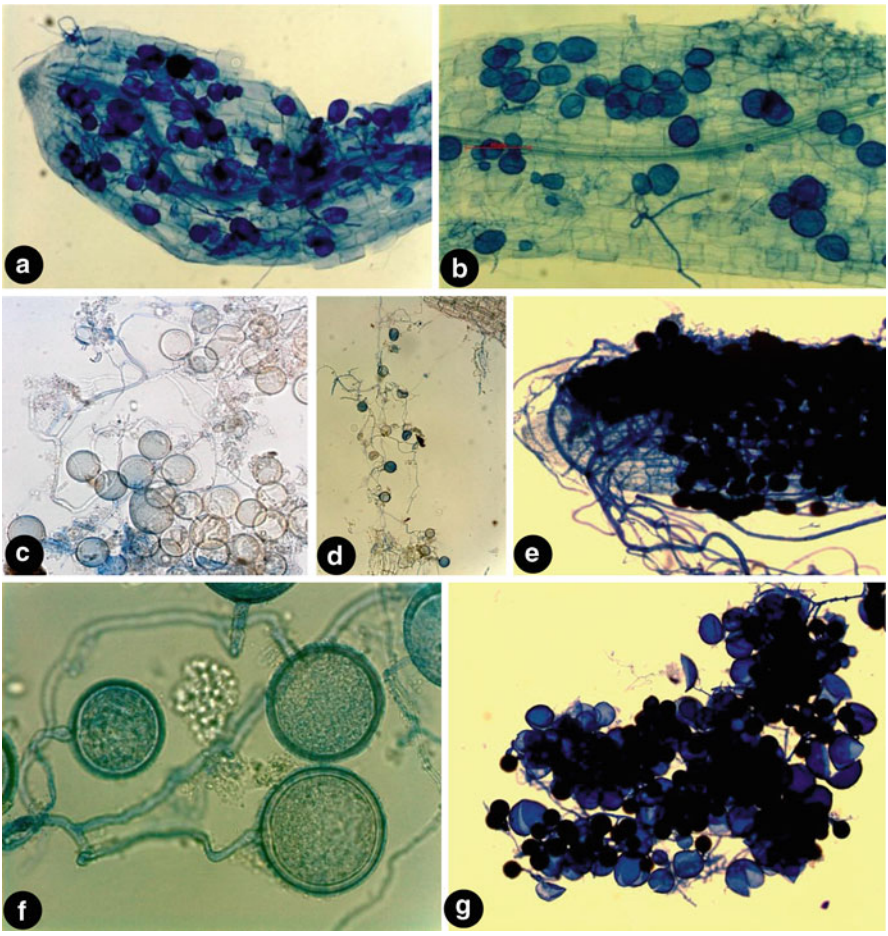

Plate 23.2 (**a**) Vesicles in tobacco root inoculated with *Glomus intraradices*; (**b**) Vesicles in tobacco root inoculated with *G. diaphanum*; (**c**–**d**) Extrammatrical azygospores formed by *G. intraradices* with in association with tobacco roots; (**e**) Vesicles ans extraradical hyphae; (**f**) Spores enlarged; (**g**) Extramatrical vesicles

formed along with the plant root system but also developed on the mesh which is generally used to block the wholes present at the bottom of the pot (Plate 23.4). The results when viewed in terms of number of such monstrous sporocarps and the mesh squares colonized have indicated that *m-orange* and *gbr-15* are the two constructs with a potential to produce maximum number of such structures (Fig. 23.2). Therefore, these findings may be of interest for mass multiplication of AM inoculum under controlled conditions. Practical steps may be initiated to manipulate genes specifically targeting the responsiveness of host plants towards a particular AM fungus or a consortium of these fungi. Furthermore, the results of this study are in line with those of Wenke and Lianfeng (2008) who have stated that when *Bt* transgenic crops are planted continuously, it may lead to a surprisingly weird results as regards

Plate 23.3 Different categories of spore aggregates depending upon the number of spores per aggregate. These categories were used to determine differences within the transgenic lines

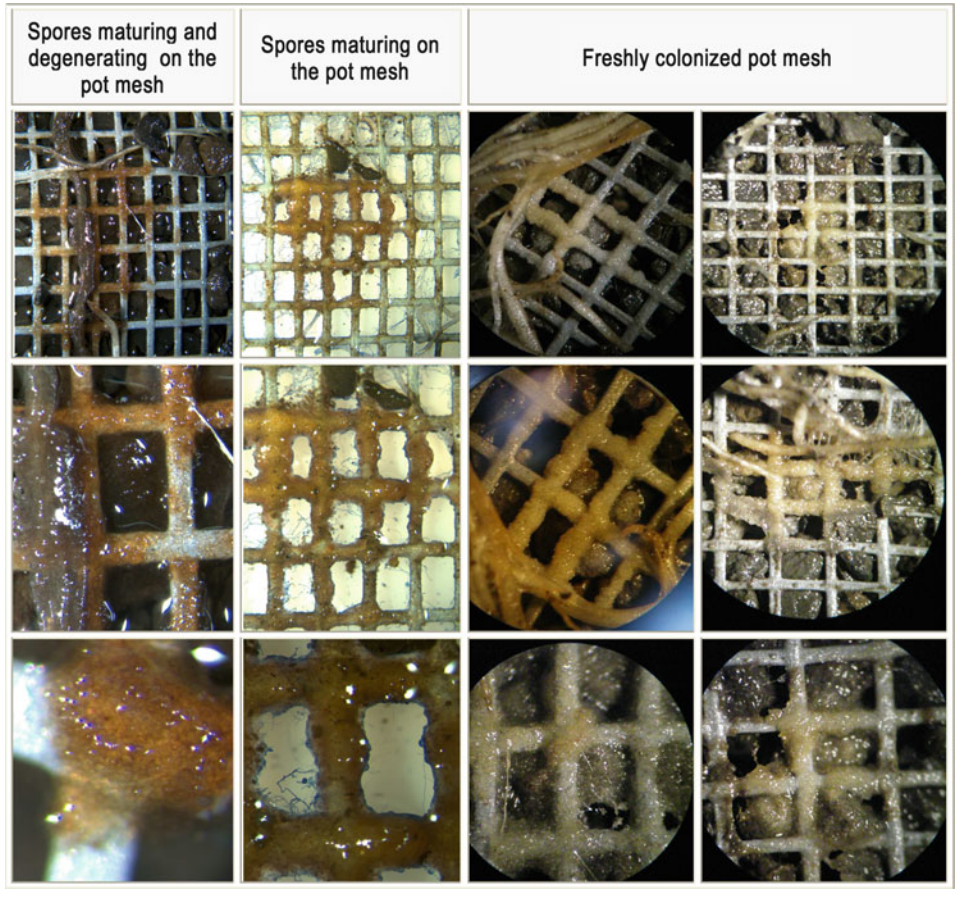

Plate 23.4 Different stages of spore colonization and maturity on pot mesh in transgenic tobacco lines. The number of squares (2×3 mm) colonized was adopted as a parameter to differentiate extent of colonization in various genetic lines

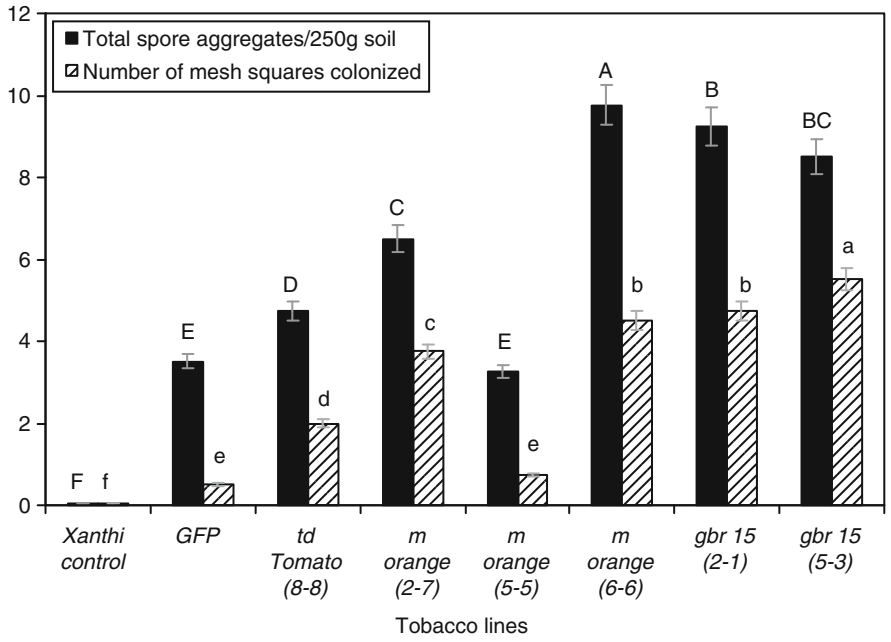

Fig. 23.2 Arbuscular mycorrhiza (AM) data of eight selected genetic lines of tobacco at first harvest after 6 weeks of inoculation. Means sharing same uppercase or lowercase letters are non-significant at 5% level

specific abundance and diversity of AM fungi. They concluded that interaction between AM fungi and *Bt* transgenic crops at individual and community level are an urgent soil ecological issue. Similarly, the results of the present study show that growing transgenic crop plants without proper risk assessment may have interesting ecological and agricultural implications.

The study presented in this session strongly suggests that the response of a species of AM fungi, *Glomus intraradices* is highly variable when inoculated to some of the fluorescent transgenic lines of tobacco, *Nicotiana tobaccum*, cv. Xanthi. The AM fungus formed monstrous sporocarp-like spore aggregates up to 2.8 cm in dimension. In view of these findings and accelerating costs of chemical fertilizers along with the environmental impact of excessive use of these agrochemicals, it is been suggested to develop transgenic strains which can promote the growth and sporulation of these beneficial fungi in pot culture for further utilization as biofertilizer for economically important plants.

These host-AM combinations may be exploited as biofertilizers for field applications. Biofertilizers are living fertilizer compounds consisting of microbial inoculants which are able to fix atmospheric nitrogen, solubilize phosphorus, decompose organic material or oxidize sulphur in the soil. On application, it enhances the growth and yield of plant yield and also improves soil fertility and reduces pollution. Biofertilizers are an alternative to mineral fertilizers for increasing soil productivity and plant growth in sustainable agriculture. Bio-inoculants like

arbuscular mycorrhizae (AM), free living and symbiotic N-fixers, phosphorus solubilizing bacteria (PSB), and plant growth promoting rhizobacteria (PGPR), could be regarded as wide spectrum biofertilizers (Gianinazzi et al. 2002).

8 Role of AM in Sustainable Agriculture

Sustainable agricultural systems employ natural processes to achieve acceptable levels of productivity and food quality while minimizing adverse environmental impacts (Harrier and Watson 2004). The concept of sustainability in agriculture emphasizes that it must be ecologically sound or environment-friendly, economically viable and socially just or simply 'sustainable agriculture' is the practice of farming using principles of ecology. It relies on the principle that we must meet the needs of the present without compromising the ability of future generations to meet their own needs. Sustainable agriculture relies on long-term solutions using proactive measures rather than reactive measures at system level (Siddiqui and Pichtel 2008). Several factors contribute towards the development of agriculture on sustainable basis. Soil fertility is very important in this regard. Soil fertility can be regulated through the control of soil-borne diseases, increasing microbial activity leading to increased competition and parasitism within the rhizosphere (Jawson et al. 1993; Knudsen et al. 1995).

The fundamental objective of agriculture is human food security. However, in the past few decades the intensification of agricultural activity has resulted into tremendous environmental degradation. Moreover, the use of agricultural chemicals has produced significant effects on the natural ecosystem. Such constraints of modern-day agriculture have given birth to new concepts in farming, such as organic farming, natural farming, biodynamic agriculture, do-nothing agriculture, eco-farming, etc., (Panwar et al. 2008). This back to natural farming or sustainable agriculture is the only option which encompasses soil and crop productivity by integration of agricultural management technology at the same time maintaining and enhancing the farm profitability and environmental quality (Panwar et al. 2008). In the concept of sustainable agriculture, plant microbe interactions particularly with the beneficial soil fungi like AM have played a significant role. AM fungal associations occur widely throughout the plant kingdom including most of the agricultural and horticultural crops (Gerdemann 1968). AM fungi are ubiquitous soil microbes, but little is known of natural ecology of these fungi. The conventional agronomic practices may adversely affect the efficiency of these microbes. The impact of soil factors such as soil pH, soil moisture contents (Redhead 1977) and light intensity (Hayman 1974) on AM fungi is also well documented. The soil conditions like salinity, waterlogging, erosion, soil types, water holding capacity, soil porosity, fertility status, and vegetation greatly influence AM fungal associations (Panwar et al. 2008).

Mycorrhizal fungi are clearly instrumental in augmenting plant nutrient availability particularly in nutrient stressed soils. Research has shown that the growth increment due to AM fungi is basically through an increased surface area for the absorption of nutrients. The increased uptake of phosphate, nitrogen, sulphur,

potassium, calcium, zinc, iron, copper, and water transfer, are most commonly reported (Allen 1991). Moreover, the inter-plant movement of nutrients has also been reported in between plants connected with AM fungi (Read et al. 1989). Mycorrhizal inoculation enhances nodulation in leguminous plants resulting into manifold increase in nitrogen fixation (Carling et al. 1978). Arbuscluar mycorrhizal fungi play an important role also in plant-water relations. The associations improve the unsaturated hydraulic conductivity of the roots either by modifying root morphology and root anatomy or indirectly by hormonal or structural changes in the host plant (Auge 2000; Augé et al. 2008.).

The role of AM fungi in improving soil quality and aggregation is also well established (Miller and Jastrow 2000). They have suggested that AM hyphae form and stabilize aggregates of soil through three distinct processes: (1) The AM hyphae physically entangle primary particles of soil; (2) roots and AM hyphae create conditions that enable microorganisms to proliferate in the soil; and (3) roots and AM hyphae enmesh and bind microaggregates and smaller macroaggregates into larger macroaggregates. This improvement in soil structure has a direct impact on the indigenous microbial community through aeration and moisture infiltration and indirectly through stimulation of plant root growth. This may make mycorrhizal plants particularly useful for reclamation of soils with problems of surface crusting and sand dunes. Glomalin, a glycoprotein produced by AM fungal hyphae plays a major role in aggregate stabilization (Wright and Upadhyaya 1998). Glomalin has been considered to enhance ecosystem productivity and carbon sequestration in soil ecosystems (Wright et al. 2000; Rilling 2004). AM fungi also contribute towards microbial biomass resulting into improvement of soil quality.

In problematic soils, the AM fungi have been reported to regulate the uptake of heavy metals and salts (Diaz et al. 1996; Augé et al. 2008.). This may be due to high metal sorption capacity of these fungi, which could 'filter' metal ions during uptake (Joner et al. 2000).

For large scale application in agricultural systems on sustainable basis, it is necessary to evaluate the efficacy of consortia of organisms ensuring better chances of survival of plants and which are able to withstand competition with the indigenous fungi. It is further needed to conserve the diversity of AM fungal flora to ensure the restoration of plant community in abandoned agricultural lands. For commercialization, it is necessary to identify genetic basis for effective nutrient transport for increasing efficacy and adaptation to crop vigor and yield. The research is needed to be done on biotechnological application of AM fungi, technical and commercial aspects of their application for micropropagated or genetically modified plants in sustainable agricultural systems.

9 Conclusion

Arbuscular mycorrhiza plays a vital role in the life of a plant. There are innumerable advantages for the plant harbouring this relationship. AM technoly may be merged with modern biotechnologies to tap greater advantage in terms of sustainable agri-

culture and food security. Cultivars or genetic lines with greater responsiveness towards mycotrophy may be evolved through integrated research efforts.

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Chapter 24
A Site-Specific Potassium Fertilization Approach to Overcome Sporadic Response of Crops

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Abstract As soils vary in adsorption characteristics depending upon their physical, chemical and mineralogical composition, it is difficult to identify a single critical value of potassium (K) concentration for a variety of crops on different textured soils. In general long experimental trials need to be conducted to develop fertilizer recommendations for a particular region in a country. Even then these proposals are often insufficient to get desired yield targets. However, fertilizer recommendations based on soil solution concentration level using sorption isotherm technique provide a good indicator of soil fertility. To this end Freundlich and Langmuir adsorption models are widely used to monitor nutrient behavior in the soil and for predicting site specific and crop specific fertilizer requirements. This approach requires short experimentation; say for example, only one trial at selected sites. Before starting the experiment, target soil solution levels are set in the laboratory based on soil characteristics using above said adsorption models. After completing the trial and to arrive at precise yield target, yield data are further evaluated using different yield response models (linear plus plateau, quadratic, exponential/Mitscherlich, Extended logistic, and Boltzman sigmoid models). In this study maize fodder crop was taken using above said approach. Total green fodder yield of 61.91 Mg ha^{-1} was obtained at a target soil solution level of 14.50 mg L^{-1} equivalent to 144 kg K ha^{-1}. However, to get maximum output with respect to fodder quality traits, a higher soil solution level need to be adjusted. This would definitely lead to higher fertilizer rate.

Keywords Freundlich • Langmuir • Fertilizer recommendation • Soil solution • Yield target

1 Introduction

Potassium is one of the essential major nutrients required both for plants and animals together with nitrogen as well as phosphorus (Oborn et al. 2005). Total K contents in soils range between 3 and 100 Mg ha^{-1} in the upper 200 cm of the soil profile. More than 95% K is bound in the mineral form, whereas less than 3% is in soil extractable phases (Sparks 1987). The essentiality of K to plant growth was first established by Von Liebig in 1840 (Sparks 2000) and since then this subject has been studied extensively (Du et al. 2004; Askegaard et al. 2005). Regardless of much study, the fundamental fate, movement, and availability to plants that govern

its chemical and physical phenomena have not been investigated completely. Earlier studies have revealed that K behaviour has a good relationship to various soil and environmental components (Munson 1985), such as the types of soil minerals present, moisture regime (Malla 2002; Surapaneni et al. 2002), cropping and fertilizer history (Askegaard et al. 2005), temperature variations (Barber 1995), and soil weathering (Cakmak 2002; Askegaard et al. 2005). No coordinated explanation has been arrived at that could link all the said variables in a logical way. That is why soil tests based K fertilization mostly remained ineffective on various types of soils resulting in unpredictable yield for various crops (Munson 1985).

For predicting fertilizer requirements, Freundlich and Langmuir models have been widely used to monitor nutrient adsorption in soil which involves development of sorption isotherm as it quantitatively predicts the exact amount of fertilizer required for maximum crop yield. It predicts in a better way how the nutrient status of a soil will change upon cropping. By knowing the critical solution level for a particular crop, the exact amount of fertilizer can be computed using nutrient-sorption isotherm technique equivalent to soil solution level needed for maximum crop yield on various types of soils. Different soil solution K levels serve as a guideline of K availability which depends upon the rate of removal by the plants as well as rapidity at which K can be desorbed from the adsorbed phase. Thus K concentration in soil solution provides a good indicator of soil fertility. K fertilization therefore, is important factor to meet the K requirements of a particular cropping system. Very few studies have been reported with respect to potassium adsorption characteristics of alkaline and calcareous soils. Keeping this in view, present overview has been prepared for: (a) to evaluate the adsorption isotherm technique for optimum yield and quality of crops, (b) prepare site-specific and crop-specific K fertilizer recommendations for a particular region. In this regard, maize fodder crop in potato-maize cropping sequence was evaluated as a test case.

2 K Categorization in Soil

Potassium in soil is found in solution, exchangeable, non-exchangeable (fixed) and inert (structural) K forms. These four forms can be easily identified with the help of appropriate laboratory methods, however, no clear-cut margins exist in the field among these forms (Sharpley 1990). The kinetics and equilibrium reactions among these four forms ultimately affect its availability to plants.

Extractable (Exchangeable + non-exchangeable) K level constitutes only a minor portion of total K, whereas the major portion is present in mineral fraction (Sparks and Huang 1985). The different forms of K availability to plants and microbes are in the order; solution > exchangeable > fixed (non-exchangeable) > mineral (Sparks and Huang 1985; Sparks 1987, 2000).

The relative amount of these four forms depends upon soil factors like types and amount of clay, K input and output from the field, leaching losses as for instance in sandy soils and K fixation in particularly in the illitic dominated soils. A brief description of these four K forms is given below:

2.1 Solution K

The solution K is the form which is not only taken up by the plants and microbes directly but also subjected to leaching losses in soil. The replenishment as well as depletion from exchangeable and non-exchangeable K sites at any given time determines the K content of this fraction. The processes that cause the reduction in the solution K concentration are also a cause for the release of K from non-exchangeable sites. In normal agricultural soils the level of solution K may vary from 3 to 30 mg $K\,L^{-1}$ (During 1984; Haby et al. 1990). Soil moisture content, bivalent cations in both solution and exchangeable sites and reactions occurring between different forms of K leading to equilibrium and kinetic reactions affect the level of K in the soil solution (Sparks and Huang 1985; Sparks 2000). Similarly, clay minerals and soil buffering capacity cause variations in the amount of K level in the solution. Weakly buffered allophanic clays replenish solution K slowly while well-buffered clay minerals like micas and vermiculites maintain the level of solution K rapidly and fairly unchanged (Parfitt 1992).

2.2 Exchangeable K

It is the portion of extractable K which occupies exchange sites of soil colloids (Malavolta 1985). Exchangeable K is held with different bonding forces at particular adsorption sites (planer, edge, wedge, cracks etc.) of the clay minerals. Although fairly a constant number of exchange sites are present on clay particles through isomorphic substitution yet, the exchange sites on humus colloids and amorphous clay minerals increase as pH increases mainly due to the dissociation of H^+ from weak acid groups. The kinetic and thermodynamic factors are responsible for holding a particular amount of K^+ at exchange sites of clay minerals (Parfitt 1992). The displacement of exchangeable K^+ by Ca^{2+} is particularly important in soils where Ca^{2+} is added in large quantities through phosphatic fertilizers and gypsum. These additions of Ca^{2+} may be responsible for leaching of K^+ due to the selective adsorption of Ca^{2+} at exchange sites (Edmeades 1982). Exchangeable K is generally less than 2% of total soil K and ranges between 10 and 400 mg kg^{-1}.

2.3 Non-exchangeable or Fixed K

Non-exchangeable or interlayer fixed K differs from structural K as in the former K is not a part of the crystal structure of minerals. It is held between adjacent tetrahedral layers of dioctahedral and trioctahedral micas, vermiculites, and intergrade clay minerals such as chloritized vermiculite (Sparks and Huang 1985; Sparks 1987). Fixation of potassium on clay colloids depends upon the relative differences in forces between K^+ ions with clay surfaces and hydration forces among K^+ ions

and dominancy of the former causes its fixation. This results in a partial collapse of the crystal structures and the K^+ ions are physically trapped to varying degrees, making K release a slow, diffusion controlled process (Sparks 1987). The weathered wedge zones of micas and vermiculites are also source of fixed (non-exchangeable) NH_4^+ and H_3O^+ ions, due to their size similar to K^+, can exchange K from wedge zones only, while hydrated Ca^{2+} and Mg^2 cations whose sizes are larger than K^+ are unable to exchange K from wedge zones. This fixed (non-exchangeable) K may also get released to exchangeable form when a reduction in exchangeable/soil solution K occurs as a result of crop removal, leaching or increased microbial activity (Sparks et al. 1980; Sparks 2000). Non-exchangeable K is fairly to moderately available to plants (Mengel 1985; Sparks and Huang 1985; Sparks 1987).

2.4 Structural K

Structural K is known by a number of alternative names like mineral K, un-weathered K, native K, matrix K, or inert K. It generally makes up the bulk of the total K in most soils, however soil parent material as well as formation stage determine the exact amount of total K in soils (Sparks and Huang 1985). In K-bearing minerals such as biotite/muscovite micas, orthoclase/microcline feldspar and volcanic glasses it is mostly bonded covalently within their crystal structures (Metson 1968a). These minerals are generally found in the coarser fractions because of their partial weathering/alteration during soil formation stages, as the particle size decreases the degree of alteration increases. The replacement of K^+ with Mg^{2+}, Ca^{2+} or Al^3 from the interlayer spaces of micas results in the commencement of weathering of micas which in turn lead to the formation of illite, vermiculite, smectite and other interstratified minerals. For example, smectites may form with the complete removal of K^+ from interlayer spaces of mica in an environment of abundant Ca^{2+} and Mg^{2+}, whereas vermiculite may form if environment is changed to more acidic condition.

3 General Fertilizer Recommendations

Soil testing is an important tool for preparing fertilizer recommendations, but it is little used by farmers due to the lack of supportive research. Though standard soil testing methods are good tools to monitor soil fertility status but it often fails to predict the precise requirement of fertilizer for a particular crop. In order to predict the response of a particular crop to any fertilizer based on soil tests require thorough understanding of the mechanism of bioavailability of nutrient present in soil as well as target crop yield. The significant variations due to soil conditions at different sites do not allow to develop a strong relationship between available nutrient pool and crop yield. For this purpose people generally use the critical value i.e., that amount of available nutrient above which the probability of a crop response to

applied fertilizer is poor and below which the reverse occurs. Recommendation of nutrient fertilizers merely on the basis of soil test demands strongly to find the critical amount of nutrient present in the soil because it varies with crop type. Fertilizer recommendations in general are obtained from simple experiments and extrapolated on generalized soil properties but when made by using the adsorption technique with Freundlich or Langmuir models, it results in higher yields, greater economic return, and balanced fertilization in alkaline calcareous soils (Hannan 2008). Thus, there is an urgent need for more site specific nutrient recommendations that can be readily transferred to and which meet farmer's production goals and resources. The response of crop to applied K is erratic due to lack of refined critical level of soil K and this unpredictability is further aggravated due to different adsorption characteristics of various soils for K. The fate of added K as fertilizer depends upon the initial level of soil K and concentration of K in soil solution depends upon the rate of removal by the plants and the rapidity at which K can be desorbed from the adsorbed phase, whereas adsorption equilibrium solution K levels serve as an index of K availability. This depicts that equilibrium K concentration provide a better index of soil fertility using sorption isotherm technique.

As adsorption characteristics vary from soil to soil so knowledge about the factors which affect the K adsorption characteristics of the soils is necessary to predict the fate of added K fertilizers and to arrive at precise K fertilizer recommendations (Sparks and Huang 1985). As stated by Grewal et al. (1992), the yield of potato can be increased up to 50% through an improvement in nutrient management. Thus, big chances exist to enhance crop yield and quality by improving nutrient management.

4 Types of Exchange Sites on the Clay Minerals

A number of researchers have explained the exchange behaviour of K with respect to Mg and Ca on illite by assuming the presence of three types of exchange sites for K, each with its own exchange coefficient (Sumner and Bolt 1962; Bolt et al. 1963). These are (a) planner sites that occur on the outer surface of the crystal structures, (b) edges sites, and (c) interlattice sites situated between layers of the minerals. Bolt et al. (1963) showed that interlattice sites showed the highest Gapon selectivity coefficient (KG), where as planar sites exhibit the lowest kG. Goulding (1987) postulated various types of adsorption for K on clay minerals such as planar, edge, interlayer, wedge, crack, and step potassium exchange sites in 2:1 layer silicate minerals.

5 Potassium Sorption Isotherms Versus Nutrient Bioavailability

Sorption isotherm describes the relation between the amount of substance that is adsorbed by the solid phase of the soil and its equilibrium amount in soil solution. Among numerous chemical processes occurring in soil, sorption is one of most

important processes which describe the fate and mobility of nutrients in the soil as well. A number of essential elements like P, K, S, Cu, Zn, can be studied practically through sorption isotherm (Hunter 1980). Sorption isotherm further explores whether applied plant nutrients react, fix or make complex with the soil. Without having a comprehensive knowledge regarding the characteristics of nutrient sorption isotherm of a particular experimental site it is inevitable to establish fertilizer optima for enhancing crop productivity, therefore understanding the nutrient sorption characteristics of the soil of the experimental site is inevitable. This approach helps to envisage a safe level of any one of the nutrients in the soil because it can be adjusted in the available pool of the soil at a level in which it is neither deficient nor toxic to the crops (Hunter 1980). This makes the potassium sorption isotherms a part of quantity-intensity approach (Neiderbudde 1986), that is used to evaluate soil K supply to plants and explain the exchange of K by other ions mostly Ca (Bedrossian and Singh 2002) and to determine interchangeable potassium in order to understand its dynamics in soil (Yunda et al. 1997).

Of all the equations, Freundlich and Langmuir adsorption models give a closer description of the real adsorption phenomenon in the soil system.

5.1 Amount of Nutrient Adsorbed

$$\frac{x}{m} = [Ci - Cf]V \times 10 / W \tag{24.1}$$

Where:

$\frac{x}{m}$ is the amount of the nutrient adsorbed per unit weight of soil (mg kg^{-1})

Ci is the initial concentration of the nutrient added (mg L^{-1}),
Cf is the concentration of that nutrient in the equilibrium condition (mg L^{-1}),
V is the volume of solution taken (ml),
W is the weight of soil sample used (g)

The theoretical modifications made in the Eqs. (24.1, 24.2, 24.3, and 24.4) for the fertilizer application are given as under: Freundlich equation:

$$\frac{x}{m} = aC^{b} \tag{24.2}$$

Where x/m and C are same as discussed above.

By rearranging:

$$\frac{x}{m} = \log a + b \log C \tag{24.3}$$

$$\frac{x}{m} = \log a + b \log C + \log Tc \tag{24.4}$$

Where Tc is the target nutrient concentration which will be adjusted by adding the amount of fertilizer corresponding to its specific soil solution level.

$$\text{Fertilizer dose } \left(\text{mg kg}^{-1}\right) = \text{ antilog } \left(\frac{x}{m} = \log a + b \log C + \log Tc\right) \tag{24.5}$$

$$\text{Fertilizer dose } \left(\text{kg ha}^{-1}\right) = \text{ Fertilizer dose } \left(\text{mg kg}^{-1}\right) \times 2 \tag{24.6}$$

Finally the Eq. 24.6 is used to compute fertilizer rates against specific soil solution K levels. The 'a' and 'b' values are taken from the intercept and slope of the Freundlich model, with b values of $0<n<1$. These parameters have no physical meaning, although Sposito (1980) presented 'b' as measure of heterogeneity of adsorption sites on the adsorbent surface. Equation 24.3 estimates the total amount of nutrient needed to be added just to satisfy adsorptive surfaces of the soil. The amount added in addition to that (Eq. 24.4) will contribute to that which will contribute to its concentration that will come into soil solution. By developing adsorption isotherm in the laboratory, different levels of solution (say for example for potassium 0, 3, 6, 9, 12, 15, 18, 21, 24, and 27 mg L^{-1}) can be worked out theoretically using Freundlich model and then equivalent to these specific solution levels K fertilizer doses can be calculated as given above. For micronutrients or over narrow range of nutrient concentration Langmuir equation can be used in the same way.

Langmuir equation:

$$\frac{x}{m} = \frac{\kappa cb}{1+\kappa c} + Tc \tag{24.7}$$

$$\text{Fertilizer dose } \left(\text{mg kg}^{-1}\right) = \text{ antilog} \left(\frac{x}{m} = \frac{\kappa cb}{1+\kappa c} + Tc\right) \tag{24.8}$$

$$\text{Fertilizer dose } \left(\text{kg ha}^{-1}\right) = \text{ Fertilizer dose } \left(\text{mg kg}^{-1}\right) \times 2 \tag{24.9}$$

These equations help in resolving sporadic nutrient response in the agricultural soils of Asia and to find out specific soil solution level required for maximum yield for a given crop over a given set of environmental conditions. In spite of certain limitations; as these provide rough estimate of the buffering capacity of the soil which might not reflect the true situation in the field due to mineralization of organic pool, rhizophere and mycorrhizal effects; but on overall basis these effects contribute to a very limited extent because in adsorption isotherm the net change in the charge of the soil either due to fluctuations in pH or the presence of different organic acids has also been counted for (Xu et al. 2005). Several examples prove the

capacity of this model to predict adsorption phenomena of various elements by soils, such as Pal et al. (1999) for K, Erdem et al. (2004) for Cu, Co, Zn, and Mn, Imtiaz et al. (2006) for Zn; and Fontes and Coelho (2005) for Mo.

6 Yield Response Models

High K application rates are unwanted because of economic reasons and environmental pollution problems, for instance, higher K rate does not improve yield in potato (Hannan 2008; Lal and Singh 1983; Rhue et al. 1986, and Chapman et al. 1992). Negative affects are assigned to salt damage (Bilski et al. 1988; Maier et al. 1994), and physiological changes (Westermann et al. 1994), while both reduction in potato yield and small-sized tubers are attributed to insufficient K supply (McDole 1978; Satyanarayana and Arora 1985). Fertilizer recommendations thus must optimize crop yield and quality for maximizing profit, and would cut down the risk of environmental pollution; therefore different yield response models can be evaluated to identify the optimum K rate for maximum output. A brief summary of commonly used yield response models is given below:

6.1 Linear Plus Plateau Model

$$Y = a + bx \quad \text{if } x < c$$

$$Y = P \quad \text{If } x \geq c$$

Where 'Y' is the yield (Mg ha^{-1}) and x is K rate (kg ha^{-1}); 'a' represents intercept, 'b' denotes linear coefficient, 'c' is the optimum K rate at the juncture of the linear response and the plateau lines, while 'P' is the plateau yield.

6.2 Quadratic Model

$$Y = a + b\,x + cx^2$$

Where 'Y' is the yield (Mg ha^{-1}) and 'x' is K rate (kg ha^{-1}); 'a' is intercept, 'b' is linear coefficient and 'c' is quadratic coefficient.

6.3 The Exponential/Mitscherlich Model

$$Y = M\left(1 - \exp^{-c(x+b)}\right)$$

Where 'Y' is the yield in Mg ha^{-1}, 'x' is K rate in kg ha^{-1}, 'M' is the highest yield. The 'c' stands for Mitscherlich constant and the 'b' constant calculates the quantity of extractable K.

6.4 Extended Logistic Model

$$Y = Y_{max} / \left(1 + \exp\left(b - c * x\right)\right)$$

Where 'Y'$_{max}$ is the highest yield in Mg ha^{-1} and 'x' is the K rate in kg ha^{-1}, 'a' is intercept, 'b' is the linear coefficient and 'c' stands for logistic coefficient. R^2 values for models were calculated through regression analysis.

6.5 Boltzman Sigmoid Model

$$Y = \frac{Y\,\min + (Y\,\max - Y\,\min)}{(1 + \exp(Y50 - X)/b)}$$

Where 'Y'$_{max}$ is the maximum and 'Y'$_{min}$ is the minimum yield (Mg ha^{-1}), 'X' is K rate in kg ha^{-1}, 'b' is linear coefficient, 'Y'$_{50}$ is the yield halfway between 'Y'$_{max}$ and 'Y'$_{min.}$ Coefficients of determination (R^2 values) were calculated through regression analysis.

7 Example of Site-Specific K Fertilization – A Case Study

7.1 Potassium Adsorption

Three soils were selected before sowing at 0.3 m depths and their physical and chemical attributes were determined (Table 24.1). The potassium adsorption data of the selected soils best fitted to Freundlich ($R2 \geq 0.95$**) as given in Table 24.2. The reason might be that Freundlich model assumes unlimited sorption sites of heterogeneous medium which give better correlation due to mixed mineralogy of the soil with illite as the dominant clay mineral. These findings are supported by the results obtained by Pal et al. (1999). The data shows that though all soils had altogether different fertilizer requirements to maintain the same soil solution level, in this study Freundlich adsorption isotherm technique was thus used to predict the quantity of added K required for the adjustment of soil solution to achieve prescribed isotherm based soil solution K levels because K concentration in the soil solution has a more universal applicability. Such studies can provide a suitable basis for determining K fertilizer requirements, especially on soils with varying K adsorption characteristics. In terms of fertilization (Table 24.3), Typic Ustochrepts soil possessed greater K adsorption capacity and required large K application

Table 24.1 Physical and chemical characteristics of selected soils

Parameters	Typic Halorthids	Typic Camborthids	Typic Ustochrepts
EC (dS m^{-1})	1.02	0.94	1.33
pH	8.16	8.29	8.22
O.M (%)	0.49	0.81	0.43
Extractable P (mg kg^{-1})	6.45	7.89	7.26
Extractable K (mg kg^{-1})	242	81	65
CEC ($cmol_c$ kg^{-1})	6.26	6.78	8.78
$CaCO_3$ (%)	7.93	9.15	9.78
Clay (%)	16	14	31
Textural class	Sandy clay loam	Sandy loam	Silty clay loam
FAO world map	Orthic solonchaks	Cacaric gleysols	Cacaric cambisols

Table 24.2 Parameters of Freundlich equations for the adsorption data of three soils

Site	a	b	R^2
Typic Ustochrepts	0.4253	1.1681	0.96*
Typic Halorthids	0.2732	0.9731	0.98*
Typic Camborthids	0.345	1.024	0.95*

*Significant at $P=0.01$

Table 24.3 Model based K rates[a] for the four soils against different soil solution levels

Target soil solution K level	Typic Ustochrepts	Typic Halorthids	Typic Camborthids
(mg L^{-1})	K rate kg ha^{-1}		
0	Control	Control	Control
3	19	11	14
6	43	21	28
9	69	32	42
12	97	42	56
15	126	52	71
18	156	62	85
21	187	73	100
24	218	83	115
27	250	93	129

[a]Rates calculated with Freundlich model

i.e. 250 kg ha^{-1} . The other two soils had almost similar clay contents and other properties but differed only in available K. This factor again leads to varied fertilizer requirement to maintain the same soil solution level.

7.2 Maize Fodder Yield and K Concentration

The model based K rates applied to first and second maize fodder crops (Fig. 24.1), revealed that interaction was non-significant with respect to yield, dry matter and crude protein for first and second crop, therefore the data of two crops was pooled.

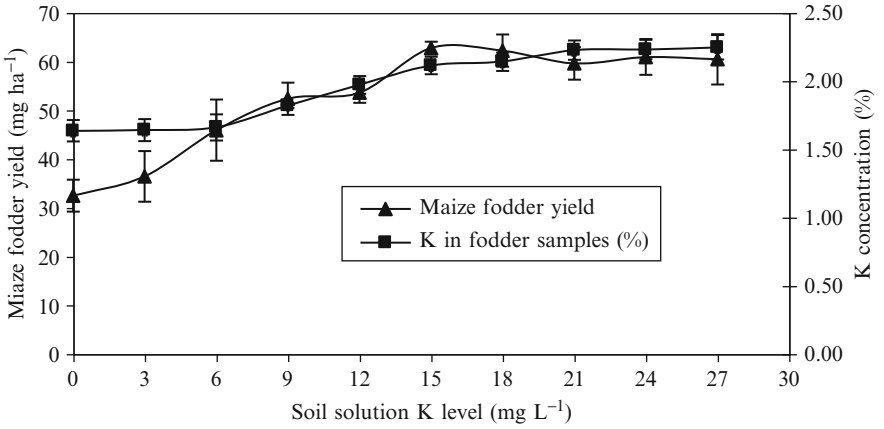

Fig. 24.1 Maize fodder yield and K concentration in plant samples as affected by various soil solution K levels

Green fodder yield increased significantly ($P<0.05$) in response to K application attaining maximum yield of 62.76 Mg ha^{-1} with T_6 where K was applied at 144 (average of 145–152) kg ha^{-1}. Adjusted soil solution K level for maximum yield was 15 mg L^{-1}. This treatment resulted in 47.99% higher green fodder yield as compared to control. Further increase of K rate could not improve maize green fodder yield and treatments remained statistically at par. A closer look on the data indicated that green fodder yield was over 60 Mg ha^{-1} with T_6 to T_{10} where corresponding K fertilizer rates were 145–192 kg ha^{-1}. This increase in fodder yield with K is a big achievement in a country where average green fodder yield is very low i.e. 22 Mg ha^{-1} (Economic Survey of Pakistan 2007–2008), and the existing livestock is facing 54% fodder shortage at national level.

Response of maize fodder to K application was found linear (Fig. 24.1), in which K concentration increased from 1.64% (please check) to the level best of 2.20% on highest K rate of 206 kg ha^{-1}. K concentration was 2.04% in T_6 when K was used at 152 kg ha^{-1}, coinciding with the maximum fodder yield (62.76 Mg ha^{-1}). From T_7 to T_{10} fodder yield remained statistically at par but dry matter content decreased from 26.87% (T_7) to 26.11% (T_{10}) when K concentration increased from 2.07% to 2.20%. Potassium is known to support plant turgidity in the guard cells surrounding stomata followed by an influx of K that reduces the aperture of the stomata, thus contributing to the regulation of water uptake and rate of transpiration; this factor might explain the fact of stagnant green fodder yield (T_7 to T_{10}) and decreasing trend of dry matter content with increasing level of K concentration in plant samples. The results are also comparable to the findings of Vicente-Chandler et al. (1962) who described that maximum dry matter yield of maize fodder was observed at 450 kg K ha^{-1} year^{-1} but K uptake continued to increase up to 900 kg ha^{-1} year^{-1} indicating luxury consumption which is not desirable as livestock particularly cattle need K in low concentration (NRC 1984). Luxury consumption of K may be avoided by split application yearly. McLeod (1965) however reported that K uptake increased from 0.8% to 3.4%

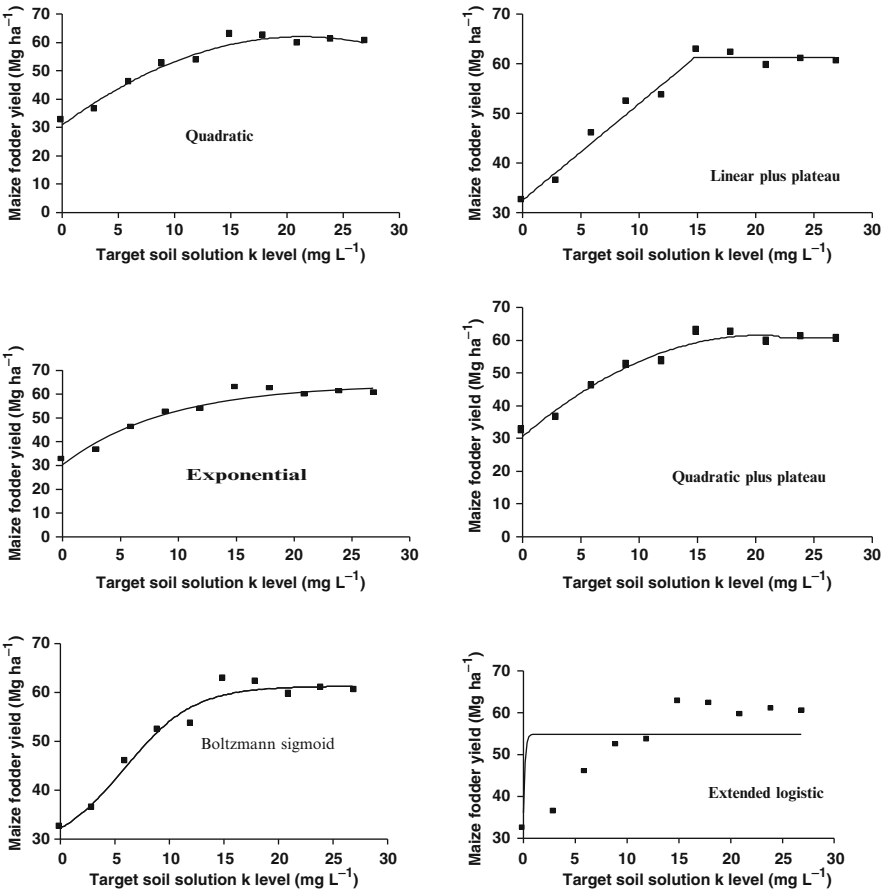

Fig. 24.2 Evaluation of maize fodder yield by different yield response models

with increased K rate but it could not affect forage yield. In addition to K supply the relatively high forage yields of the fertilized maize fodder crops were due to the factors like adequate supply of good quality canal water throughout the growing season, optimum supply of other plant nutrients, favorable temperature etc. As soil was deficient in available K status, the response of maize to K fertilization was anticipated.

7.3 Evaluation of Yield Response Models to Predict Optimum K Rate

In order to find out optimum K rate, maize fodder yield data was evaluated by various yield response models (Fig. 24.2 and Table 24.4). The optimum K rates varied greatly among the tested models. Slight difference among all models' R^2 was

Table 24.4 Optimum soil solution K levels predicted by various models

Model name	Optimum soil solution K level (mg L^{-1})	Coefficient of determination (R^2)	Coefficients of equations		
			a	b	c
Quadratic	21.33	0.97**	30.80 (1.77)	2.92 (0.30)	−0.068 (0.010)
Boltzmann sigmoid	18.50	0.97**	27.58(6.68)	61.27 (1.28)	505 (201)
Linear plus plateau	14.50	0.98**	1.94 (0.20)	32.49 (1.43)	61.19 (0.83)
Exponential	20.50	0.95**	–	5.74 (1.76)	0.11 (0.030)
Extended logistic	[a]	0.40 ns	−0.65 (0.77)	7.30 (5.16×10^8)	–
Quadratic plus plateau	18.50	0.97**	30.51 (2.01)	3.03 (0.045)	−0.07 (0.02)

*Values in parenthesis are standard error of estimate (significant at $P = 0.05$)
[a]Predicted rate was less than the tested minimum K rate

observed ($R^2 \geq 0.92$**) with the exception of extended logistic model (Table 24.4). With similar R^2 values, however, large variations in calculated optimum K rates were obtained. The coefficient of determination therefore should not be used alone to find out optimal K rate. This matches the findings of Colwell (1994). Although models differed in their estimated standard error, yet linear plus plateau model expressed less biasness with respect to R^2 and S.E., therefore it was used for optimum K rate calculation in all other parameters.

7.4 Effect of K Rate on Dry Matter (DM) and Crude Protein

The DM increased to the plateau level of 26.87% with T_7 (160 kg K ha^{-1}) (Fig. 24.3) but it then decreased thereafter possibly due to dilution effect in both the crops. The DM content (%) was slightly higher in second than that of first crop. This might be the result of elevated residual K coupled with higher K fertilizer rate, which goes against the findings of Lioveras et al. (2001) who observed no significant effect of different K fertilizer rate (0, 42, 83, 166 and 332 kg ha^{-1}) on alfalfa DM yield under Mediterranean climate. Pravinchandra and Kotecha (2006), however, reported an enhancement in DM to the tune of 13.3% at 300 kg K ha^{-1}.

Crude protein (CP) is a key nutrition that must be considered in adequate amounts in various animal diets. Crude protein varied from 9.37% (T_1) to statistically significant level of 12.06% with T_9 while it declined to 12.03% in T_{10}. Increase upto 22.28% higher than control was observed with T_9 where 169 kg K ha^{-1} was added equivalent to solution K level of 24 mg L^{-1} (Fig. 24.3). CP concentration with both T_9 and T_{10} approached 12%, which is acceptable limit for dry cow rations (Thomas et al. 1998). For maize fodder, the plateau yield of CP to K rate was agreed with

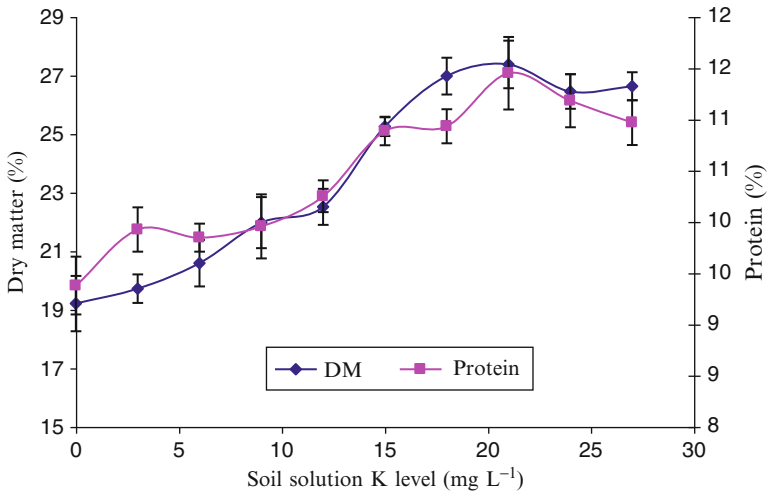

Fig. 24.3 Dry matter and crude protein content as affected by various soil solution K levels

work of Pravinchandra and Kotecha (2006) who reported 17.6% higher CP in Lucerne when 300 kg K ha^{-1} was used.

7.5 Optimum Soil K Solution Level for Maximum Output

For maximum green fodder yield a target soil solution K level required was found to be 14.50 mg L^{-1} (Table 24.5) which could be adjusted by adding 144 kg K ha^{-1} and predicted yield corresponding to 14.50 mg L^{-1} level was 61.19 Mg ha^{-1}. As no significant variation was observed regarding dry matter and crude protein contents both for soil solution and K rate, these were pooled together for the sake of convenience. To get maximum output with respect to dry matter and crude protein, a soil solution level of 20 mg L^{-1} would be sufficient and its equivalent K rate was 165 kg ha^{-1}. A different K rate is required for yield and quality parameters (dry matter and crude protein contents).

8 Conclusions

1. K fertilizer application based on soil solution level worked out through adsorption model provided the basis in resolving sporadic K response of crops on soils of mixed mineralogy as adsorption based K rates were different for the four selected soils
2. Linear plus plateau model predicted economic K rate with greater accuracy
3. Owing to the high K fertilizer prices, integrated approach of using adsorption and yield response models proved very useful in optimizing K fertilizer application

Table 24.5 Optimum Soil solution K levels for maximizing various maize fodder quality parameters

Variables	Soil solution K level (mg L^{-1})	Optimum K rate (kg ha^{-1})[a]	R^2	Predicted value*
Maize fodder yield (Mg ha^{-1})	14.50[a]	144[b]	0.98*	61.19[c]
Dry matter (%)	19.25[a]	164[b]	0.98*	26.83[c]
Crude protein content (%)	20.75[a]	167[b]	0.95*	11.19[c]
Cumulative average of dry matter and protein content	20	165	–	–

*Significant at $P = 0.01$

[a]Average soil solution level for fodder yield, dry matter and crude protein content using adsorption model

[b]Values determined by linear plus plateau yield response model

[c]Predicted yielded parameters

in alkaline calcareous soils, for instance it required only 144 kg ha^{-1} (soil solution level of 14.50 mg KL^{-1}) to maximize fodder yield, however, considerably higher rate would be needed to harvest crop with maize fodder of best quality in terms of dry matter and crude protein, (soil solution level vary from 19.25 and 20.75 mg L^{-1})

4. The growers would have to decide one option out of quality or quantity depending upon their marketing system because one cannot have both maximum quantity and supreme quality at the same time.

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Chapter 25
Optimal Supply of Micronutrients Improves Drought Tolerance in Legumes

Muhammad Yasin Ashraf, Khalid Mahmood, Muhammad Ashraf, Javed Akhter, and Faqir Hussain

Contents

Abstract Legumes are of considerable importance for providing food and feed world over. In comparison to cereal grains, legume seeds are rich in protein and thus provide highly nutritive food. Legumes are grown on a wide range of soils varying in texture and fertility. Most of the soils of arid and semi-arid regions, being low in soil moisture content, are also low in fertility. So to maximize plant productivity, proper supply of macro- and micro-nutrients to crops is essential. As a general practice, optimal supply of macronutrients to crops is usually ensured but that of

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micronutrients is ignored. In view of a plethora of literature, it is now well established that application of micronutrients is effective in alleviating the adverse effects of abiotic stresses such as salinity and drought. The involvement of micronutrients in different physiological and biochemical activities of the legume plants is well documented because correlations between micronutrient supply and crop growth and productivity have been often observed. Use of micronutrients like zinc (Zn), iron (Fe), boron (B), manganese (Mn), molybdenum (Mo), copper (Cu), cobalt (Co) and nickel (Ni) has now become a common practice to increase crop yield especially under adverse environmental conditions. Plants deficient in micronutrients may become susceptible to diseases and abiotic stresses. Rapid leaching of acids in sandy soils tends to produce a deficiency of tightly held nutrients such as Zn, Fe, Cu or B. Therefore, problem soils such as acid, alkaline or sandy soils are often deficient in one or more micronutrient elements. Micronutrient application not only improves the stress tolerance potential indirectly (because micronutrients deficient plants exhibit an impaired defense response) but also results in improving a number of metabolic phenomena. Thus, application of micronutrients as foliar or soil amendment is recommended to achieve optimum crop productivity from the soils having inherent micronutrient deficiency and low moisture contents. In this chapter, the role of micronutrient management in improving the drought tolerance potential and productivity of legumes is reviewed and critically discussed.

Keywords Micronutrients • Legumes • Drought tolerance • Metabolic changes and plant productivity

1 Introduction

Economically effective and sustainable modern agriculture requires high yielding crops necessary to get reasonable profit so as to meet the high demand for food of the growing population of the world. Although scientists have developed high yielding crop varieties, deficiency of food is still there, which is attributed to unavailability of sufficient irrigation water for crops. Water shortage is a worldwide problem, which affects the growth and productivity of crops through reducing the availability of macro- and micro-nutrients necessary for photosynthesis and other metabolic phenomena (Mahajan and Tuteja 2005; Miletic et al. 2010). Mineral nutrients play an active role in minimizing the photo-oxidative damages caused by generation of reactive oxygen species (ROS) in plants growing under drought conditions (Cakmak 2005; Wang et al. 2005). Therefore, limited supply of water adversely affects plant growth and metabolic processes of the plants (Ashraf et al. 1998) and proper application of macro- and micro-nutrients leads to higher plant productivity under drought conditions (Hussain et al. 2004). Many reports are available which prove strong relationships between micronutrient availability, plant growth and productivity, and recommend proper supply of micronutrients such as zinc (Zn), manganese (Mn), copper (Cu), molybdenum (Mo), iron (Fe), and boron (B) (Mann

et al. 2002; Bhadoria et al. 2003; Rashid and Ryan 2004; Welch and Graham 2004; Gupta 2005; He et al. 2005; Liang et al. 2007; Waraich et al. 2011). Several studies have shown that proper supply of micro-nutrients to crops resulted in more vigorous seedlings, lower vulnerability to plant diseases, and improved crop drought resistance/tolerance (Frossard et al. 2000; Bouis 2003; Welch and Graham 2004). Introduction of high yielding crop varieties and liberal applications of irrigation water and fertilizers containing major nutrients (NPK), have increased the prevalence of micro-nutrient deficiencies in soils, and consequently, in human nutrition (Imtiaz et al. 2010).

In Pakistan, 22 Mha of cultivated area comprises predominantly alluvial soils which are alkaline, calcareous and deficient in micronutrients and organic matter (Imtiaz et al. 2010). Due to calcareous nature and high pH of soils, nutrients are fixed and become less available to plants. The most widespread deficiency is of Zn which is observed in 70% of the soils of Pakistan. Boron deficiency is another major nutritional disorder which severely affects growth and crop productivity in Pakistan (Rashid 2005). The third commonly prevalent disorder is Fe chlorosis which has been recorded in peanut, chickpea, mungbean and other legumes (Rourke et al. 2007). Copper and Mn deficiencies are also observed in some areas of Pakistan but the problem is of localized nature (Rashid 2005; Imtiaz et al. 2010).

Leguminous crops are grown all over the world for food (Franca et al. 2000). They contain considerable amount of protein, starch, oils and vitamins necessary for human growth and health. Legumes are mostly grown in the arid and semi-arid regions, where availability of water for crops is limited and yield losses due to water stress are very high (Farias-Rodriguez et al. 1998). Many reports (e.g. El-Hamdaoui et al. 2003; Ashraf and Iram 2005; Arrese-Igor et al. 2011) have shown that nodulation and nitrogen fixation of the legumes were severely affected by the water stress. Photosynthesis and stomatal conductance were also reduced due to the drought stress resulting in decreased biomass and productivity of legumes (Flexas et al. 2002; Taiz and Zeiger 2002; Purcell 2009). This chapter discusses the influence of micronutrients, particularly Zn, Fe, B, Mn, Cu, Mo, Co and Ni, on improvement in drought tolerance and productivity of plants for sustainable agriculture in drought-prone areas of the world.

2 Zinc

Zinc (Zn) is the most deficient micronutrient in the soils of many countries including Bangladesh, India, Nepal and Pakistan (Nayyar et al. 2001; Imtiaz et al. 2010), so its application either as foliar or soil amendment is recommended. Foliar application of Zn could be of great importance to plants grown under sandy soil conditions as most sandy soils suffer from micronutrients deficiency particularly that of Zn. Sandy soils in many semi-arid regions are known to limit mobility and availability of soil-Zn to plant roots (Marschner 1993; Imtiaz et al. 2006). Foliar application with Zn showed a significant effect on growth, yield, and yield components and seed quality of groundnut under sandy soil conditions (Gobarah et al. 2006).

Earlier, Karaman et al. (1999) reported that biomass production increased with increasing Zn concentrations applied to bean (*Phaseolus vulgaris* L.) plants. Foliar application of Zn or urea and their combination on mungbean improved the plant height by producing the tallest plants and increased the number of branches per plant as a result of which yield per plant improved (Abd-El-Lateef et al. 1998; Malakouti 2007; Rajaiea et al. 2009). Krishna (1995) also found that foliar application of Zn significantly enhanced the dry biomass, seed and straw yield, and seed protein contents in mungbean.

Foliar application of Zn showed a positive effect on growth, yield and yield components in mungbean grown under stress environments (Thalooth et al. 2006). Kassab (2005) indicated that foliar application of Zn, Mg, Mn and Fe significantly increased growth, yield and yield components of mungbean plants grown in soils with low moisture contents. Under arid condition, foliar application of Zn was effective in enhancing the growth and yield of mungbean (Gupta et al. 2003; Kassab 2005). Foliar application of Zn and K was found effective in improving the leaf area, number of leaves, biomass, number of pods and seed weight in mungbean grown in soils having limited moisture contents (Thalooth et al. 2006). Earlier, Basole et al. (2003) conducted an experiment on hormones and nutrient management of soybean and recorded a significant improvement in growth and yield of soybean when treated with Zn or Zn + hormones. Ali and Mowafy (2003) reported that application of Zn (2%) as foliar spray improved yield and seed quality of peanut when grown in sandy soils. Similarly, there are many reports which indicated that foliar application of Zn had a significant effect on growth, yield and seed quality of groundnut under sandy soil conditions (Gobarah et al. 2006; El-Tohamy and El-Greadly 2007). The above discussed findings of different workers clearly indicate that proper application of Zn to either soil or foliage is effective in enhancing the stress tolerance potential in different legume crops as a result of which enhanced crop yield can be achieved. The recommendations of various workers regarding Zn application in legumes to obtain optimal crop productivity are summarized in Table 25.1.

3 Iron

Iron (Fe) is essential micronutrient for a number of key enzymes of the nitrogenase complex in legume plants, and also for the electron carrier, i.e. ferredoxin (Rashid 2005; Imtiaz et al. 2010). Legumes also have a relatively higher iron requirement for the synthesis of heme, the component of haemoglobin, and nodule formation. Under iron deficiency, nodule mass, leghaemoglobin content, number of bacteroids and nitrogenase activity are drastically reduced (Tang et al. 1992; Scherer et al. 2008). Under water deficit conditions, iron deficiency caused chlorosis in peanut and nodule formation also failed until adequate iron supply through foliar applications. Parkpian and Boonkerd (1989) reported that nodule development in groundnut was decreased under iron deficiency in black calcareous soils. However, population of bacteria (*Bradyrhizobium*) in the soil and rhizosphere was not

Table 25.1 Recommendations and impact of Zn application on improving growth, yield and other parameters of legumes under drought conditions

Legume species	Recommended rate of Zn	Effect of Zn fertilizers	Reference
Chickpea (*Cicer arietinum*)	2.5 μg Zn g^{-1} soil	Improved biomass production, water relations and osmotic adjustment	Khan et al. (2004)
Snap bean (*Phaseolus vulgaris*)	Zn (chelated form 13%), 0.3–0.5 g L^{-1} foliar application	Increased chlorophyll and carbohydrate contents in pods and reduced fibers	El-Tohamy and El-Greadly (2007)
Lentil (*Lens culinaris*) and chickpea (*Cicer arietinum*)	12–15 kg $ZnSO_4$ ha^{-1} through soil	Improved growth and yield	Ramakrishna et al. (2000)
Lentil (*Lens culinaris*) and chickpea (*Cicer arietinum*)	25 kg $ZnSO_4$ ha^{-1} through soil	Improved nodulation, root growth, yield and uptake of Zn, B, Fe and P	
Mungbean (*Vigna radiata*)	0.5% Fe, Zn and Mn applied as foliar spray	Improved chlorosis caused by abiotic stress and increased number of pods and seed yield	Unkasem and Tawonsok (1988)
Mungbean (*Vigna radiata*)	300 mg kg^{-1} Zn-EDTA as foliar spray	Improved yield and yield components	Thalooth et al. (2006)
Groundnut (*Arachis hypogaea*)	Foliar spraying with Zn, tap water (control), 0.50, 0.75 and 1.00 g Zn L^{-1}	Increased oil percentage, protein and seed yield	Gobarah et al. (2006)
Groundnut (*Arachis hypogaea*)	1,000 mg L^{-1} $ZnSO_4$ as foliar spray	Increased oil and protein contents in seeds and improved overall yield	Darwish et al. (2002)

influenced by Fe deficiency and root infection by *Bradyrhizobium* was also not affected. Foliar application of Fe resulted in greater number of nodules and thus increased the nodule mass in plants. As a result of Fe application, nodules in sprayed plants contained 200 times higher bacteroids per unit weight, and 14 times higher leghaemoglobin content. Deficiency of Fe delayed the nitrogenase activity in all the tested groundnut varieties (Marschner 2011).

Iron deficiency in crop plants can be overcome commonly by foliar application of inorganic salts or chelated compounds of Fe. Five foliar applications of 0.5% $FeSO_4$ solution at 10-day intervals starting at 10 days after emergence was found a very effective measure in alleviating iron chlorosis in peanut plants with low moisture contents, however, yield was also substantially improved (Ratanarat et al. 1990). Chlorotic symptoms are sometimes commonly observed in mungbean cultivars grown on calcareous soils. Mungbean plants readily recovered from chlorosis after a foliar application of nutrient solution containing 0.5% Fe, Zn and Mn and also produced greater number of pods (Unkasem and Tawonsook 1988). The method of foliar applications has the drawback that translocation of applied Fe within the plant is poor. The translocation rate is species-dependent and hardly exceeds 50% of the Fe applied to a given leaf or leaflet. Under field conditions, spraying is often required at frequent intervals to provide adequate Fe to the developing canopy, because the Fe translocation from previously treated areas is insufficient (Chen and Barak 1982). Mahmoudi et al. (2005) conducted studies with lentil and chickpea in solution culture experiment to examine the influence of iron on growth and chlorosis caused due to Fe deficiency. They reported a significant improvement in growth and reduction in chlorosis by the application of 30 μM Fe in both legumes. However, the effect was more pronounced in chickpea than in lentil. Production of legumes especially groundnut was reduced due to the Fe deficiency when grown on black calcareous soils of Thailand. Although these are considered fertile soils, due to $CaCO_3$ and alkaline pH, deficiency of Fe may result in legume crops (Osotsapar 2000). Therefore, all the earlier mentioned studies prove that the Fe-deficient or calcareous soils with high pH reduce the uptake of Fe which results in reduced crop photosynthesis, growth and yield. So, a proper supply of iron is necessary to have optimal legume crop yield from soils with low moisture contents (Table 25.2).

4 Boron

Boron (B) is an essential micronutrient for plants because it is involved in the activation of enzymes necessary for starch synthesis and hence for cellulose production (Blevins and Lukaszewski 1998; El-Hamdaoui et al. 2003; Hänsch and Mendel 2009). It plays an active role in the transport of sugars from the site of synthesis to meristem regions of stem and roots (Nable et al. 1997; Bolaños et al. 2006). So, the transport of sugars is retarded in boron-deficient plants as a result of which plant growth is reduced (O'Neill et al. 2004). Boron is also involved in cell multiplication and nitrogen metabolism (Sinha and Chatterjee 2003). Nutrient

Table 25.2 Recommendations and impact of Fe application on improving growth, yield and other parameters of legumes under drought conditions

Legume species	Recommended rate of Fe	Effect of Fe fertilizers	Reference
Lentil (*Lens culinaris*)	0.5 kg $FeSO_4$ ha^{-1} through soil	Improved yield and nutrient uptake	Ramakrishna et al. (2000)
Lentil (*Lens culinaris*) Chickpea (*Cicer arietinum*)	Application in growth medium, 30 μM Fe	Decreased chlorosis and enhanced growth of both the legumes	Mahmoudi et al. (2005)
Mungbean (*Vigna radiata*)	0.5% Fe, Zn and Mn applied as foliar spray	Reduced chlorosis caused by water stress and increased number of pods and seed yield	Unkasem and Tawonsok (1988)
Groundnut (*Arachis hypogaea*)	Foliar spray of 0.1% aqueous solution of Fe, 2 kg Fe ha^{-1} as iron citrate or Iron sulfate 400 g ha^{-1}	Improved yield and yield components	Singh and Joshi (1997)
Groundnut (*Arachis hypogaea*)	Foliar spray of 0.5% $FeSO_4$	Alleviated chlorosis caused by water stress in plants and improved yields	Ratanarat et al. (1990)
Peanut (*Arachis hypogaea*)	Foliar application of 0.5% $FeSO_4$	Reduced chlorosis and increased growth	Parkpean et al. (1986)

uptake is generally reduced in soils having low moisture contents. Application of B activates the absorption of other nutrients, photosynthetic efficiency, phosphorus uptake, hormone synthesis and fat metabolism (Dell and Huang 1997; Tewari et al. 2009). All these metabolic processes are helpful in sugar transport throughout the plant body (Brown and Shelp 1997; Boaretto et al. 2008; Hänsch and Mendel 2009). Boron availability in soils varies and is related with the type and degree of weathering of the parent material. Sandy soils are often deficient in B while clay soils with high organic matter contain enough amount of B (Barber 1995). Crops suffer boron deficiency commonly during the periods of drought probably because the supply of B to the root is reduced as a result of reduction in water flow to the roots. Decrease in soil moisture may cause a proportionate reduction in the rate of B diffusion to the root (Barber 1995; Boaretto et al. 2008; Tewari et al. 2009). Boron deficiency in legumes is common under drought conditions. Rerkasem et al. (1993) reported that deficiency of B decreased the seed yield up to 45% in peanut, 60% in soybean and 93% in chickpea. They also noted the depression resembling to hollow heart on the inner surface of cotyledons of soybean and peanut seeds, which was 50% in peanut and 17% in soybean seeds while black gram seeds did not show these types of symptoms. Application of B significantly reduced or even eliminated these symptoms in some varieties of the above-mentioned legume species (Rerkasem et al. 1993; Marsalis et al. 2009).

Extensive studies were conducted on uncovering the effect of boron (B) on peanut and soybean on a Typic Tropaqualf in Northern Thailand. For a comparison, sunflower, green gram, black gram, wheat, and rice were also included in B rate trial. Boron omission induced the hollow heart symptom in 10% of peanut kernels; the incidence of symptoms was correlated with B contents of the affected kernels. On the other hand, omission of B did not affect the appearance of soybean seed or the grain yield of both crops (soybean and peanut). In the experiment on B application rate, omission of B reduced grain yield by 50% in sunflower and by 40–80% in black gram. B deficiency symptoms in green gram and the hollow heart symptom in peanut kernels were observed in the B omitted treatment, but the grain yield of soybean, peanuts, rice, or wheat was not affected. Reduction in grain yield in black gram and green gram under B deficiency was apparently due to delay or inhibition of reproductive development and thus reduced pod set (Rerkasem et al. 1993; Osotsapar 2000).

Keerati-Kasikorn et al. (1987) reported boron deficiency in 28% of peanut crops studies in dry season in Khon Kaen province of Thailand. Hollow heart symptom was recorded in about 50% seeds of the B deficient crops during the dry season and 30% of seeds in wet season. Ninety-five percent of normal seeds had higher B content (>12 μg B g^{-1}), whereas 70% of the seeds with hollow heart had lower B content. The average B content of normal peanuts was 16.4 μg B g^{-1} compared with the seeds with hollow heart having 10.6 μg B g^{-1}. A comparative study on the susceptibility to B deficiency of various soybean, peanut and black gram cultivars indicated a wide

range of variation related to the genotypic response. In B-deficient soils, depression in seed yield was 30–60% in soybean, 45% in peanut and 93% in black gram. Boron deficiency also induced a localized depression on the internal surface of one or both cotyledons of some soybean seeds, resembling the symptom of hollow heart in peanut seeds. It induced 50% hollow heart in peanut, 17% in soybean, while black gram seeds had no symptoms. The addition of boron reduced or eliminated the symptoms (Rerkasem et al. 1993; Marsalis et al. 2009).

El-Hamdaoui et al. (2003) investigated the effect of different levels of B (from 9.3 to 93 μM B) and Ca (from 0.68 to 5.44 mM Ca) on growth, nitrogen fixation, and mineral composition of pea (*Pisum sativum* L., cv. Argona) grown in saline conditions. Addition of B and Ca in the growth medium alleviated the adverse effects of salinity (75 mM NaCl) on plant growth, nodulation and nitrogen fixation. The increase in salt tolerance of symbiotic plants mediated by B and Ca can be correlated with the availability of nutrients because both B and Ca contents reduced in shoots and in nodulated roots due to salinity. Increase in K and Fe was also recorded in shoot and root of pea plants due to addition of B and Ca. So, application of B and Ca is beneficial in improving the growth and nutrient uptake in pea under saline conditions. Recommendations of different workers regarding B for enhancing crop yield and stress tolerance potential in different legumes are summarized in Table 25.3.

5 Manganese

Manganese (Mn) is a very important micronutrient because it catalyses various enzymatic activities and plays an active role in electron transport. Supplemental Mn stimulates the N_2-fixation in soybean under water deficit conditions (Purcell et al. 2000; Vadez et al. 2000; Sinclair et al. 2003; Khoshgoftarmanesh et al. 2010). It is also involved in antioxidative metabolism (Polle et al. 1992; Khoshgoftarmanesh et al. 2007). Manganese deficiency is very common in arid or semi-arid soils, and the cultivation of leguminous crops is severely affected on these soils. However, Mn supplementation is effective in improving crop yield (Purcell et al. 2000) through regulating nitrogen fixation because Mn is also important in the ureide degradation in leaves of soybean. Increases in the N_2-fixation by Mn application in soybean under water deficit was also reported by Vadez et al. (2000) and Sinclair et al. (2003). Drought sensitive cultivars required higher amount of Mn than that did the tolerant ones (Todd et al. 2006; Khoshgoftarmanesh et al. 2007). In a drought sensitive soybean cultivar, xylem flow reduced due to low water potential resulting in decrease in Mn delivery in leaves necessary for degradation of ureide enzymes as a result of which ureides build-up takes place. Use of Mn for effectively enhancing growth and yield of leguminous crops suggested by various researchers is summarized in Table 25.4.

Table 25.3 Recommendations and impact of B application on improving growth, yield and other parameters of legumes under drought conditions

Legume species	Recommended rate of B	Effect of B fertilizers	Reference
Peanut (*Arachis hypogaea*)	Soil application 0.25–0.50 kg B ha^{-1}	Increased pod and seed number as well as seed yield per plant	Keerati-Kasikorn et al. (1987)
Lentil (*Lens culinaris*) and chickpea (*Cicer arietinum*)	0.5–1.0 kg B ha^{-1} as soil application	Improved yield and nutrient uptake	Ramakrishna et al. (2000)
Black gram (*Vigna mungo*)	Foliar spray of 50 g ha^{-1} borax at the time of flowering and at pod setting	Improved seed size and yield	Rerkasem (1989)
Green gram (*Vigna radiata*) and black gram (*Vigna mungo*)	Soil application of 4–32 kg ha^{-1} borax in case of green gram and 4–16 kg ha^{-1} borax in black gram	Increased seed yield	Rerkasem et al. (1990)
Pea (*Pisum sativum* cv. Argona)	Foliar spray of 55.8 μM B	Increased number of nodules and nitrogen fixation in pea under water deficit conditions induced by salinity	El-Hamdaoui et al. (2003)
Pea (*Pisum sativum*)	9.3–93 μM B	Improved germination and seedling development	Bonilla et al. (2004)
Pea (*Pisum sativum*)	Foliar spray of 55.8 μM B	Improved nitrogen fixation and Fe uptake	Bolaños et al. (2006)
Soybean (*Glycine max*)	Foliar application of 30 μM B	Improved growth, biomass, pod and seed yield, concentration of boron and reduced ribonuclease peroxidase, acid phosphatase and starch phosphorylase activities	Sinha and Chatterjee (2003)

Table 25.4 Recommendations and impact of Mn and Mo application on improving growth, yield and other parameters of legumes under drought conditions

Legume species	Recommended rates of Mn or Mo	Effect of micronutrient fertilizers	Reference
Soybean (*Glycine max*)	Foliar application of 52.8 mM Mn	Improved growth and nitrogen fixation under water stress conditions	Vadez et al. (2000)
Soybean (*Glycine max*)	Addition of 90 μM Mn in nutrient solution	Decreased ureides accumulation and improved growth and N_2-fixation	Izaguirre-Mayoral and Sinclair (2005)
Faba bean (*Vicia faba*)	Foliar application of 0.1% Mn	Improved growth and productivity under adverse conditions created by NaCl salinity	El-Fouly et al. (2001)
Soybean (*Glycine max*)	Soil application of 390–780 g Mo ha^{-1}	Increased nodulation and number of effective nodules per plant	Tenywa (1997)
Cowpea (*Vigna unguiculata*) Bambara groundnut (*Vigna subterranean*) Groundnut (*Arachis hypogaea*)	Soil application of 0.5 kg Mo ha^{-1}	Increased nitrogen fixation	Yakubu et al. (2010)
Common bean (*Phaseolus vulgaris*)	Inoculation with 12 g Mo kg^{-1} of seeds	Improved number of pods per plant, number of seeds per plant, 100-seed weight and seed yield	Bambara and Ndakidemi (2010)
Groundnut (*Arachis hypogaea*)	Foliar application of 100 g Mo ha^{-1} as MoO_3	Increased number of nodules, nodule weight and nitrogenase activity	Hafner et al. (1992)

6 Molybdenum

Molybdenum (Mo) regulates the enzymatic action in oxidation reduction reactions in plants (Mendel and Hansch 2002; Tan et al. 2010). It is also involved in N_2-fixation in leguminous plants (Williams and Frausto da Silva 2002). Molybdenum deficiency resulted in poor growth and decrease in chlorophyll content in plants (Marschner 1995; Kaiser et al. 2005). Application of Mo in deficient soils improved the nodulation and N_2-fixation by enhancing the nitrogenase activities (Adams 1997; Bambara and Ndakidemi 2010). So, deficiency of Mo resulted in deficiency of N which can be observed from the N deficiency symptoms and application of NH_4 or Mo is beneficial to regulate all the above mentioned metabolic activities. Similarly, supplemental application of Mo is necessary for active nodule system and optimal plant growth and productivity. Chongpraditnun et al. (1997) reported that the foliar application of Mn increased Mo uptake and translocation by reducing the adsorption of Mo to the surface of cell wall or cell membrane of leaves which may increase the movement of Mo into the phloem. Foliar application of ammonium molybdate significantly increased biomass and N_2-fixation in clover (El-Bably 2002) and in cowpea (Ndakidemi 2005). Molybdenum is an essential component of two major enzymes, nitrogenase and nitrate reductase in plants (Beevers and Hagenman 1983; Khan et al. 1990; Ashraf et al. 1995; Westermann 2005). Nitrogen fixation is regulated by nitrogenase which also helps the bacteria in fixing N_2 (Mengel and Kirkby 1987; Thibaund 2005; Westermann 2005) and for these activities Mo is necessary. Hafner et al. (1992) reported that Mo application increased the growth and symbiotic N_2-fixation in groundnut (*Arachis hypogaea* L.). Application of fertilizer containing 0.1% Mo was effective in enhancing the pod yield from 37% to 86% and total N from 53 to 108 kg N ha^{-1}. Foliar spray of Mo (100 g Mo ha^{-1} as MoO_3) increased nitrogenase activity by 2–4 times as compared to non-sprayed control plants. The increase in nitrogenase activity was mainly due to increase in nodule dry weight and to a lesser extent to increase in specific nitrogenase activity per unit nodule dry weight. The recommendations of various workers regarding Mo application through soil or as foliar spray are given in Table 25.4.

7 Copper

Calcareous or sandy soils are usually deficient in copper (Cu) because they have low moisture contents (Aref 2011). As most of the plants require very low amount of Cu, soil scientists do not recommend its application which may be the reason of its deficiency. The effect of Cu deficiency on crops is a significant reduction in yield and sometimes complete failure of a crop (Nascimento et al. 2003). However, copper application resulted in yield increase under stress environments (Khoshgoftar and Hajimozaffari 2006; Khoshgoftarmanesh et al. 2007). It was recorded that 53–62% of Cu uptake in some legumes is due to uptake by mycorrhizal hyphae (Barber 1995).

Peanut plants have been reported to depend on vesicular abuscular mycorrhizae for Cu supply in degraded soils (Bell et al. 1990; Quilambo 2003). Its concentration, 1–1.5 mg kg^{-1} (dry weight basis), in the shoot tips promotes vegetative growth and yield of peanut (Bell et al. 1990). Higher rates of phosphate fertilizer application may enhance the Cu deficiency (Tiaranan et al. 1985). Marked reduction in nodule development and N_2-fixation was recorded in the Cu-deficient legumes (Snowball and Robson 1980). It was also observed that foliar application of Cu increased dry matter yield and water use efficiency, respectively (El-Bably 2002). The level of Cu in soil solution decreases with increasing pH due to stronger adsorption (Reddy et al. 1995; Georgaka and Spanos 2010). If the deficiency is due to pH imbalance, addition of micronutrients can adversely affect the plant and metabolic activities because the level of individual micronutrients may affect the level of other micronutrients in the plant through antagonism. To get benefits from Cu application, recommendations are given in Table 25.5. On the other hand, toxicity can occur when micronutrients such as Cu are applied in excess.

8 Cobalt

Cobalt (Co) is considered to be very essential element for legumes because its proper supply is necessary for nodule formation and N_2-fixation processes (Bonilla and Bolaños 2010). Bolacer et al. (2003) reported its active role in the symbiotic N_2-fixation mechanism. The presence of cobamide coenzymes in the nodules of many legumes and nitrogen fixing non-legumes also confirmed its involvement in N_2-fixation (Gad 2006). Earlier, Castro et al. (1997) reported that *Phaseolus* seeds treated with Co showed a significant effect on nodulation, dry weight, physiological quality, vigour, protein and nutrient content of seeds. Due et al. (1999) and Tenywa (2003) observed that Co application enhanced nodulation and number of effective nodules in soybean plants and N contents in shoots. Sharma and Bhandari (2002) showed that seed inoculation with *Bradyrhizobium*, farmyard manure (FYM) and minerals significantly increased the number of nodules, their fresh weight, leghaemoglobin content of nodules and chlorophyll content in cowpea plants. The highest performance of all these parameters was recorded in Co alone or Co + FYM treatments. Abd El-Moez and Gad (2002) reported that the addition of Co along with inorganic and organic fertilizers increased the uptake of macro- and micro-nutrients in cowpea plants. Howell and Skoog (1955) observed that Co promoted stem coleoptile elongation, opening of hypocotyle hooks, and leaf expansion and bud development in peas. Gad (2006) conducted pot and field experiments to examine the effect of Co on growth and nutrient uptake in pea plants and noted that application of Co (8 mg L^{-1}) significantly enhanced the plant height, number of branches and leaves, leaf area, stem diameter, and root length. Cobalt application improved nodule formation process and increased the efficiency of organic and inorganic nitrogen fertilizers (urea and ammonium nitrate) and peanut compost in pea plants (Tenywa 2003). Addition of Co in the growth media significantly improved nutrients

Table 25.5 Recommendations and effects of Cu, Co and Ni application on improving growth, yield and other parameters of legumes under drought conditions

Legumes species	Recommended rate of micronutrients	Effect of micronutrients fertilizers	Reference
Peanut (*Arachis hypogaea*)	20 kg $CuSO_4$ ha^{-1}	Total dry matter and seed yields were greatly improved	Chew et al. (1979)
Peanut (*Arachis hypogaea*)	40 kg $CuSO_4$ ha^{-1}	Increased pod yield , haulm yield and oil yield	Desai (1994)
Soybean (*Glycine max*)	Copper ore tailings 600 g ha^{-1}	Increased seed and oil yield and uptake of all nutrients	Virupaksh (1995)
Chickpea (*Cicer arietinum*)	Copper ore tailings 600 g ha^{-1}	Improved chickpea grain yield and nutrient contents/uptake	Jadi (1997)
Soybean (*Glycine max*)	454 g Co ha^{-1}	Increased nodulation and number of effective nodules per plant	Tenywa (1997)
Pea (*Pisum sativum*)	8 mg Co L^{-1} through fertigation	Increased N fertilizer use efficiency, growth, biomass and pod size and seed yield	Gad (2006)
Peanut (*Arachis hypogaea*)	50 mg Co kg^{-1} of soil	Altered the activities of antioxidant enzymes i.e. catalase, peroxidase and polyphenol oxidase	Jaleel et al. (2008)
Groundnut (*Arachis hypogaea*)	Soil application, 0.21 kg Co ha^{-1}	Increased 10% kernel yield	Basu and Bhodaria (2008)
Soybean (*Glycine max*)	Fertigation with 5–8 mM Ni	Increased growth, urease and hydrogenase activities under Ni deficient environments	Klucas et al. (1983)
Soybean (*Glycine max*.)	Ni 1 μg L^{-1} in nutrient solution	Improved growth, urease and hydrogenase activities under adverse environmental conditions	Eskew et al. (1983)

uptake in plants even under low moisture availability conditions. Its application can reduce the requirement of organic and inorganic fertilizers up to 67% and 25%, respectively, of the recommended doses. It also enhanced the fresh and dry weight of shoots and roots, nodule number and weight, uptake of N, P, K, Fe, Mn and Zn as well as yield, number of pods and seeds quality (Tenywa 2003). Recommendations regarding foliar or soil application of Co are summarized in Table 25.5.

9 Nickel

Nickel (Ni) is an essential element for several biological processes, like H_2 oxidation and urea hydrolysis in plants (Bromilow et al. 1993; Zahran 1999; Coutinho and Mazo 2005; Zobiole et al. 2010). Klucas et al. (1983) reported that the application of Ni @ 5 to 8 mM is effective in increasing the 7- to 10-fold urease activity in soybean grown with nitrate or symbiotically; it significantly increased the hydrogenase activity in leaves and isolated nodule bacteroids. Zobiole et al. (2010) also reported that free-living *Rhizobium japonicum*, cultured under chemolithotrophic conditions, needed Ni application for growth and activation of hydrogenase activity. Ni is essential micronutrient element for the proper growth of soybean plant and *R. japonicum* (Ureta et al. 2005). Eskew et al. (1983), Taiz and Zeiger (1998) and Ureta et al. (2005) reported that soybean plants deficient in Ni showed higher accumulation of urea and as a result necrotic lesions appeared on their leaflet tips. Nickel deficiency resulted in delayed nodulation and reduction in early growth. Application of Ni @ 1 μg L^{-1} to the nutrient media prevented urea accumulation, necrosis, and reduction in growth. This proved that Ni is essential for soybeans and possibly for all legumes. The available recommendations regarding Ni application for crop improvement are given in Table 25.5.

10 Conclusion

Micronutrients are essential for legumes grown in soils with low moisture contents. Their application is recommended for most legume species particularly to counteract deleterious effects of drought stress on growth, yield and metabolic activities. Application of micronutrients increases plant growth, nitrogen fixation, dry matter and seed yield of legumes. Moreover, they are essential component of enzymes, photoelectron chain and activate various metabolic activities in legumes in response to drought stress. Overall, application of micronutrients through foliage or soil is necessary to obtain optimal productivity of crops grown in soils with low moisture contents. Proper application of micronutrients to crops may thus help meet the food demands of ever-growing population of the world.

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Chapter 26
Potential of Rhizobia for Sustainable Production of Non-legumes

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Abstract Rhizobia are familiar as the symbiotic associates of legumes, forming N_2-fixing nodules. However, it has been proven that these bacteria also have the ability to survive and colonize the roots of non-legumes as efficiently as they colonize the roots of their legume host. Although example of N_2-fixation in non-legume parasponia has been observed, the researchers are yet unable to extend this nitrogen-fixation symbiosis to major cereals of the planet. Only nodule-like structures or hypertrophies or outgrowths have been recorded with rhizobia on the roots of non-legumes yet without significant N_2-fixation. Generally, three types of rhizobial interactions with non-legumes have been demonstrated i.e. interactions which result in growth and yield promotion of the interacting non-legumes, those which result in poor or detrimental effect on the growth and yield of inoculated non-leguminous plants and those which cannot result in any increase or decrease (missing effect/no effect/neutral effect) in the plant growth. Like other PGPR, rhizobia can affect the non-legumes beneficially by solubilizing sparingly soluble organic and inorganic phosphates, by releasing phytohormnes, enzymes, siderophores, lumichromes, lipo-chito-oligosaccharides, exo-polysaccharides and riboflavins. They can also promote the growth of non-legumes by inhibiting the growth of pathogens by sequestering the iron in the rhizosphere with siderophore production, by releasing the antibiotics and/or by the production of cell wall degrading enzymes. They can also play a significant role in alleviating the deleterious effects of various environmental stresses. Even more, they can improve the growth of non-legumes by changing the host-plant susceptibility by releasing different bio-stimulatory agents. However, plant variety, cultural conditions, native micro-flora, soil and other ecological factors have been reported affecting the degree to which rhizobial association benefit the non-legumes. But it has also been recognized that the potential of PGPR strains of rhizobia (regarding colonization to a variety of plants, adoption under variable soil and environmental conditions and against the pathogens that can attack the host plants) can be improved further through dual/mix inoculation with other beneficial microorganisms. Finally, it is recognized that competent rhizobial strains could be used as biofertilizers, stress regulators, and as biocontrol agents to non-legumes to increase their production. However, much more research efforts are needed to develop rhizobial strains which can effectively improve crop productivity under a variety of environments.

Keywords Rhizobia • Biofertilization • Phytostimulation • Bioprotection • Phytoremediation • N_2-fixation • Non-legumes

1 Introduction

Worldwide, the population depends on agricultural plants as they are food for humans and feed for animals. Demand for food and feed is gradually increasing day-by-day. To meet the demand, farmers apply additional inputs in soil in the form of chemical fertilizers, pesticides, herbicides, etc. But these chemicals disturb the

environment, subvert ecology, degrade soil and mismanage water resources. Other anthropogenic activities (i.e. manufacturing industries, mining of natural resources and energy production) further accelerate these biospheric pollutions. Hence, soil fertility maintenance and remediation of contaminated soils was ever in need of an effective and affordable technological solution. Current trends in agriculture are focused on reduction in the use of chemical pesticides and inorganic fertilizers, compelling the search for alternatives that enhance environmental quality (Haggag 2002). Improving the soil fertility and crop production without the use of chemical fertilizers is a difficult task (Ray et al. 2000; Bera et al. 2006). Plant growth promoting rhizobacteria (PGPR) - mediated remediation of contaminated soils (Jing et al. 2007; Khan et al. 2009), improvement in soil fertility, enhancement in plant growth, control of plant pests and diseases is an alternative eco-friendly approach (Kloepper 1993).

Originally, the "rhizobacteria" that exert a beneficial effect on plant growth (referred to as PGPR) when reintroduced as plant inoculant in a soil containing competitive micro-flora were restricted to only free-living bacteria. But this restriction was uplifted during the last couple of decades when the work of a lot of researchers clearly indicated that rhizobia can associate with the roots of non-legumes also, without forming true nodules, and can promote their growth by using various direct and indirect mechanisms of actions. The original definition of beneficial rhizobacteria restricted to only free-living bacteria is now changed as "any root-colonizing bacteria". Generally, growth promotion as a result of N_2-fixation in legume nodules by rhizobia is not considered as a PGPR mechanism of action, but when these associative bacteria do not exhibit morphological modification of the host plant are considered as PGPR.

Recently, PGPR have been classified as phytostimulators, biofertilizers, biopesticides and rhizoremediators depending on their activities (Somers et al. 2004). Rhizobia, like other PGPR, also exert their beneficial effects on the growth of non-leguminous plants (Hoflich et al. 1995; Noel et al. 1996; Yanni et al. 1997) by producing phytohormones (i.e. auxins, abscisic acid, gibberellic acid and cytokinins, etc.), increasing solubility and availability of nutrients (i.e. mineral P solubilization through organic acid production and mineralization of organic P through release of acid phosphatases). They are also helpful in inhibiting the growth of plant pathogens via competition (i.e. out-competing the pathogens by sequestering the iron in the rhizosphere with siderophore production), antibiosis (i.e. production of an antibiotic namely peptide trifolitoxin whose spectrum of activity includes plant pathogen bacteria) and parasitism (i.e. production of enzymes like chitinase which usually degrade the cell wall of the plant pathogenic fungi). Rhizobia play a significant role in changing the host-plant susceptibility by producing various biostimulatory agents (i.e. lipopolysaccharide production) or in interaction with other beneficial microorganisms. Also, rhizobia are able to protect plants from the deleterious effects of various environmental stresses by producing different metabolites (i.e. rhizobia can mitigate the effects of water stress by decreasing leaf stomatal conductance via abscisic acid and lumichrome production). In addition, rhizobia can produce other beneficial chemicals which can promote the growth of inoculated plants directly or

indirectly e.g. lipochito-oligosaccharides (LCOs), lumichrome, rhiboflavin, enzymes, siderophores or exopolysaccharides in the rhizosphere (Mehboob et al. 2009). In addition, certain rhizobial strains containing ACC deaminase activities could be used to ameliorate the stresses of phytoremediator plants (Glick and Pasternak 2003; Glick 2010) which they experience during remediation of contaminated soils. Conclusively, it could be inferred that rhizobial species are very helpful in stimulating plant growth, protecting them from different pathogenic attacks, environmental stresses, in sustaining fertility level and reviving the health of contaminated soil through remediation without disturbing/degrading the environment. However, proper exploitation of these organisms is a pre-requisite.

2 Rhizobia-non-legume Association: An Overview

Rhizobia belong to *Rhizobiaceae* family. *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium* are their common genera (Tan et al. 2001a; Sessitsch et al. 2002; West et al. 2002; Mia and Shamsuddin 2010) which are collectively referred to as rhizobia (Chaintreuil et al. 2000; Shaukat and Siddqui 2003; Dakora 2003; Okazaki et al. 2004; Matiru and Dakora 2005a, b). By nature they are gram-negative, chemo-organotrophic or chemo-lithotrophic organisms (Werner 1992). Generally, they are famous for their unique ability to establish specific associative interaction with leguminous plants and form nodules in which they fix atmospheric nitrogen (Schloter et al. 1997). However, nonspecific associative interactions of rhizobia with roots of non-legumes have also been investigated (Reyes and Schmidt 1979; Chabot et al. 1996).

Generally, plant-associated bacteria are of two types: those that are closely associated and those that are loosely associated with plants. They may be endophytic (which reside in the internal tissue of the plant), phyllospheric (which reside on the above-ground plant parts i.e. leaves, stems, blossoms and fruits) or rhizospheric (which reside within the rhizosphere) (Weyens et al. 2009). Of these interactions, there are associative interactions between plant roots and bacteria, which exert effects on growth and yield of many plants (Lemanceau 1992; Yanni et al. 1997). These interactions may be stimulating or inhibiting depending upon the rhizosphere survival and colonizing ability of the bacteria and their phyto-effective metabolic characteristics (Hoflich et al. 1994; Antoun et al. 1998).

The ability of rhizobia to survive and colonize the roots of non-legumes as efficiently as they colonize the roots of their legume host has been proven (Gaur et al. 1980; Ladha et al. 1989; Al-Mallah et al. 1990; Yanni et al. 1995; Chabot et al. 1996; Antoun and Prevost 2000; Bhattacharjee et al. 2008). Moreover, rhizobia are now considered endophytes or rhizobacteria of non-leguminous plants (Sessitsch et al. 2002). There is plethora of literature available in which rhizobial inoculants have been recognized as endophytes [those microorganisms that live within host plants for at least part of their life and do not cause apparent symptoms of diseases (Mei and Flinn 2010)] in the roots of non-legumes (McInroy and Kloepper 1995;

Prayitno et al. 1999; Sturz et al. 1999; Biswas et al. 2000a; Matiru et al. 2000; Hilali et al. 2001; Yanni et al. 2001; Reiter et al. 2002; Peng et al. 2002; Lupwayi et al. 2004; Zahir et al. 2004; Singh et al. 2006) such as rice (Balley et al. 1983; Roger and Watanabe 1986; Ladha et al. 1989; Khush and Bennett 1992; Natalia et al. 1994; Ueda et al. 1995; Yanni et al. 1997; Chaintreuil et al. 2000; Engelhard et al. 2000; James et al. 2000; Perrine et al. 2001; Tan et al. 2001b; Peng et al. 2002; Anyia et al. 2004; Matiru and Dakora 2004; Naidu et al. 2004; Singh et al. 2005; Mano and Morisaki 2008; Bhattacharjee et al. 2008; Senthilkumar et al. 2008, 2009), wheat (Sabry et al. 1997; Biederbeck et al. 2000; Anyia et al. 2004; Kaci et al. 2005; Afzal and Bano 2008), maize (Schloter et al. 1997; Gutierrez-Zamora and Martinez-Romero 2001; Reiter et al. 2002; Rosenblueth and Martinez-Romero 2004; Hossain 2007; Mehboob et al. 2008; Cassan et al. 2009), lettuce (Chabot et al. 1993, 1996; Noel et al. 1996), cotton (McInroy and Kloepper 1995; Martinez-Romero et al. 2000; Egamberdiyeva et al. 2004; Hafeez et al. 2004), barley and canola (Lupwayi et al. 2000; Peix et al. 2001; Humphry et al. 2007), mustard (Chandra et al. 2007), oilseed rape (Lupwayi et al. 2000), sorghum, millet, Sudan grass (Matiru et al. 2005), sunflower and okra (Ehteshamul-Haque and Ghaffar 1993; Alami et al. 2000; Sheikh et al. 2006), potato (Sturz et al. 1999), and tobacco (Ji et al. 2010).

A strong evidence exists which indicates that in the presence of germinating seed and root developing systems, rhizobia could flourish in a similar way with legume and non-legumes (Pena-Cabriales and Alexander 1983). Under particular conditions, nodule-like structures or hypertrophies formed by rhizobia on the roots of non-legumes without significant N_2 fixation has also been reported by a number of researchers (Al-Mallah et al. 1989; Bender et al. 1990; De Bruijn et al. 1995; Jing et al. 1992; Li et al. 1991; Rolfe and Bender 1990; Ridge et al. 1992; Trinick and Habdodas 1995; Naidu et al. 2004). Furthermore, for the analysis of rhizobial attachment to the roots of rice and wheat seedlings in terms of dynamic equilibrium model such as Langmuir adsorption isotherm was reported by Shimshick and Hebert (1979) in which the maximum number of binding sites was $8 \times 10^9/g$ of fresh root (gfr) at 22°C. As an evidence, Wiehe and Hoflich (1995) reported a range of more than log 5 to less than log 3 CFU/gfr regarding maize root colonization by rhizobia. Whereas, Chabot et al. (1996) during investigation on the potential of maize and lettuce root colonization by rhizobia reported that root population of rhizobia was averaged log 4.1 CFU/gfr on maize roots 4 weeks after seeding and log 3.7 CFU/gfr on lettuce roots 5 weeks after seeding. They verified the competence of rhizobia to colonize and survive on maize and lettuce roots. Wiehe et al. (1994) reported that rhizobia can colonize not only the rhizoplane but also the cortex and root cap intercellular spaces on the non-leguminous plants. To characterize the associative colonization of the non-leguminous plants by rhizobia, a time-course study was also performed by Schloter et al. (1997) in which they indicated the presence of rhizobia in lysed cells of the root cortex as well as in intracellular spaces of central root cylinder cells. Endophytic invasion of rhizobia in rice roots through cracks in the epidermis and fissures created during emergence of lateral roots was also indicated by Reddy et al. (1997). Whereas, Sabry et al. (1997) reported invasion of rhizobia between cells of the cortex, within the xylem and the root meristem of wheat.

Surface colonization of the rhizoplane followed by endophytic colonization within roots and then ascending endophytic migration into the stem base, leaf sheath and leaves of rice have been reported by Chi et al. (2005). An evidence also exists that for the crack entry invasion of emerging lateral roots of wheat, rice, maize and oilseed rape by rhizobia (Cocking et al. 1994; Gough et al. 1996; Webster et al. 1997; O'Callaghan et al. 2000; Hoflich 2000; Anyia et al. 2004). Rhizobial attachment to asparagus, oat, rice and wheat has been described by Shimshick and Hebert (1979), Ladha et al. (1989) and Terouchi and Syono (1990). Whereas, Wiehe and Hoflich (1995) demonstrated the multiplication and survival of rhizobia in the rhizosphere of wheat, corn, rape and *Brassica napus*. Rhizobial presence at the root epidermal surfaces as well as inside the tissues of inoculated sorghum and millet plants has been shown by Matiru et al. (2005). Pena and Reyes (2007) detected *Rhizobium* as rhizosphere colonizer of lettuce. Prayitno et al. (1999) studied the root colonization pattern of *Rhizobium* sp. and reported that the rhizobia preferentially colonize rice root surface, or at the emerging lateral root zones and at the root tips. Also, Singh et al. (2006) demonstrated that rhizobial cell can go inside and colonize the rice root interiors. Even so, Perrine-Walker et al. (2007b) showed that *Rhizobium* spp. can infect rice roots and colonize the intercellular spaces of the rice roots.

As far as phyto-effective metabolic characteristics are concerned, rhizobia have been recognized to release many powerful phyto-effective metabolites through which they affect fundamental processes in non-leguminous plant development. There are many metabolites of rhizobia recognized so far such as cytokinins (Phillips and Torrey 1972; Puppo and Riguard 1978; Upadhyaya et al. 1991; Hoflich et al. 1995; Noel et al. 1996), abscisic acid (Phillips and Torrey 1970; Hirsch et al. 1997; Law and Strijdom 1989; Atzorn et al. 1988; Minamisawa et al. 1996), indole-3-acetic acid (Law and Strijdom 1989; Hunter and Kuykendall 1990; Minamisawa et al. 1996; Antoun et al. 1998; Vessey 2003; Naidu et al. 2004; Dazzo et al. 2005; Matiru et al. 2005; Boiero et al. 2007; Chandra et al. 2007; Pandey and Maheshwari 2007; Pena and Reyes 2007; Venieraki et al. 2011), gibberellic acid (Atzorn et al. 1988; Yanni et al. 2001; Humphry et al. 2007; Chi et al. 2005; Afzal and Bano 2008), ethylene (Boiero et al. 2007), lipo-chito-oligosaccharides (Lopez-Lara et al. 1995; Dyachok et al. 2000; Prithiviraj et al. 2000; Smith et al. 2002), lumichrome (Phillips et al. 1999; Yang et al. 2002; Beveridge et al. 2003; Dakora 2003; Matiru and Dakora 2005a), siderophore (Schwyn and Neilands 1987; Chabot et al. 1993; Derylo et al. 1994; Dudeja et al. 1997; Antoun et al. 1998; Carson et al. 2000; Arora et al. 2001; Deshwal et al. 2003; Hossain and Martensson 2008), rhiboflavin (West and Wilson 1938; Phillips et al. 1999; Dakora et al. 2002; Dakora 2003), exopolysaccharide (Alami et al. 2000; Reitz et al. 2000; Kaci et al. 2005; Santaella et al. 2008; Berge et al. 2009), phenolic acids (Mishra et al. 2006), nod factors (Spaink 1992; Spaink and Lugtenberg 1994; Xie et al. 1995; Zhang and Smith 2001; Smith et al. 2002), enzyme ACC-deaminase (Belimov et al. 2001; Trott et al. 2001; Yasutu et al. 2001; Kaneko et al. 2002; Sullivan et al. 2002; Ma et al. 2003a, b; Okazaki et al. 2003; Belimov et al. 2005; Hafeez et al. 2008; Duan et al. 2009), antibiotics (Chakraborty and Purkayastha 1984; Ehteshamul-Haque and Ghaffar 1993; Breil et al. 1993), and lipopolysaccharides (Reitz et al. 2000) have been known for a long

time. In general, the metabolites produced as a result of beneficial interaction between rhizobia and non-leguminous plants have been shown to cause increase in seed germination, rate of radical elongation, seedling vigor, root architecture (length, branching, biovolume, surface), shoot growth, photosynthetic activity, leaf area, chlorophyll contents, stomatal conductance, harvest index, grain yield, nutrient uptake, protein content, tolerance to abiotic stresses (Hilali et al. 2001; Hafeez et al. 2004; Siddiqui 2007; Reimann et al. 2008; Hossain and Martensson 2008).

Rhizobial interaction with non-legumes does not always lead to the desired results. Their interaction, at certain time or site, event may result in poor, missing or detrimental effect on the growth and yield of inoculated plants (Mehboob et al. 2009). As evidence, Antoun et al. (1998) have reported deleterious effects of some of the rhizobial species due to the overproduction of IAA and related compounds. Whereas, O'Sullivan and O'Gara (1992) and Alstrom and Burns (1989) showed harmful effects due to the production of HCN by rhizobial inoculants. Similarly, Perrine-Walker et al. (2007a) reported inhibitory effects of rhizobial inoculants on growth and development of non-leguminous plants due to the production of high concentration of auxin and nitrate. Also, suppression of non-legumes with rhizobial inoculation due to the production of growth inhibitor by the strain used has been described by El-Tarabily et al. (2006), whereas Perrine-Walker et al. (2009) reported rice growth inhibition by a rhizobial strain (i.e. *Sinorhizobium meliloti* 1021) which produces bacteriocin-like substance.

It is now widely accepted that the degree to which these associations between rhizobia and non-leguminous plants benefit their growth varies with plant variety, inoculated bacteria, cultural conditions, native microflora, soil and other ecological factors (Lynch 1990; O'Sullivan and O'Gara 1992; Antoun et al. 1998; Biswas et al. 2000b; Hilali et al. 2001; Dobbelaere et al. 2003; Depret et al. 2004; Mehboob et al. 2008; Hussain et al. 2009). Overall, rhizobial strains have been classified into three general groups; strains that promote growth, those that inhibit growth, and strains having no influence in their association with non-leguminous plants (Prayitno et al. 1999; Perrine et al. 2001, 2005).

Finally, the above-cited research evidences have not only provided the better understanding about rhizobial interaction with non-legumes, but also recognized that competent rhizobial strains could be applied to non-legumes to increase their production. However, much more research efforts are needed to develop rhizobial strains specific to crop, variety, soil and environment.

3 Ecophysiology of Rhizobia-Plant Interaction

3.1 Plant Growth Enhancement – Mode of Action

Microbial population in the soil around the roots [Hiltner (1904) for the first time called this soil "rhizosphere"] is 19–32 times more than the root free soil (Bodelier et al. 1997) because of the release of organic compounds such as amino acids,

sugars, vitamins, organic acids, auxins and flavonoides by the plant roots, which can be rapidly utilized by the microorganisms (Somers et al. 2004; Dardanelli et al. 2008, 2009; Raaijmakers et al. 2009). Microorganisms are attracted to this nutritious environment and use these plant root exudates for growth and multiplication on the root surface and in the adjacent rhizosphere soil (Lynch and Whipps 1991; Dakora and Phillips 2002). Actually, the complex exchanges that take place around the plant roots lead to the establishment of rhizosphere interaction. In other words, this complex molecular signaling regulates all the beneficial, deleterious and neutral relationships between microorganisms and the plant roots. The study of these systems has led to the discovery that plants and bacteria communicate by using chemical signals, which are involved in a successful interaction (Bolton et al. 1986; Dardanelli et al. 2008, 2009). Generally, the most abundant microorganisms in the rhizosphere are bacteria. The competent bacteria that aggressively colonize the plant roots are termed as "rhizobacteria" (Antoun and Kloepper 2001) which can have a neutral, deleterious or beneficial effect on plant growth. Deleterious microorganisms are injurious to the plant, but beneficial microorganisms can save the plant from biotic and abiotic stresses, whereas the neutral microorganisms neither harm the plant nor benefit it. Of these types of rhizobacteria, the beneficial could be only 2–5%, which can have beneficial effect on the growth of plants and have been termed as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978). Plant growth promoting rhizobacteria can affect plant growth either directly by providing plants with a compound synthesized by the bacterium or by facilitating the uptake of certain nutrients from the environments or indirectly by decreasing or preventing the deleterious effects of one or more phytopathogenic organisms (Glick et al. 1995). Originally, only free-living bacteria were included in the PGPR definition and not nitrogen fixing symbiotic rhizobia (Kloepper 1993). But now rhizobia are also considered as PGPR (Chandra et al. 2007) when they do not exhibit morphological modification of the host plant. It is widely reported that rhizobia, like other PGPR, can also behave like PGPR with non-legume plants as well (Hoflich et al. 1995; Noel et al. 1996; Yanni et al. 1997; Antoun et al. 1998; Rodriguez and Fraga 1999; Sessitsch et al. 2002) as they exert their positive effects on plant growth through various mechanisms different from nitrogen fixation (Dobbelaere et al. 2003). The well known PGPR strains of rhizobia include members of the genera *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium and Sinorhizobium*. PGPR strains of rhizobia modulate the growth of non-legumes by direct or indirect modes of action described as under:

3.1.1 Direct Growth Promoting Activities

Generally, rhizobacteria cause direct growth promotion of plants in the absence of pathogens by producing phyto-effective metabolites (Lugtenberg and Kamilova 2009). Rhizobia can promote the growth of non-leguminous plants directly by producing plant hormones like auxins, gibberellins, abscisic acid and cytokinins, lowering of plant ethylene levels by producing the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC)

deaminase, providing bio-available phosphorus for plant uptake, sequestering iron for plants by siderophores and other useful compounds like lipo-chito-oligosaccharides (LCOs), lumichrome and rhiboflavin, etc. (Mehboob et al. 2009). Direct growth promotion of non-legumes by rhizobia has also been earlier reported (Hoflich et al. 1995; Chabot et al. 1996; Noel et al. 1996; Yanni et al. 1997).

Phytohormones are the substances that stimulate plant growth at micromolar concentration (or even lower). Also, they regulate essentially all physiological and developmental processes during the life cycle of a plant (Chiwocha et al. 2003). Phytohormones that are produced by the rhizobia in response to seed or root inoculation with non-legumes, such as indole-3-acetic acid, cytokinins, gibberellins and abscisic acid can stimulate the growth and yield of non-leguminous plants. The most studied phytohormone released by rhizobia during interaction with non-legumes is indole-3-acetic acid (IAA). This adds to plant growth and development by improving root/shoot growth and seedling vigor. Rhizobial strains producing IAA include *Azorhizobium caulinodans, Bradyrhizobium japonicum, Bradyrhizobium elkanii, Mesorhizobium loti, Rhizobium japonicum, Rhizobium leguminosarum, Rhizobium lupine, Rhizobium meliloti, Rhizobium phaseoli, Rhizobium trifolii* and *Sinorhizobium* spp. (Dullaart 1970; Badenoch-Jones et al. 1982; Wang et al. 1982; Kaneshiro et al. 1983; Garcia-Rodriguez et al. 1984; Wheeler et al. 1984; Ernesten et al. 1987; Kittell et al. 1989; Hoflich et al. 1995; Minamisawa et al. 1996; Noel et al. 1996; Antoun et al. 1998; Biswas et al. 2000a; Yanni et al. 2001; Naidu et al. 2004; Dazzo et al. 2005; Boiero et al. 2007; Chandra et al. 2007; Afzal and Bano 2008; Weyens et al. 2009; Senthilkumar et al. 2009; Vargas et al. 2009; Chi et al. 2010). Although rhizobia produce other plant hormones as well such as cytokinins, gibberellins and abscisic acid, much less information is available in this regard in the literature. Cytokinins usually stimulate cell division and cell enlargement. So far, strains of only *Rhizobium* spp. have been recognized for cytokinin production (Phillips and Torrey 1970, 1972; Newcomb et al. 1977; Puppo and Riguard 1978; Wang et al. 1982; Upadhyaya et al. 1991; Hoflich et al. 1994; Caba et al. 2000; Senthilkumar et al. 2009). Also, gibberellin production by rhizobia has been less widespread yet the strains of *Rhizobium phaseoli, Sinorhizobium meliloti and Rhizobium leguminosarum* have been isolated and demonstrated that these can promote the growth and yield of non-leguminous plants via gibberellin production (Atzorn et al. 1988; Yanni et al. 2001; Chi et al. 2005; Boiero et al. 2007; Humphry et al. 2007; Afzal and Bano 2008). Very little information regarding enhancement in growth of non-legumes through the production of abscisic acid is available. However, Dangar and Basu (1987) and Boiero et al. (2007) have reported *Bradyrhizobium japonicum* as growth promoter of non-legumes via abscisic acid production.

Lowering of plant ethylene levels by producing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzyme is also considered a growth promoting mechanism in non-leguminous plants by rhizobial inoculation. Actually, these rhizobia take up the immediate precursor of the ethylene i.e. ACC and convert it into α-ketobutyrate and NH_3 in order to use it as a source of nitrogen and carbon. Generally, inoculation with ACC-deaminase producing rhizobial strains may result in longer roots and may relieve several forms of stresses such as heavy metals, pathogens, drought, and

salinity, etc. Many rhizobial strains have been found to produce ACC-deaminase enzyme such as *Rhizobium leguminosarum* bv. *viciae, Rhizobium hedysari, Rhizobium japonicum, Bradyrhizobium japonicum, Bradyrhizobium elkani, Rhizobium gallicum, Mesorhizobium loti* and *Sinorhizobium meliloti* (Kaneko et al. 2000; Sullivan et al. 2002; Ma et al. 2003a, b, 2004; Okazaki et al. 2004; Uchiumi et al. 2004; Madhaiyan et al. 2006; Hafeez et al. 2008; Duan et al. 2009).

Increase in growth and yield development by providing bio-available phosphorus uptake via rhizobial inoculation is also a well studied mechanism. In fact, a large portion of soil phosphorus as well as of applied phosphorus immobilized either in organic or inorganic form and becomes unavailable to plants depending upon the type and pH of soil. Some rhizobial strains have the ability to solubilize such bound phosphorus from either organic form through releasing phosphatases (Abd-Alla 1994) or inorganic form by releasing organic acids such as 2-ketogluconic acid, glutamic acid, sulphuric acid, nitric acid and carbonic acids (Halder et al. 1990; Halder and Chakraborty 1993; Chabot et al. 1996; Antoun et al. 1998; Dazzo et al. 2000; Arora et al. 2001; Mikanova and Novakova 2002; Alikhani et al. 2006; Jayasinghearachchi and Seneviratne 2006; Afzal and Bano 2008) thereby increasing its uptake and facilitating plant growth. *Rhizobium* genera have been recognized to have the most powerful phosphate solubilizing strains (Abd-Alla 1994; Rodriguez and Fraga 1999). However, the rhizobial strains that have been investigated to solubilize or mineralize phosphate in the rhizosphere of non-legumes include *Rhizobium leguminosarum, Rhizobium meliloti, Mesorhizobium mediterraneum, Bradyrhizobium japonicum* and *Bradyrhizobium* sp. (Wood and Cooper 1984; Halder et al. 1990; Halder and Chakraborty 1993; Chabot et al. 1996; Rodriguez and Fraga 1999; Antoun et al. 1998; Peix et al. 2001; Vessey 2003; Egamberdiyeva et al. 2004; Hara and de Oliveira 2004; Puente et al. 2004; Rodriguez et al. 2006; Fernandez et al. 2007; Afzal and Bano 2008)

Rhizobial sequesterations of iron by producing siderophores are assumed to serve as a source of plant available iron under iron deficient environment. Actually, siderophores are low-molecular-weight compounds produced to sequester Fe^{3+} as they have very high Fe^{3+} affinity constants (10^{25}–10^{52}) (Matzanke et al. 1989) and are used to mobilize iron (Plessner et al. 1993). Iron which is needed by plants for chlorophyll synthesis is often present in the highly insoluble form of ferric hydroxide. Many rhizobia such as *Rhizobium meliloti, Rhizobium tropici, Rhizobium leguminosarum* bv. *viciae, Rhizobium leguminosarum* bv. *trifolii, Rhizobium leguminosarum* bv. *phaseoli, Sinorhizobium meliloti and Bradyrhizobium* sp. produce siderophores that can bind insoluble Fe^{3+}, and reduce it to soluble Fe^{2+}, which is preferred by plants (Schwyn and Neilands 1987; Carson et al. 1992; Guerinot 1991, 1994; Chabot et al. 1993; Plessner et al. 1993; Derylo et al. 1994; Jadhav et al. 1994; Dudeja et al. 1997; Antoun et al. 1998; Carson et al. 2000; Arora et al. 2001).

Lipo-chito-oligosaccharides (LCOs) are bacteria-to-plant signal molecules produced by rhizobia during rhizobia-legume symbiosis (Spaink 1992). In the absence of auxins and cytokinins, LCOs have the ability to restore or resume cell division and embryogenesis in non-legumes (De Jong et al. 1993; Dyachok et al. 2000). Also, rhizosphere application of LCOs at low concentration (10^{-7} to 10^{-9} M)

can promote seed germination, early seedling, root mass and root length, whereas leaf spray with micromolar concentration of LCOs (10^{-6}, 10^{-8} or 10^{-10}) can increase photosynthate production and grain yield of non-legumes (Smith et al. 2001, 2002; Miransari and Smith 2009). Rhizobia of the genera *Azorhizobium, Bradyrhizobium, Rhizobium, Mesorhizobium and Sinorhizobium* have been observed to improve seed germination and seedling development in non-legumes. These are also known for increase in foliar photosynthetic rates, induction in expression of flavonoid genes, cell division and embryogenesis via releasing LCOs in the rhizosphere (Spaink 1992; Spaink and Lugtenberg 1994; Xie et al. 1995; Zhang and Smith 2001; Smith et al. 2002).

Likewise, lumichrome is also a signaling compound that can encourage growth of non-legumes and has been identified from the culture filtrate of *Sinorhizobium meliloti* cell (Phillips et al. 1999; Yang et al. 2002; Dakora 2003). It can stimulate seedling development and also help in alleviation of water stress (Phillips et al. 1999; Matiru and Dakora 2005a).

Riboflavin is a vitamin which enzymatically or photochemically may be changed into lumichrome (Yagi 1962; Yanagita and Foster 1956; Phillips et al. 1999) that in turn stimulates plant growth (Dakora et al. 2002) via motivating root respiration (Phillips et al. 1999). Only, few researchers i.e. West and Wilson (1938), Rodelas et al. (1993), Sierra et al. (1999), Phillips et al. (1999), Dakora et al. (2002), Yang et al. (2002) have recognized the riboflavin as growth promoter of non-legumes produced by *Rhizobium, Sinorhizobium meliloti* and *Rhizobium leguminosarum* bv. *viciae*.

3.1.2 Indirect Growth Promoting Activities

Rhizobia promote the growth of non-leguminous plants indirectly by lessening or preventing the injurious effects of one or more phytopathogenic organisms through a variety of mechanisms. These include the ability to produce anti-pathogen metabolites such as antibiotics, fungal cell wall-degrading enzymes or production of volatiles such as hydrogen cyanide, which suppress the growth of pathogens. Rhizobia can produce siderophores that chelate iron, making it unavailable to pathogens. They may also have ability to successfully compete with pathogens for nutrients, the ability to stimulate host plant defense mechanism through induced systemic resistance (ISR), and the ability to increase root adhering soil (RAS).

To prevent the proliferation of phytopathogens via synthesizing antibiotics is one of the most effective mechanisms of PGPR. Rhizobia are capable of producing antimicrobial compounds known as antibiotics, as well as fungal cell-wall-degrading enzymes that are inhibitory to the growth and/or activities of non-legume pathogens. Many rhizobia such as *Rhizobium leguminosarum* bv. *trifolii, Rhizobium leguminosarum* bv. *viciae, Rhizobium meliloti, Rhizobium trifolii, Sinorhizobium meliloti and Bradyrhizobium japonicum* have been reported to secrete antibiotics and cell-wall-degrading enzymes that can inhibit/kill the pathogens of non-leguminous plants (Schwinghamer and Belkengren 1968; Antoun et al. 1978; Tu 1978, 1979;

Parveen and Ghaffar 1991; Breil et al. 1993; Parveen et al. 1993; Ehteshamul-Haque and Ghaffar 1992, 1993; Ehteshamul-Haque et al. 1996; Siddiqui et al. 1998, 2000; Siddiqui and Mahmood 2001; Ozkoc and Deliveli 2001; Shaukat and Siddqui 2003; Bardin et al. 2004; Chandra et al. 2007).

Rhizobial strains have also been found capable of displacing pathogens through better competition for nutrients. For example, rhizobia can inhibit a widely occurring plant pathogen *Macrophomina phaseolina* by producing high-affinity iron binding siderophores (Arora et al. 2001) under iron limiting conditions which sequester iron in the rhizosphere and make it less available to deleterious microorganisms. Rhizobia thus out-compete the pathogens for available iron, thus causing death of pathogens or limit their growth.

Furthermore, rhizobia can inhibit pathogens or deleterious microorganisms by producing HCN (Wei et al. 1996; Antoun et al. 1998; Vidhyasekaran and Muthamilan 1999; Chandra et al. 2007). Some reports show that the rhizobial strains such as *Rhizobium leguminosarum* bv. *phaseoli, Rhizobium leguminosarum* bv. *trifolii*, *Rhizobium leguminosarum* bv. *viciae* and *Mesorhizobium loti* are able to produce HCN (Antoun et al. 1998; Chandra et al. 2007).

Defensive capacity of plants can be enhanced when they are stimulated appropriately. Use of selected PGPR strains could trigger resistance in plant parts upon inoculation. Non-leguminous plants such as rice, sunflower, okra and potato can be protected against viral, bacterial or fungal attack through physiological immunity which can be induced with the colonization of their roots by certain rhizobial species (Ehteshamul-Haque and Ghaffar 1993; Abdelaziz et al. 1996; Nautiyal 1997, 2000). This process has been termed as induced systemic resistance (ISR). In ISR process, it is considered that the inducing rhizobacteria in the plant roots generate signal, which spreads systemically inside the plant and boosts the defensive capacity of the distant tissues from the succeeding infection by the pathogens. Various rhizobial species such as *Rhizobium etli, Rhizobium leguminosarum* bv. *phaseoli* and *Rhizobium leguminosarum* bv. *trifolii* can induce systemic resistance in different non-legumes by producing various biostimulatory agents (Yanni et al. 2001; Peng et al. 2002; Mishra et al. 2006; Singh et al. 2006).

The importance of root adhering soil (RAS) is well established because of the uptake of water and nutrients required by the plants for their growth uptaken from the soil. Therefore, around the root system, soil structure and aggregate stability is even more important. *Rhizobium alamii* sp. nov., *Rhizobium phaseoli, Rhizobium trifolii, Rhizobium meliloti, Rhizobium* and *Rhizobium leguminosarum* are able to produce compounds such as exopolysaccharides (EPS) (Martens 1982; Alami et al. 1998, 2000; Kaci et al. 2005; Berge et al. 2009). These rhizobial EPS can improve plant growth by increasing RAS through better control of water and nutrient uptake.

Conclusively, growth and yield of non-leguminous plants could be triggered by rhizobial inoculation directly via producing phyto-effective metabolites such as plant hormones, enzymes, lipo-chito-oligosaccharides, siderophores, lumichrome, riboflavin, etc. by increasing the uptake of nutrients or by increasing resistance in them against biotic and abiotic stresses. Whereas, rhizobia can indirectly promote the growth of non-leguminous plants by inhibiting the phytopathogens via producing

antibiotics, fungal cell wall-degrading enzymes and hydrogen cyanide or through starvation via siderophore production. Also, rhizobia can increase the growth and yield of non-legumes indirectly through improving their defense mechanism or by increasing root adhering soil.

3.2 Single and Mixed Population of Microbes

No doubt, the exploitation of single microbe is easy and economical. Also, the commercial formulations of single inoculants are more stable than consortium inoculants. But it is also a proven fact that a single PGPR agent may sometimes account for the inconsistent performance because of its inadequate colonization, inadaptability under variable soil environments or inability against all pathogens that attack the host plant, as reported by Raupach and Kloepper (1998) that a single PGPR agent may not perform well at all times in all kinds of soil environment. In contrast, mixture of PGPR agents that need diverse pH, moisture and temperature conditions, may adapt better to the environmental changes that occur throughout the growing season, may colonize roots more aggressively, may protect the plant against a broader range of pathogens and utilize a wider array of plant growth mechanisms. Earlier, Hoflich et al. (1994) reported that a combination of microorganisms with different metabolic capacities (N_2-fixation, P-mobilization; production of phytohormones and antibiotics) can partly surpass the effect of single inoculations, or can produce a positive effect where single inoculations are ineffective. In other words, it could be said that plant benefits derived from the co-inoculation/mixed inoculation will be superior to that of plant inoculated with single PGPR agent (Xavier and Germida 2002; Adesemoye et al. 2008). Conclusively, despite exploiting their individual plant growth promoting capacity, the potential of selected rhizobia can be improved further through dual/mix inoculation with other microorganisms for additive and/or synergistic effects (Dobbelaere et al. 2003). In general, the advantages of mixture of PGPR agents include broad spectrum of actions, improved efficacy, reliability and allowance of combination of various traits without genetic engineering (Janisiewicz 1996). But sometimes the incompatibility of mixture inoculants may arise because of which inhibition of each other as well as target pathogens may occur. Hence, for the development of mixture inoculants, compatibility of microorganisms is a prerequisite.

Rhizobial potential to promote the growth of non-legumes can be improved further through dual inoculation with other PGPR. For example, Berg (2009) has reported that on the basis of beneficial effects of PGPR and rhizobia, studies using inoculant mixtures were very promising. Also, for providing a more balanced nutrition to plants and to improve the performance of plants in nutrient-deficient and degraded habitats, a compatible combined inoculation of rhizobia, arbuscular mycorrhizal (AM) fungi and PGPR strains is more effective than a single microorganism (Algawadi and Gaur 1988; Belimov et al. 1995; Requena et al. 1997; Galleguillos et al. 2000; Prasad and Chandra 2003; Gunasekaran et al. 2004). Similarly,

Valdes et al. (1993) revealed that rhizobia and VAM fungi when used together synergistically stimulate plant growth. A number of researchers have reported improved yields of many non-leguminous crops such as sorghum, barley, rice, maize, wheat, sugar beet, okra and lettuce by using rhizobia in combination with other PGPR (Algawadi and Gaur 1992; Chabot et al. 1993; Hoflich et al. 1994; Belimov et al. 1995; Yanni et al. 1997; Galal 2003; Sahin et al. 2004; Han and Lee 2005; Sheikh et al. 2006; Afzal and Bano 2008).

Different combinations of rhizobia with other PGPR which have been studied include *Rhizobium*, and *Azotobacter* (Barea et al. 1975), *Rhizobium* with *Azospirillum* or with *Azotobacter* (Burns et al. 1981; Iruthayathas et al. 1983; Plazinski and Rolfe 1985a, b; Sarig et al. 1986; Rodelas et al. 1996; Burdman et al. 1998, 2000). *Bradyrhizobium japonicum* and *Aeromonas* and *Pseudomonas* spp. (Polonenko et al. 1987), *Rhizobium leguminosarum* and *Pseudomonas putida, Pseudomonas fluorescens, Pseudomonas fonticola* (Chanway et al. 1989), *Rhizobium leguminosarum* and *Azospirillum* (Vidhyasekaran and Muthamilan 1995), *Bradyrhizobium japonicum*, *Rhizobium* sp. and mycorrhizal fungus *Glomus mosseae* (Xie et al. 1995), *Rhizobium etli, Rhizobium tropici* and *Azospirillum brasilense* (Burdman et al. 1996), *Bradyrhizobium* and *Pseudomonas proteamaculans, Pseudomonas liquefaciens* (Zhang et al. 1997), *Enterobacter* and *Bradyrhizobium* sp. (Gupta et al. 1998), *Bradyrhizobium japonicum* and *Serratia proteamaculans* (Dashti et al. 1998), *Sinorhizobium meliloti* and *arbuscular mycorrhizal fungi* i.e. *Glomus intraradices* (Galleguillos et al. 2000), *Mesorhizobium* and *Pseudomonas* (Goel et al. 2002; Sindhu et al. 2002), *Fluorescent Pseudomonas* and *Rhizobium* (Kumar et al. 2001; Chebotar et al. 2001), *Bradyrhizobium japonicum* and *Bacillus thuringiensis* (Bai et al. 2003), *Rhizobium leguminosarum* (*Rhizobium* sp. strain Thal-8/SK8) and phosphorus solubilizing bacteria (*Pseudomonas* sp. strain 54RB) (Afzal and Bano 2008).

Finally, the applicability of rhizobia in combination with other beneficial bacteria to improve growth of non-legumes has been proven more effective, however, the research is very rare in this regard. Hence, there is a need to explore a wide range of combinations of rhizobia with other PGPR that interact synergistically in order to result promising yields of various important non-legumes.

3.3 Nitrogen Fixation in Non-legumes

Fixation of atmospheric nitrogen in leguminous plants by symbiotic bacteria is a popular phenomenon. But the extension of nitrogen-fixing symbiosis to cereals has been a long-standing goal in the field of biological nitrogen fixation. So, among the efforts, there are some investigations which have shown that the N_2-fixing microorganisms are fixing N_2 through exogenous or endogenous symbiosis with some of non-leguminous plants. The most recognized example is of Parasponia (a non-leguminous plant) in which *Rhizobium* forms nodules and fixes N_2 in a similar fashion as in leguminous plants (Trinick 1979; Werner 1992) and of oilseed rape nodulation

stimulation by *Rhizobhium parasponium* RP 501 and *Bradyrhizobium* CP 283 (Cocking et al. 1992). Moreover, nodule-like structures or hypertrophies or outgrowth on roots formed by rhizobia have also been observed in oilseed rape, rice, *Arabidopsis thaliana*, and other non-legumes (Al-Mallah et al. 1989, 1990; Bender et al. 1990; Rolfe and Bender 1990; Jing et al. 1990, 1992; Li et al. 1991; Ridge et al. 1992; Spencer et al. 1994; De Bruijn et al. 1995; Trinick and Habdodas 1995). Furthermore, Velazquez et al. (2005) reported that coexistence of symbiosis and pathogenicity-determining genes has been shown to occur in strains of *Rhizobium rhizogenes* which enabled them to induce nodules or tumors depending on plant species. Also, Sabry et al. (1997) observed increased dry weight and nitrogen contents as a result of nitrogenase activity of *Azorhizobium caulinodans* in inoculated wheat grown in pot under controlled conditions and demonstrated the involvement of symbiotic nitrogen fixation. Naidu et al. (2004) also observed nitrogenase activity in rice plants inoculated with *Azorhizobium caulinodans* in a hydroponic study. Low level of nitrogen fixation was also observed by Chen et al. (1992), Yu and Kennedy (1995) and Cocking et al. (1995) in wheat inoculated with rhizobia. These discoveries encouraged to make attempts to extend the host range of *Rhizobium* from legumes to non-legumes. But a number of inoculation experiments failed to show a substantial contribution of biological nitrogen fixation to plant growth in most cases. For example, Spencer et al. (1994) did not detect nitrogenase activity in studies of the interaction between rhizobia and cultured potato tissues. Riggs et al. (2001) have reported that inoculation with different strains of diazotrophs did not relieve the N-deficiency symptoms of unfertilized maize in either field or greenhouse assays. However, Quispel (1991) has suggested that only in endophytic systems are the prerequisites for effective nitrogen fixation likely to be fulfilled in interactions between non-legumes and diazotrophic bacteria.

Presently, the endophytic diazotrophs for which evidence exists that their plant stimulation effect is related to their ability to fix N_2 include the *Azoarcus* sp., *Burkholderia* sp., *Gluconacetobacter diazotrophicus* and *Herbaspirillum* sp. (Vessey, 2003). Several other studies have also indicated the contribution of biological nitrogen fixation (BNF) in the growth promotion of non-leguminous plants through associative interaction with diazotrophs. For example, in a study conducted by Ladha et al. (1987) it was reported that out of total needed nitrogen by the rice plants, 20–25% was supplied by associative nitrogen fixation. Higher contribution in required nitrogen level to rice plants via biological nitrogen fixation (BNF) was reported by Baldani et al. (2000) when rice seedlings were inoculated with strains of *Burkholderia* spp. Whereas, Sevilla and Kennedy (2000) have revealed the involvement of the biologically fixed nitrogen in the observed growth promotion of rice upon inoculation with *Acetobacter diazotrophicus*. Christiansen-Weniger (1997) also reported the colonization of 2,4-D-induced para-nodules of rice by *Azospirillum*. Some other diazotrophs such as *Pantoea* sp., *Ochrobactum* sp., *Herbaspirillum* sp., *Pantoea agglomerans* YS19 and *Azoarcus* sp. have been proposed to be responsible for the supply of biologically fixed nitrogen to their host rice plant (Ladha et al. 1997; Verma et al. 2004; You et al. 2005; Feng et al. 2006; Reinhold-Hurek et al. 2006). Furthermore, Gantar and Elhai (1999) elaborated the

possibility of colonization of wheat para-nodules by Cyanobacterium *Nostoc* spp. strain 2S9B and of N_2 fixation. Also, increase in fixed nitrogen in 2,4-D induced para-nodulating maize plants by *Azospirillum* have been stated by Christiansen-Weniger and Vanderleyden (1994). Moreover, enhanced nitrogenase activity in wheat with *Azospillum brasilense* and *Azospirillum lipoferum* was reported by Kennedy and Tchan (1992), whereas the same activity was noticed by Saikia et al. (2004) in maize plants treated with *Azospirillum*. Regarding various attempts which have been made so far, to extend the host range of *Rhizbium* from legumes to non-legumes through plant genetic manipulation have seen little or no success in achieving the induction of symbiosis between cereals and diazotrophs (Saikia and Jain 2007), however, various N_2-fixing diazotrophs found in close association with non-leguminous plant roots such as *Azospirillum lipoferum*, *Azospirillum brasilense* and *Azospirillum amazonense* have been isolated from the roots of rice (Ladha et al. 1982; Pereira et al. 1988).

No doubt, the evidence regarding biological nitrogen fixation in non-legumes has been reviewed in the above discussion, yet the work is in its infancy and requires much more advances.

4 Practical Applications

4.1 Biofertilization

The use of plant growth promoting rhizobacterial inoculants, as biofertilizers, is now common in many regions of the world with the aim of reducing the application of potentially polluting industrial fertilizers and pesticides (Burdman et al. 2000). Whereas, "Biofertilizer" designates the biological product or a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, the microorganisms colonize the rhizosphere or the interior of the plant and promote growth by increasing the supply or availability of primary nutrients to the host plant (Vessey 2003). So, a biofertilizer increases crop growth through combinations of biological nitrogen fixation, growth promoting or hormonal substances, and increased availability of soil nutrients (Cocking 2003). Hence, the importance of biofertilizers lies in their ability to supplement/mobilize soil nutrients with minimal use of non-renewable resources. The utility of rhizobial strains as biofertilizers has been demonstrated by Bardin et al. (2004) and Chi et al. (2005) so, as a biofertilizer, rhizobia should promote the growth of plants by supplying symbiotically fixed nitrogen, phytohormones and by increasing the availability/solubility of insoluble/fixed nutrients. Furthermore, Canbolat et al. (2006) recognize the biofertilizers as an alternative to mineral fertilizers, whereas Galal et al. (2001) demonstrated that biofertilization technology can minimize the costs of production and at the same time, avoid the environmental hazards. There are many investigations which are confirming the ability of rhizobia to colonize the non-legumes (Gaur et al.

1980; Ladha et al. 1989; Al-Mallah et al. 1990; Yanni et al. 1995; Hoflich et al. 1995; Chabot et al. 1996; Schloter et al. 1997; Webster et al. 1997; Prayitno et al. 1999; Antoun and Prevost 2000; Gopalaswamy et al. 2000; Bhattacharjee et al. 2008; Ji et al. 2010; Chi et al. 2010), produce phyto-effective hormones (Phillips and Torrey 1970, 1972; Puppo and Riguard 1978; Atzorn et al. 1988; Law and Strijdom 1989; Hunter and Kuykendall 1990; Upadhyaya et al. 1991; Hoflich et al. 1995; Noel et al. 1996; Minamisawa et al. 1996; Hirsch et al. 1997; Antoun et al. 1998; Yanni et al. 2001; Vessey 2003; Naidu et al. 2004; Chi et al. 2005; Dazzo et al. 2005; Matiru et al. 2005; Boiero et al. 2007; Chandra et al. 2007; Pandey and Maheshwari 2007; Pena and Reyes 2007; Humphry et al. 2007; Afzal and Bano 2008; Naher et al. 2009; Senthilkumar et al. 2009), and increase uptake of nutrients (Amara and Dahdoh 1997; Khokhar and Qureshi 1998; Alami et al. 1999; Biswas et al. 2000a; Yanni et al. 2001; Hafeez et al. 2004; Etesami et al. 2009a, b). Rodriguez and Fraga (1999) showed the commercial production of biofertilizers containing the strains of *Rhizobium* and their usage with different crops. Improvements in the growth and yield parameters of different non-leguminous crops (Table 1) as a result of rhizobial biofertilization through production of phyto-effective metabolites and by mobilization or increased uptake of nutrients, which are discussed below:

The efficacy of various rhizobial strains to enhance the growth and yield of wheat through hormone production or by increasing uptake of nutrients has been assessed in a number of studies (Hoflich et al. 1994; Amara and Dahdoh 1997; Galal et al. 2001; Anyia et al. 2004; Egamberdiyeva et al. 2004; Lupwayi et al. 2004; Afzal and Bano 2008; Etesami et al. 2009a, b). These authors have reported that rhizobial strains used as a biofertilizer on wheat promoted the growth via increased uptake of nutrients (such as N, P, K, Na, Zn, Fe and Cu), whereas, in some other studies it has been reported that the increased growth and yield when wheat was biofertilized with rhizobia was via production of phyto-effective metabolites (Kavimandan 1985; Law and Strijdom 1989; Hoflich 2000; Monteleone et al. 2003).

The growth promoting ability of rhizobial inoculants in maize has been examined in a number of studies via increased uptake of nutrients (such as N, P, Mg, Fe, Cu) (Gaur et al. 1980; Fyson and Oaks 1990; Chabot et al. 1993, 1996, 1998; Pineda et al. 1994; Hoflich et al. 1994; Schloter et al. 1997; Hoflich 2000; Prevost et al. 2000; Peng et al. 2002; Egamberdiyeva et al. 2004; Matiru and Dakora 2005b). In some other studies, increased maize growth and yield has been reported via hormone (i.e. auxin, cytokinine) production by using rhizobial biofertilizers (Hoflich et al. 1994; Gutierrez-Zamora and Martinez-Romero 2001; Souleimanov et al. 2002).

Likewise, a number of researchers have reported an increased growth and yield in rice upon rhizobial inoculation via hormone production (Reddy et al. 1997; Yanni et al. 1997, 2000; Biswas et al. 2000a, b; Perrine et al. 2001; Chi et al. 2005) such as auxin, and gibberellic acid. Whereas, in some other studies increased growth and yield of rice plants has been reported by rhizobial inoculation via improved uptake of nutrients such as N, P, K, Ca, Mg, Zn, Na, Mo and Fe (Bashan et al. 1990; Yanni et al. 2001; Biswas 1998; Biswas et al. 2000a; Peng et al. 2002; Singh et al. 2006).

Improvement in growth of canola and lettuce with rhizobial inoculation has been observed by Chabot et al. (1993) possibly with the production of indole-3-acetic

Table 26.1 Yield improvement by *Rhizobium* inoculation and proposed mechanism(s) of action in non-legumes

Host plant	Rhizobium	Growing conditions	Proposed mechanism(s) of action	% increase over control	Reference
Rice	*Rhizobium leguminosarum* bv. trifolii strain E11 and E12	Field trial	Production of indole-3-acetic acid and gibberellin (GA7)	Grain yield by 46 and 42%	Yanni et al. (1997)
	Azorhizobium caulinodans strains (TAL-1926 and IBRG-42)	Pot trial	Not described	Shoot dry weight by 120 and 58%, %N by 28 and 19 and total N by 140 and 68%	Khokhar and Qureshi (1998)
	Rhizobium sp.	Laboratory and greenhouse	Root colonization, N_2-fixation	Bacterization promoted rice plant growth	Prayitno et al. (1999)
	R. leguminosarum bv. trifolii E11, *Rhizobium* sp. IRBG 74 and *Bradyrhizobium* sp. IRBG271	Pot trial	Indole-3-acetic acid	Grain yield up to 22%, straw yield up to 19% and NPK uptake up to 28%	Biswas et al. (2000a)
	R. leguminosarum bv. trifolii E11, *Rhizobium* sp. IRBG74	Laboratory and greenhouse	Production of indole-3-acetic acid	Grain yield by 17 and 12% while straw yield by 20 and 15%, respectively.	Biswas et al. (2000b)
	Bradyrhizobium spp.	Greenhouse	Root colonization, N_2-fixation, auxin production	Grain yield and shoot growth by 20%	Chaintreuil et al. (2000)
	Rhizobium leguminosarum bv. trifolii E11	Axenic conditions	Production of indole-3-acetic acid	Significant increase in plant biomass	Dazzo et al. (2000)
	Azorhizobium caulinodans	Gnotobiotic and field	Colonization and N_2-fixation	Plant height (13%), shoot dry weight by 8%, fresh weight by 7%.	Nieuwenhove et al. (2000)
	Rhizobium leguminosarum bv. trifolii, *Rhizobium leguminosarum* bv. viciae	Laboratory and greenhouse	Production of auxin and nitrate, and root colonization	Inoculation could either promote, inhibit or have no influence on plant growth	Perrine et al. (2001)

Rhizobium leguminosarum bv. trifolii strain E12, IRBG74, *Bradyrhizobium* sp. strain IRBG271	Pot trial	Not described	Grain yield up to 16%, photosynthetic rate up to 12%	Peng et al. (2002)
Rhizobium sp.	Pot trial	Production of indole-3-acetic acid, gibberellin and root colonization	Grain yield by 20-69%, shoot dry mass 11-43%	Chi et al. (2005)
Rhizobium leguminosarum bv. phaseoli (RRE6), *Rhizobium leguminosarum* bv. trifolii (ANU 843)	Greenhouse	Biocontrol/production of phenolics	Root/shoot dry weight up to 26%, grain yield up to 15%	Mishra et al. (2006)
Rhizobium sp.	Greenhouse	N_2-fixation and root colonization	Strains RRE3, RRE5, and RRE6 increased biomass up to 21%, grain yield up to 13%	Singh et al. (2006)
Rhizobium leguminosarum bv. trifolii strain R4	Magenta jars	N_2-fixation	Strain R4 improved plant shoot and root dry mass compared to other strains	Perrine-Walker et al. (2007a)
Rhizobium phaseoli, Rhizobium leguminosarum, Mesorhizobium ciceri	Pot trial	Not described	*R. phaseoli* strain LSI 29 increased the paddy yield and biomass by 43 and 18%, respectively	Hussain et al. (2009)
Bradyrhizobium strains UPMR48, UPMR29	Laboratory assay	Not described	Dry biomass by 6 and 11%, vigor index by 21 and 20%, respectively	Mia and Shamsuddin (2009)
Rhizobium sp.	Axenic trial	Rhizosphere and root colonization, and N_2-fixation	Plant biomass by 36%, tissue N by 4.47%	Naher et al. (2009)

(continued)

Table 26.1 (continued)

Host plant	Rhizobium	Growing conditions	Proposed mechanism(s) of action	% increase over control	Reference
	Azorhizobium caulinodans strain ORS 571	Gnotobiotic and hydroponics	Production of indole-3-acetic acid, cytokinins and nitrogenase activity	Biomass and total N increased up to 70 and 50%, respectively	Senthilkumar et al. (2009)
	Rhizobium sp.	Axenic condition	Production of IAA, siderophores and P-solubilization	Dry seedling weight and germination vigor increased up to 58 and 43%, respectively	Vargas et al. (2009)
	Sinorhizobium meliloti 1021	Axenic and greenhouse	Nutrient uptake and production of indole-3-acetic acid	Shoot growth was increased up to 44%	Chi et al. (2010)
Wheat	*R. leguminosarum* bv. Trifolii strain R39	Greenhouse and field trials	Production of auxins and cytokinins	Strain R39 resulted in significantly higher wheat yield by 8%	Hoflich et al. (1994)
	Rhizobium sp.	Greenhouse	Nutrient solubilization/ uptake	Grain yield by 106%	Amara and Dahdoh (1997)
	Rhizobium leguminosarum bv. trifolii	Greenhouse	Not described	Strain IAT168 increased the shoot dry matter by 24%	Hilali et al. (2001)
	Rhizobium strain (KYGT207)	Greenhouse	Production of exopolysaccharides	Strain KYGT207 increased shoot and root dry mass by 85 and 56%, respectively	Kaci et al. (2005)
	Rhizobium leguminosarum strain (Thal 8)	Pot trial	P-solubilization	Grain yield up to 30%	Afzal and Bano (2008)
	Rhizobium leguminosarum var. phaseoli, Rhizobium leguminosarum var. viciae	Greenhouse trial	Production of indole-3-acetic acid and nutrient solubilization	Root dry weight was increased up to 24%	Etesami et al. (2009a)
	Rhizobium sp.	Pot trial	Production of indole-3-acetic acid	Shoot and root weight was increased up to 37%	Etesami et al. (2009b)

Maize/Corn	*Rhizobium* sp.	Laboratory and Field trials	P-solubilization, Siderophores and auxins production	*Rhizobium* sp. increased lettuce yield only by 11%	Chabot et al. (1993)
	R. leguminosarum bv. trifolii strain R39	Greenhouse and field trials	Production of auxins and cytokinins	Inoculation increased maize yield up to 10%	Hoflich et al. (1994)
	Rhizobium leguminosarum bv. phaseoli	Laboratory and pot trials	P-solubilization	Strain P31 increased the maize shoot dry matter by 8% with full dose of P fertilizer, strain P31 increased lettuce dry matter by 15% with half P dose	Chabot et al. (1996)
	R. leguminosarum bv. Phaseoli	Pot trial	P-solubilization and root colonization	Inoculation improved maize yield up to 30% in high P soil	Chabot et al. (1998)
	Bradyrhizobium japonicum	Greenhouse	Not described	Strain 532C increased the shoot dry matter yield by 8.66%	Prevost et al. (2000)
	Rhizobium strain NCCB 100053, 100054	Greenhouse	Low nutrient solubilization	Plant growth not affected or was inhibited	El-Tarabily et al. (2006)
	Mesorhizobium ciceri, *Rhizobium leguminosarum*, *Rhizobium phaseoli*	Axenic conditions	Not described	*Rhizobium* sp. increased the root/shoot length and seedling biomass up to 48, 21 and 61%, respectively	Mehboob et al. (2008)
Barley	*Bradyrhizobium japonicum*	Hydroponic conditions	Not described	Barley root length and fresh weight by 12.9 and 6.3%, respectively	Carletti et al. (1994)

(continued)

Table 26.1 (continued)

Host plant	Rhizobium	Growing conditions	Proposed mechanism(s) of action	% increase over control	Reference
	R. leguminosarum bv. Trifolii strain R39	Greenhouse and field trials	Production of auxins and cytokinins	Strain R39 resulted in higher yield i.e. up to 16%	Hoflich et al. (1994)
	Mesorhizobium mediterraneum	Growth chamber	P-solubilization	Plant biomass by 56% and P uptake by 100%	Peix et al. (2001)
	Rhizobium radiobacter strains 204	Laboratory and pot trials	Production of indole-3-acetic acid and gibberellic acid	Shoot and root length by 63 and 37%, respectively	Humphry et al. (2007)
	Bradyrhizobium japonicum strain 532C	Laboratory	Lipo-chitooligosaccharides and Gibberellins	Barley germination was increased up to 44%	Miransari and Smith (2009)
Brassica campes-tris/napus	*Rhizobium leguminosarum*	Gnotobiotic conditions	Production of indole-3-acetic acid and cytokinin	Inoculation promoted seedling growth of *Brassica*	Noel et al. (1996)
	Mesorhizobium loti strain MP6	Laboratory and field trials	Biocontrol, production of Hydrocyanic acid, indole-3-acetic acid, and P-solubilization	Grain yield by 52%, *Sclerotinia sclerotiorum* was reduced by 75%	Chandra et al. (2007)
Lettuce	*Rhizobium* sp.	Laboratory and field trials	P-solubilization, Siderophores and auxins production	*Rhizobium* sp. increased lettuce yield by 11%	Chabot et al. (1993)
	Rhizobium sp. strains 33 and 45	Laboratory and greenhouse	Indole-3-acetic acid and P-solubilization	Inoculation showed higher weights of lettuce plantlets	Pena and Reyes (2007)

Sorghum	*Bradyrhizobium japonicum* strain (Tal 110), *Sinorhizobium meliloti* strain 1, R.I. bv. viciae Cn6 and R.I. bv. viciae strain 30.	Leonard jars	Production of indole-3-acetic and nutrient solubilization	Inoculation promoted sorghum shoot fresh growth by 11-51% and shoot dry weight by 8-55%. Shoot P and K by 17-250%	Matiru et al. (2005)
Cotton	*Rhizobium leguminosarum* bv. trifolii (E11)	Growth room conditions	Production of indole-3-acetic acid	Strain E11 increased root dry weight, shoot dry weight, biomass and N uptake by 248, 48, 75 and 57%, respectively	Hafeez et al. (2004)
Sunflower	*Rhizobium* sp. strain (YAS34)	Pot trial	Production of exopolysaccharides	Strain YAS34 showed up to 50 and 70% increase in shoot and root diameter in normal and water stressed- conditions, respectively	Alami et al. (2000)
Radish	*Rhizobium, Bradyrhizobium*	Growth cabinets/ Greenhouse	Production of indole-3-acetic acid and siderophores, P-solubilization	On an average *Rhizobium* and *Bradyrhizobium* improved 25% radish dry matter	Antoun et al. (1998)
Potato	*Rhizobium etli* strain G12	Pot trial	Biocontrol	Growth of potato improved, Nematode infection reduced by 43%	Reitz et al. (2000)
Tomato	*Bradyrhizobium japonicum*	Hydroponic conditions	Not described	Total root length of tomato increased by 87%	Carletti et al. (1994)

acid and cytokinin (Noel et al. 1996; Chabot et al. 1996; Pena and Reyes 2007) and with the increased mineral uptake (Han and Lee 2005). Hoflich et al. (1994) assessed the growth improvement of barley plants by rhizobial inoculation via mobilization of phosphorous by the inoculants. Similarly, Peix et al. (2001) reported a significant improvement in barley yield and uptake of P, N, K, Ca and Mg upon inoculation with *Mesorhizobium mediterraneum* strain. Growth improvements in barley have also been observed by Lynch and Clark (1984) and Humphry et al. (2007) via growth promoting molecules (such as gibberellic acid) released by rhizobia upon inoculation. Inoculation of sunflower with *Rhizobium* sp. promoted its growth via enhanced uptake of nitrogen (Alami et al. 2000). Increase in radish dry matter yield with *Bradyrhizobium japonicum* was recorded by Antoun et al. (1998). Increased growth of sugar beet via indol-3-acetic acid has been reported by Loper and Schroth (1986) when they used rhizobia as inoculant to these plants. Induced growth and yield of *Brassica campestris* by inoculation with *Mesorhizobium loti* MP6 strains has been demonstrated by Chandra et al. (2007). They recorded the production of indole acetic acid and phosphate solubilization ability of the strains. *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* promoted cotton growth (Hafeez et al. 2004) through efficient uptake of nutrients (Egamberdiyeva et al. 2004). Matiru et al. (2005) investigated growth promotion of sorghum and finger millet seedlings as cultured aseptically in Leonard jars as a result of inoculation with *Rhizobium* spp. They also reported the production of indole acetic acid by the strains examined.

Finally, it could be inferred that rhizobia possess the characteristics on the basis of which it could be used safely and economically as biofertilizer and hence can improve growth and yield of non-legumes through biological nitrogen fixation, hormonal substances and increased availability of plant nutrients.

4.2 Bioprotection

Any condition under which a practice whereby survival or activity of a pathogen is reduced through the agency of another living organisms (except by man himself) resulting in the reduction of incidence of disease caused by pathogens, is termed as biocontrol (Garrette 1965). Whereas, Handelsman and Stabb (1996) defined the biocontrol as a process which involves harnessing of disease-suppressive microorganisms to improve plant health.

More simply, it could be said that biocontrol is a process by which one living organism (which is beneficial/PGPR) is used to limit the growth and propagation of another (which is undesirable one/pathogen) or a pathogenic organism is maintained at low inoculum density or is controlled or eradicated by beneficial organisms.

Several PGPR strains of rhizobia serve as potential biocontrol agents and exhibit numerous mechanisms of biocontrol. These mechanisms include competition (Arora et al. 2001), production of metabolites, which affect the pathogen directly such as antibiotics (Schwinghamer and Belkengren 1968; Antoun et al. 1978; Chakraborty and Purkayastha 1984; Deshwal et al. 2003; Bardin et al. 2004;

Chandra et al. 2007), cell wall degrading enzymes (Ehteshamul-Haque and Ghaffar 1993; Ozkoc and Deliveli 2001), siderophores (Schwyn and Neilands 1987; Derylo et al. 1994; Dudeja et al. 1997; Carson et al. 2000; Deshwal et al. 2003), and HCN (Wei et al. 1996; Antoun et al. 1998; Vidhyasekaran and Muthamilan 1999; Chandra et al. 2007). Some of the PGPR strains of rhizobia do not produce metabolites against the pathogens rather they induce systemic resistance in the host plant (Yanni et al. 2001; Peng et al. 2002; Mishra et al. 2006; Singh et al. 2006) which account for the disease protection.

A variety of rhizobial strains have been used as biocontrol agents for disease/pathogen suppression of various non-leguminous plants. *Bradyrhizobium japonicum, Rhizobium meliloti* and *Rhizobium leguminosarum* were used to control okra and sunflower pathogen such as *Macrophomina phaseolina, Rhizoctonia solani, Fusarium solanik* and *Fusarium solani* (Ehteshamul-Haque and Ghaffar 1993). Whereas, Siddiqui et al. (1998, 2000) used *Rhizobium trifolii* and *Bradyrhizobium* sp. to control the root rot of the same crops i.e. okra and sunflower. Reitz et al. (2000) used *Rhizobium etli* strain G12 as biocontrol agent against the Cyst nematode (*Globoderma pallida*) of potato. Bardin et al. (2004) used *Rhizobium leguminosarum* bv. *viciae* as biocontrol agent against sugar beet pathogen *Pythium* causing damping-off disease. Likewise, Chandra et al. (2007) demonstrated that *Mezorhizobium loti* can be used to reduce the incidence of white rot disease of *Brassica campestris* when applied as a biocontrol agent. Also, the ability of *Sinorhizobium meliloti* and *Rhizobium trifolii* to be used as a biocontrol agent against root rot/knot diseases and a pathogen *Fusarium oxysporum* of sunflower and tomato have been demonstrated (Antoun et al. 1978; Siddiqui et al. 1998; Shaukat and Siddqui 2003). Whereas, *Bradyrhizobium japonicum, Rhizobium leguminosarum* and *Sinorhizobium meliloti* have also been reported as successful biocontrol agents against fungal pathogens belonging to the genera *Fusarium*, *Macrophomina* and *Rhizoctonia* (Ehteshamul-Haque and Ghaffar 1993; Ozkoc and Deliveli 2001). Arora et al. (2001) reported inhibition of *Macrophomina phaseolina,* a widely occurring plant pathogen, by the siderophores produced by rhizobia. Whereas Tu (1978), Ehteshamul-Haque and Ghaffar (1992) and Siddiqui et al. (2000) revealed that *Bradyrhizobium japonicum* can control a disease of mustard and sunflower known as root rot when used as biocontrol agents, whereas Parveen and Ghaffar (1991), Parveen et al. (1993) and Ehteshamul-Haque et al. (1996) elaborated that *Rhizobium meliloti* can control root knot nematodes in okra. Likewise, Mishra et al. (2006) used *Rhizobium leguminosarum* bv. *phaseoli* strain RRE6 and *Rhizobium leguminosarum* bv. *trifolii* strain ANU843 for the control of rice plant pathogen known as *Rhizoctonia solani*.

The ability of rhizobia to protect the non-leguminous plants through induction of systemic resistance against pathogens has also been recognized by various researchers. Mishra et al. (2006) have reported the *Rhizobium leguminosarum* bv. *phaseoli* and *Rhizobium leguminosarum* bv. *trifolii* who protected the rice plants during pathogenic stresses. A large number of researchers have used rhizobial inoculants to control fungal diseases via induction of systemic resistance in various non-leguminous plants such as okra, sunflower and potato (Ehteshamul-Haque and Ghaffar 1993; Nautiyal 1997, 2000; Reitz et al. 2000).

Conclusively, rhizobia could be used as biocontrol agent against some pathogens of non-legumes. However, there is a need to test this potential of rhizobia against a wider range of phytopathogens.

4.3 Phytoremediation

Land fill, thermal treatment, excavation, acid leaching and electroreclamation are the methods generally used to remediate the contaminants. The great problems in their practical application are large destruction of soil structure and fertility, their high dependence on the contaminants of concern, soil properties, site conditions, their high cost, low efficiency, and so on. Hence, for the heavy metal contaminated soils, the development of phytoremediation strategies is necessary (Chaney et al. 2000; Jing et al. 2007; Khan et al. 2009; Shao et al. 2010), because these are *in situ*, cost-effective, non-intrusive, environment caring, aesthetically pleasing and socially usual (Alkorta and Garbisu 2001; Garbisu et al. 2002).

Actually, phytoremediation includes processes (physical, chemical and biological) by which the plants sequester, extract, and/or detoxify the contaminants (Cunningham et al. 1996; Wenzel et al. 1999). Phytoremediation technologies can be applied to clean up and/or stabilize both organic and inorganic contaminants but several barriers (such as evapotranspiration of volatile contaminants, phytotoxicity of the contaminants and/or degradation intermediates of the contaminants) are still there in field-scale use of these technologies (Gerhardt et al. 2009; Weyens et al. 2009). To overcome these barriers plant growth promoting rhizopheric and endophytic bacteria having appropriate characteristics can be used (Zhuang et al. 2007). Moreover, plant growth promoting rhizobacteria are important component of phytoremediational technology (Khan et al. 2009; Glick 2010) and provide high efficiency for phytoremediation when used in combination with plants, because they increases the availability and mobility of the contaminants via redox changes, acidification, phosphate solubilization and through releasing chelating agents (Abou-Shanab et al. 2003) on one hand, while on the other hand, they exert beneficial effects on the growth and development of plants (Hoflich et al. 1995; Noel et al. 1996; Yanni et al. 1997) by producing phytohormones, increasing solubility and availability of nutrients, inhibiting the growth of plant pathogens via competition, antibiosis, parasitism and by changing the host plant susceptibility by producing various biostimulatory agents (Breil et al. 1993; Dazzo et al. 2000; Arora et al. 2001; Ozkoc and Deliveli 2001; Dakora 2003; Matiru et al. 2005; Van loon 2007). Furthermore, literature suggests that certain rhizobacterial strains containing ACC deaminase activities could be used successfully to ameliorate the stresses of phytoremediator plants which they experience during remediation of contaminated soils and promote their growth (Glick and Pasternak 2003; Glick 2010). Also, Dell'Amico et al. (2008) demonstrated that bacterial strains can alleviate the inhibitory effects of heavy metals through the production of IAA, siderophores and ACC deaminase activity. In another study, Burd et al. (1998, 2000) stated that ACC deaminase and siderophore producing

plant growth promoting bacteria can help plants to overcome many of the effects of high levels of metal. So, as rhizobial strains have the ability to improve the biomass through the production of IAA, siderophores, increasing nutrient uptake and via ACC deaminase activity of the inoculated plants explained as above in section-II hence could be tried in association with phytoremediator plants to improve their phytoremediation efficiency.

4.4 Stress Regulation

Environmental stresses such as salinity, drought, and heat affect significantly the crop yields (Mendelsohn and Rosenberg 1994; Kibblewhite et al. 2008). Likewise, heat stress is a severe threat to world agriculture (Mendelsohn and Rosenberg 1994). An accelerated production of ethylene in soil and plants have been reported under various types of stresses such as heavy metals (Reed and Glick 2005), waterlogging (Bradford and Yang 1980), drought (Apelbaum and Yang 1981), and salt (Nadeem et al. 2009, 2010). Plant growth promoting rhizobacteria having ACC deaminase activity are effective in promoting plant growth under these stress conditions by lowering the ethylene whose higher levels have inhibitory effect on root and shoot growth (Wang et al. 2000; Grichko and Glick 2001; Nie et al. 2002; Mayak et al. 2004a, b; Nadeem et al. 2007, 2009; Arshad et al. 2008; Zahir et al. 2009). There are several reports of ACC-deaminase activity in *Rhizobium leguminosarum* and *Mesorhizobium loti* (Belimov et al. 2001, 2005; Ma et al. 2003a, b; Sullivan et al. 2002). Also, it has been reported that rhizobial inoculation can alleviate the effect of water stress by altering leaf stomatal conductance, transpiration, photosynthetic capacity and root morphology of the inoculated non-legume plants (Phillips and Torrey 1970; 1972; Phillips et al. 1999; Figueiredo et al. 1999; Alami et al. 2000; Anyia et al. 2004; Matiru and Dakora 2005a; Chi et al. 2005).

No doubt, the above few demonstrations revealed that rhizobia having ACC-deaminase can protect non-legumes against various stresses, yet the extensive research is required to explore the details of the mechanisms involved.

5 Conclusion and Future Prospects

The rhizobial secretions are an important way for non-leguminous plants to respond and alter their environment. Over the last several years, research and technical advances have provided a better understanding of rhizobial strains association and promotion in non-legumes growth. These advances could be applied to agricultural systems to enhance production by increasing crop growth through combinations of biological nitrogen fixation, releasing phyto-effective substances, increasing defense responses against soil-borne pathogens, improving resistance in plants against biotic and abiotic stresses and/or for further favoring in adequate colonization, adaptability under variable soil environments or against all pathogens that attack the host plant

through association with other beneficial soil microbes. Also, remediation efforts have further contributed in improving understanding of rhizobial role under contaminated environments. Hence, switching from growing non-leguminous plants to the cultivation of plant-rhizobial communities in order to reach a high productivity under minimal energy and chemical investments and with minimal pressures on the environment is an applicable technology. Accordingly, more research is needed on genetics, molecular biology and ecology of rhizobia and non-leguminous plants to improve the performance and use of rhizobia as bacterial inoculants of non-legumes as well as for attaining sustainable site-specific rhizobial-based agro technologies. So, future research should be focused on:

1. The study of signal-exchange between rhizobia and non-leguminous plants for the understanding of mechanisms affecting colonization.
2. As such, attempts on mechanisms clearing up the synergistic interaction are required so as to discover the biochemical basis of interactions between rhizobia and non-leguminous plants.
3. Additionally, the approach to improve plant growth promoting characters of rhizobial PGPR strains by genetic manipulation through recombinant DNA technology offer charm for investigations.
4. The use of potent and crop-specific promoters that could be activated under the specific soil and environmental conditions is another important approach to successful gene expression in engineered strain that invites the researchers towards its study.
5. Also, the approach of selection of mutants with increased production of phyto-effective molecules by classical genetic methods cannot be underestimated.
6. Attention should also be given to investigations and application of new combinations of rhizobia and other PGPR for improved results.
7. The conditions are yet to be determined which can help in extending the ability of rhizobia for endophytic nitrogen fixation in non-legumes.
8. Rigorous research is also required on the assortment of native or genetically manipulated rhizobial strains showing both ACC-deaminase and other contaminant-specific genes for evolving more valuable phytoremediation schemes.
9. Even more, work on the option of strains useful equally to non-legumes and legumes, especially in the crop rotation systems is also needed.

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Chapter 27
Effect of Drip and Subsurface Drip Irrigation with Saline Water on Tomato Crop

Besma Kahlaoui, Mohamed Hachicha, Saloua Rejeb, and Mohamed Néjib Rejeb

Contents

Abstract With the purpose of improving salinity management and water use efficiency in agriculture, an experiment was carried out to study the effect of surface drip irrigation (DI) and subsurface drip irrigation (SDI) on a tomato crop (*Lycopersicon esculentum* Mill, cv. Heinz-2274) in a silty clayey soil with three irrigation water qualities: 3.0, 6.0 and 8.3 dS m^{-1}. The results did not show any difference in the crop response of the two irrigation systems, whereas, the effect of the water quality was manifested. Saline water irrigation affected the tomato growth, in particular leaf area, dry matter, as well as the shoot/root ratios and the mineral composition of leaves, stems and roots. The accumulation of Na^+ and Cl^- was associated with a

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decrease in the contents of Ca^{2+}, K^{+} and Mg^{2+}, whereas, the P content in different organs remained constant. The more the salinity of the irrigation water the more marked was the decrease in yield parameters of tomato (setting, size, and yield). This experiment shows that at normal water management (100% of crop water requirement), the SDI does not present additional advantages as compared with the DI for water whose electrical conductivity is not higher than 8.3 dS m^{-1}.

Keywords Drip irrigation • Subsurface drip irrigation • Saline water • Tomato • Silty clay soil • Tunisia

1 Introduction

Many studies addressed to more suitable irrigation systems to face water salinity problem. Indeed, aridity and water scarcity compel several countries to use saline water for irrigation, to try to optimize the use of their water resources in particular in agriculture, and to reduce water used at the farm scale. In Tunisia, despite the primary soils salinization, which concerns about 1.5 millions of hectares (about 10% of the country area), about 30% of irrigated area (400,000 ha) is affected by the secondary salinization. This salinization is generally induced by irrigation with saline water; as a matter of fact, more than 30% of the Tunisian water contains more than 3 g of salt L^{-1} (Hachicha 2007).

Moreover, as in the other Mediterranean regions, the tomato crop is economically the most important vegetable. In 2006, the cultivated area of tomato in Tunisia was about 16,800 ha. This crop is generally irrigated by submersion, but drip irrigation has become more and more widespread since the beginning of the 1990s. Drip irrigation (DI) allows a low and frequent application of water directly at the root zone (Tariq et al. 1990) and a better efficiency in comparison with surface irrigation (Bogle et al. 1989). However, irrigation with saline water by DI produces an accumulation of salt at the soil surface (Ayers and Westcot 1985; Rajak et al. 2006). According to Hanson and May (2004), salts in DI accumulate at the surface soil before migrating and reaching the root zone. To reduce this effect of salts, the subsurface drip irrigation (SDI) was developed. According to Phene et al. (1990) and Oron et al. (1998), the SDI decreases the accumulation of salts within the root zone, generating an improvement in yield and fruit quality. This was observed for tomato (Ayars et al. 2000; Hanson and May 2004), onion (Enciso et al. 2007), cotton (DeTar 2007) and bean (Gençoğlan et al. 2006). SDI improves water use efficiency (Taylor et al. 2005) by allowing the application of a small quantity of water directly at the root zone and maintaining this layer at a suitable soil moisture (Enciso et al. 2007), thus reducing the effect of the saline stress (Incrocci et al. 2005).

Salinity is a constraint which affects the growth, flowering and fructification (size reduction and apical necrosis) of several crops, which has repercussions on their yield and quality of their fruits. This was observed for tomato (Karlberg et al. 2007; Tuna et al. 2005; Flowers et al. 2005). According to Flowers et al. (2005),

improvement of salinity tolerance of tomato irrigated with saline water by DI results from the increase in irrigation frequency, which reduces the accumulation of salt in the soil, as well as from the vacuolar compartmentalization of the toxic ions, in particular of Na^+ and Cl^-. The increase in salinity involves an increase in Na^+ concentration. This toxic element competes with the major elements like, Ca^{2+} and K^+ (Juan et al. 2005; Maggio et al. 2006) and causes a nutritional disturbance inhibiting the synthesis of proteins, as well as an inactivation of enzymes (Gaines and Shennan 1999; Fernández-García et al. 2002). The objective of this work was to compare the effect of the DI and SDI systems of irrigation in relation to three different salt concentrations of irrigation water on open field cultivated tomato.

2 Material and Methods

2.1 Material and Treatments

The experiment was carried out at the Agricultural Experimental Station of Cherfech, situated in the Mejerda Low Valley, 25 km North of Tunis. The climate is Mediterranean with an average annual rainfall average and an annual evapotranspiration average 470 and 1,370 mm, respectively (Penman method). The soil has a silty-clay texture, xerofluvet with 45% of clay, 45% of silt, 10% of sand, about 1% of organic matter and 20% of calcareous. The irrigation network comprised a water basin and a main pipeline. The DI equipment was constituted of 30 cm depth buried pipelines with filter protected emitters every 40 cm. The same pipelines were used for SDI. The crop was a seasonal tomato (*Lycopersicon esculentum* Mill) variety 'Heinz-2274' adapted to the mechanical harvest. The tomato seedlings were planted at the 4-leaf stage, in simple lines, on May 27, 2005. The lines were 1 m spaced out. The parcel was divided into three blocks, one for each quality of water (Fig. 27.1). There were six planting rows in each block (three lines per treatment). Every line included 27 plants: lines 1, 3 and 5 were for the DI treatment, whereas lines 2, 4 and 6 for the SDI treatment. The irrigation waters came from Mejerda River (3 dS m^{-1}), a shallow well (8.3 dS m^{-1}) and a mixture of these two waters at the rate of 50% for each (6 dS m^{-1}). These waters are rich in NaCl. Their SAR was 7, 10.7 and 6 respectively. The chemical characteristics are presented in Table 27.1.

Soil salinity analysis: Soil salinity was determined before installation and at the end of the crop cycle at the roots level and every 20 cm up to 60 cm depth. The soil/water ratio was 1/5. A significant relationship permitted to convert the electrical conductivity EC (1/5) to the electrical conductivity of the saturated paste extract (ECe) (Jammazi and Hachicha 2002) (Table 27.2).

$$ECe = 5.853 * EC(1/5) - 0.262 \quad n = 134 \text{ and } R^2 = 0.91$$

$$\text{with } 1.2 < ECe < 8.3 \text{ dS m}^{-1} \text{ and } 0.2 < EC(1/5) < 1.4 \text{ dS m}^{-1}$$

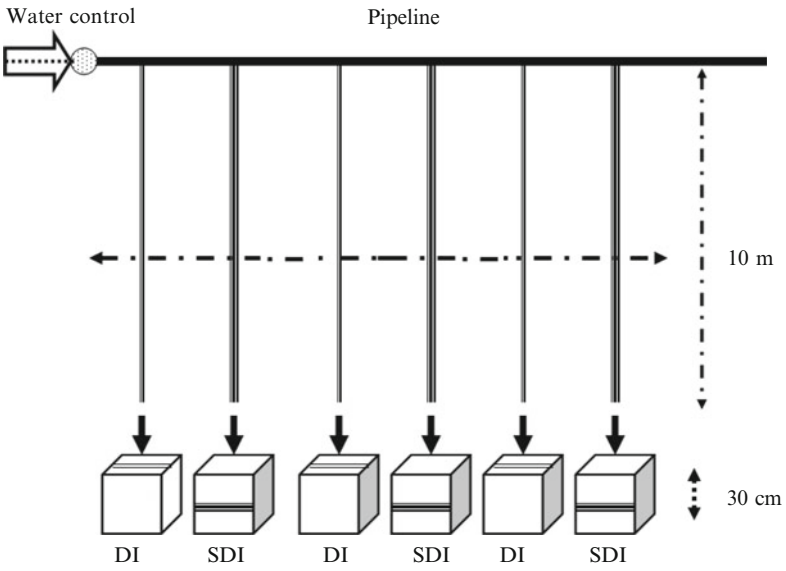

Fig. 27.1 Representation of the experimental device for every quality of irrigation water

Table 27.1 Chemical analysis of three qualities of waters used for the irrigation of tomato

				Anions (me L^{-1})			Cations (me L^{-1})				
Water quality	pH	EC (dS m^{-1})	TDS (g L^{-1})	HCO^{3-}	SO^{4-}	Cl^{-}	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}	SAR
Mejerda	7.5	3	2	0.8	4.1	19	8.1	5.6	0.2	18.3	7
Well	7.2	8.6	6	1.2	27.6	56.8	23	19.1	0.8	49	10.7
Mixture	7.4	6	4	2	8.4	44	18.6	13.5	1.7	34	6

EC electrical conductivity, *TDS* total dissolved solids

Table 27.2 Analysis of variance (ANOVA) of the soil salinity at the end of the cycle of irrigation

	Variables	ECe (dS m^{-1})	STD
System of irrigation	DI	4.9 a	1.72
	SDI	5.1 a	1.85
Water quality	Mejerda	2.9 c	0.42
	Well	4.9 b	0.44
	Mixture	7.1 a	0.35
Depth (cm)	0–20	4.9 a	1.78
	20–40	5.1 a	1.77
	40–60	4.9 a	1.85

2.2 Crop Determinations and Analyses

The following determinations were made on the tomato crop:

- Length growth: values are the average measured on 15 plants.
- Setting rate: ratio of the number of fruits set to number of flowers bloomed. (A fruit was considered set when its diameter reached 3 cm at least).
- Fresh and dry biomass: plants were harvested, divided into roots, stems and leaves. Roots were cleared of the soil particles and washed with water. Every part was weighed to determine the fresh matter. The dry matter was determined after a drying at 80°C for 48 h.
- Shoot/root ratio: allowed estimation of the allocation of assimilates between absorbing organs and photosynthetic organs.
- Leaf area: it was measured by a planimeter (Delta-T Devices Ltd). It was expressed in cm^2.
- Fruit yield: 90 plants were used to measure yield parameters. The yield was expressed per plant.
- Fruit size: Seven fruit- grade classes were adopted: C0<30 mm, 30 mm<C1< 35 mm, 35 mm<C2<40 mm, 40 mm<C3<45 mm, 45 mm<C4<50 mm, 50 mm<C5<55 mm, 55 mm<C6<60 mm.

Besides these determinations, chemical analyses were done on roots, leaves and stems for six types of elements. The extraction of ions was performed by HNO_3 at room temperature for at least 48 h:

- Dosage of Cl: by colorimetry with Buchler chloridimeter in the presence of acetic acid-nitric acid tampon and gelatin destined to agglomerate the precipitate of AgCl;
- Dosage of P: by colorimetry with vanado-molybdate;
- Dosage of K and Na: by flame- emission photometry;
- Dosage of Ca and Mg: by atomic absorption spectrophotometry;

Data analysis: The software STATITCF Version 5 (Beaux et al. 1991) was used for the statistical analyses of the data. The two-factor analysis of variance was computed by Neuman and Keuls' comparison for all pair of means.

3 Results

The soil study was extensively presented in a separate paper by Ben Necib (2008). Before irrigation, the salinity of soil was lower than 1 dS m^{-1}, and it increased at the end of the irrigation cycle. We observed significant mean differences between the qualities of irrigation water and none between DI and SDI. For the tomato crop, three aspects were studied: growth, yield and mineral composition of the plant.

3.1 Effect on Crop Growth

During the experiment, symptoms attributed to salinity were observed on the plants of tomato. From 4 $g L^{-1}$ of salt, there were tanning as well as, necrosis and coil of leaves, revealing a disruption of the mineral nutrition and this ocurred at the same time for DI and SDI. Leaf area was affected strongly by the salinity: compared to the control (Mejerda water, 2 $g L^{-1}$), the reduction being about 50% for 4 $g L^{-1}$ and 60% for 6 $g L^{-1}$. This effect was thus similar for DI and SDI (Fig. 27.3). Whatever the irrigation system, the salinity had a depressive effect on the fresh matter yield (FM) of different organs of the tomato (Fig. 27.2). This effect was higher as the salinity of the irrigation water increased. The yield reduction became significant from 4 $g L^{-1}$. Compared to the control, the reduction concerning 6 $g L^{-1}$ was about 26% for leaves, 34% for stems and 22% for roots, under all irrigation systems. The same effects were observed for the dry matter (DM). More differences were found for leaves and stems than for roots (Fig. 27.2). The reduction become significant at 6 $g L^{-1}$ for DI and SDI, it was 53% for leaves and 23% for stems. The shoots/roots ratio showed a significant reduction at the significance level of 5% when the salt concentration of the irrigation water was high (Fig. 27.3), passing from 14 for 2 $g L^{-1}$ to 12 for 4 $g L^{-1}$ and 10 for 6 $g L^{-1}$. So, the shoot, in particular leaves, was more sensible to the salinity than the roots. However, no significant difference was observed between DI and SDI.

3.2 Effect of the Salinity on Tomato Yield

Unlike the marked effect of the salinity of the irrigation water, the crop yield did not show any significant difference between DI and SDI. The rate of fruit setting (Fig. 27.4) was reduced by salt. The reduction was significant at the significance level of 5%; it was about 20% at 4 $g L^{-1}$ and 40% at 6 $g L^{-1}$. Concerning the size of fruits (Fig. 27.5), the irrigation with Mejerda water gave the best proportions of marketable fruits of good size. Sizes C4, C5 and C6 were dominant, their proportions being 17%, 23% and 22% respectively for DI, and 20%, 22% and 21% for SDI. Sizes C1, C2 and C3 were more represented in plants irrigated by the mixture of waters (4 $g L^{-1}$). At 6 $g L^{-1}$, the size C0 was the most dominant, with about 40%. In comparison with those irrigated by the well water and the mixture, plants irrigated with Mejerda water had a better fruit setting and a good vegetative development, indicating that an important proportion of assimilates was allocated to fruits. As regards the yield in fresh weight/plant (Fig. 27.4), it was the result of the number of flowers by plant and the percentage of fruit setting. Therefore, factors that affect these two parameters had an impact on yield. The effect of the salinity was significant at the significance level of 5%; an increase in the salinity of the irrigation water induced a reduction in the crop yield.

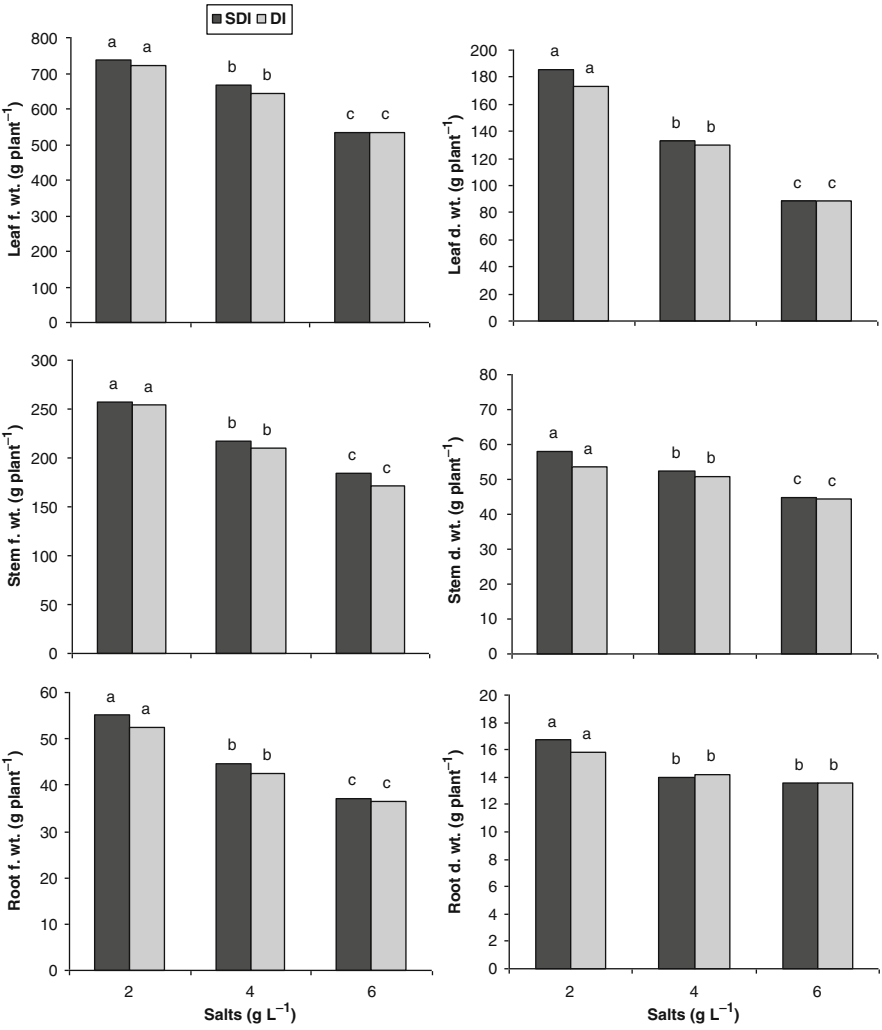

Fig. 27.2 Fresh and dry weight of different organs of irrigated tomato as a function of three salt concentrations of water and two irrigation systems (DI and SDI). Values followed by the same letter are not significant at the 5% level of significance according to the test of Neuman and Keuls

3.3 Effect on the Mineral Composition

Chemical analyses did not show any significant difference between DI and SDI. Conversely, the salinity effect of the irrigation water was manifest. For either irrigation system, DI or SDI, the presence of salt in the irrigation water resulted in an increase of the contents of Na^+ and Cl^- in different organs of tomato (Fig. 27.6).

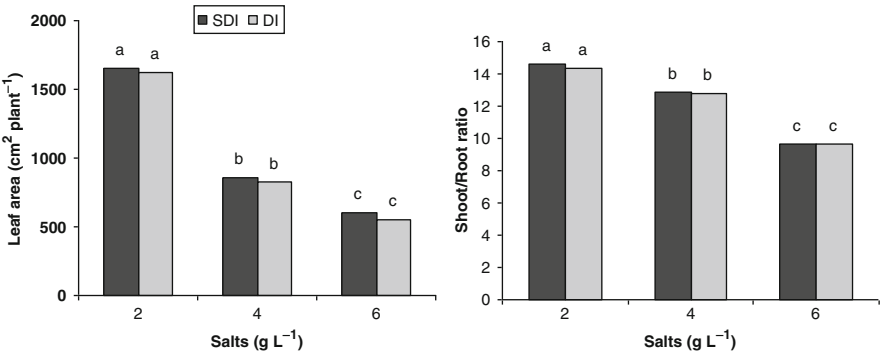

Fig. 27.3 Leaf area and shoot/root ratio of irrigated tomato as a function of three salt concentrations of water and two irrigation systems (DI and SDI). Values followed by the same letter are not significant at the 5% level of significance according to the test of Neuman and Keuls

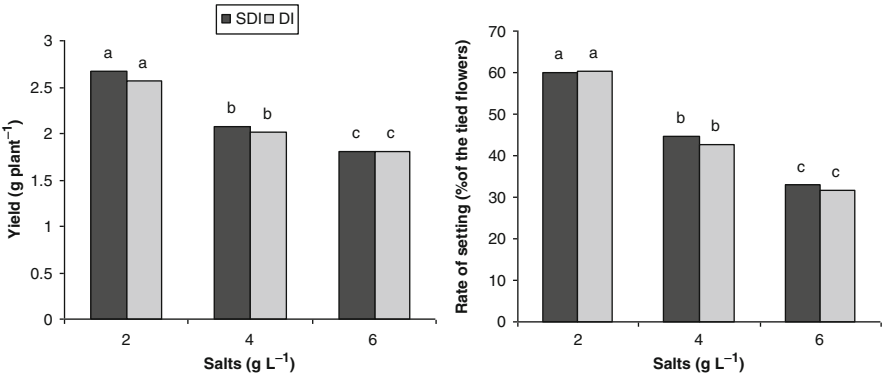

Fig. 27.4 Setting rate and yield of irrigated tomato in relation to three salt concentrations of water and two irrigation systems (DI and SDI). Values followed by the same letter are not significant at the 5% level of significance according to the test of Neuman and Keuls

This increase was higher when the concentration in salts was increased. The presence of salt in the irrigation water induced a reduction of potassium contents for all crop organs (Fig. 27.7). This reduction was proportional to the water salinity. At 6 g L^{-1}, the reduction was 40% for leaves, 11% for stems and 51% for roots. The presence of salt in the irrigation water induced also a reduction in calcium contents for the different organs (Fig. 27.7). This reduction was 34% for leaves and reached 69% for roots. For magnesium, stems and leaves were relatively not affected by the water salinity (Fig. 27.8). Only roots were affected by 4 g L^{-1}. The reduction was manifest at 6 g L^{-1} about 82%. Concerning phosphorus, a small reduction of contents in leaves, stems and roots was observed, but this effect was not significant at the significance level of 5% (Fig. 27.8).

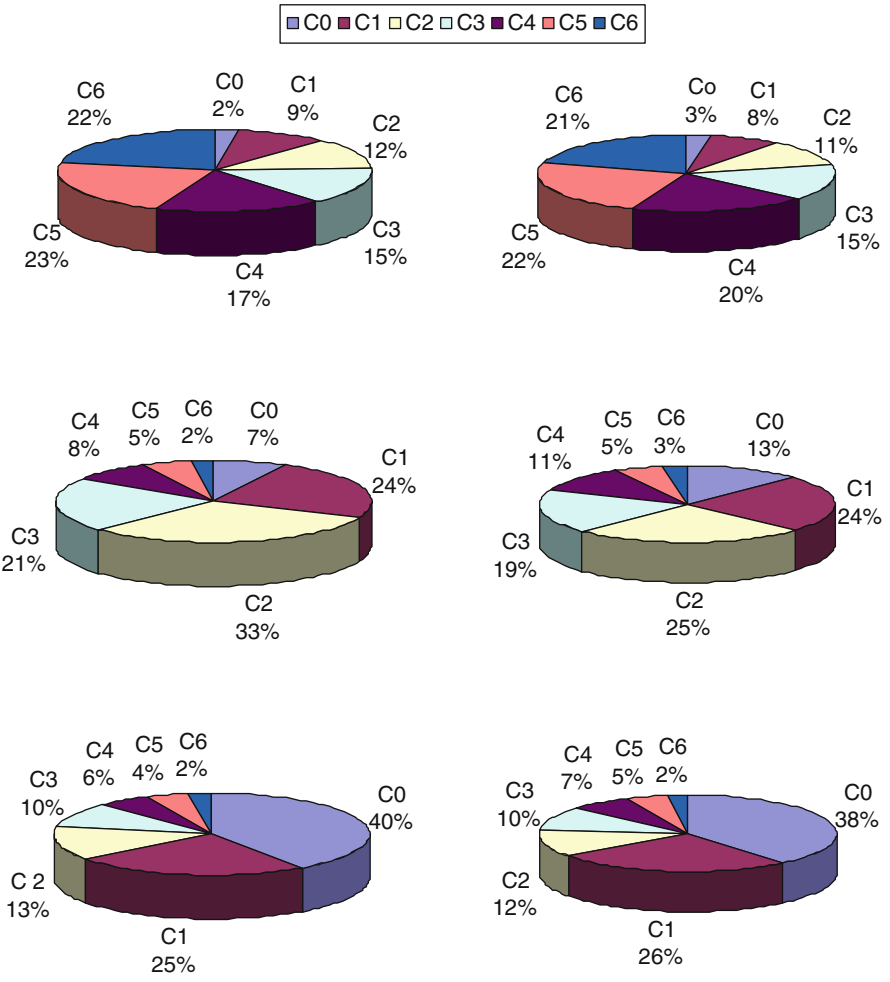

Fig. 27.5 Effect of waters of three salt concentrations (2, 4 and 6 g L^{-1}) and two irrigation systems (DI and SDI) on the fruit diameter of irrigated tomato

4 Discussion

The growth of tomato is affected by the salinity at different degrees, depending on the salt concentration in the irrigation water and the crop organ (Zhang et al. 2004). In the present study, leaf area decreased greatly under saline stress for SDI and DI. This reduction was more marked at 6 g L^{-1}, and it resulted from a general reduction of the crop growth and from the fall of leaves from the bottom part of plants, induced

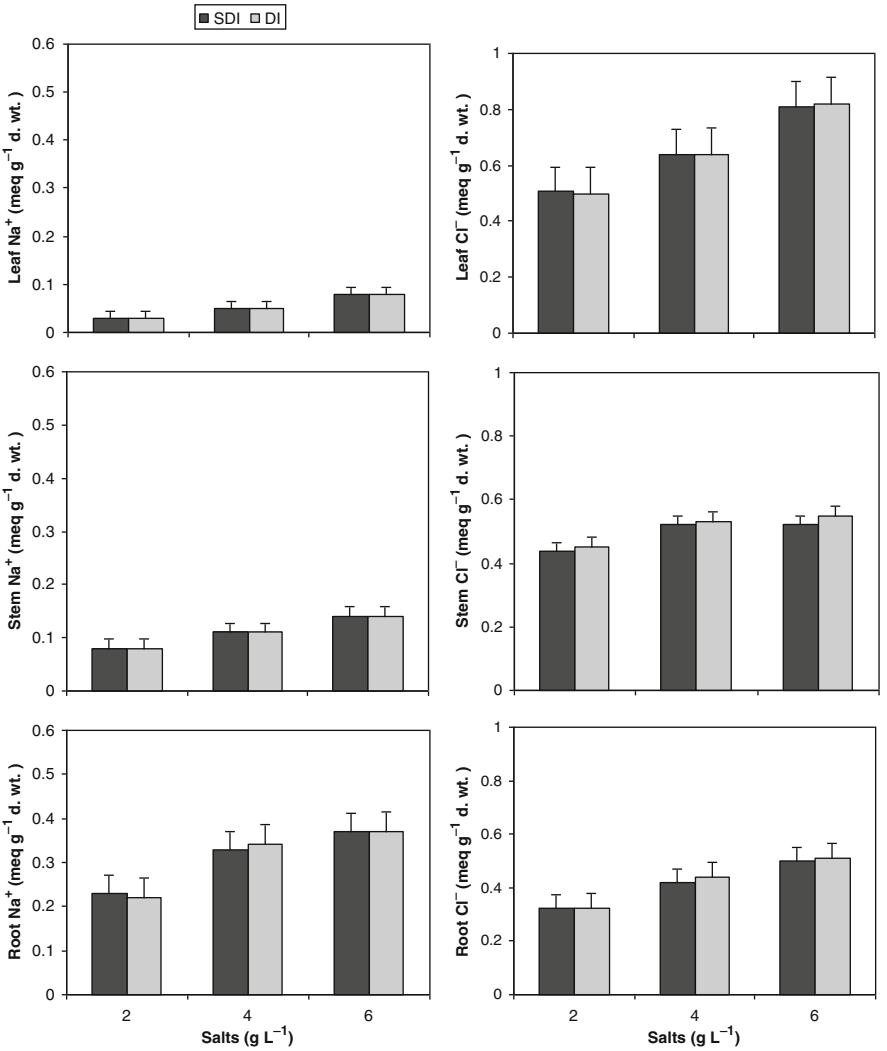

Fig. 27.6 Na^+ and Cl^- content in the dry matter of different organs of irrigated tomato in relation to three salt concentrations of water and two irrigation systems (DI and SDI)

by a premature senescence. These results are analogous to those observed by Taleisnik (1987). However, Karlberg et al. (2007) showed that the increase of salinity in irrigation water (6 gL^{-1}) produced an increase of leaf area of the tomato cultivar Daniella when DI system was used. With regard to the biomass, the results revealed that the salinity limited the growth, whatever the applied treatment may have been. This effect depends on the salt concentration and the type of organ. So, the high salt concentrations (6 gL^{-1}) reduced the biomass significantly. The reduction in growth

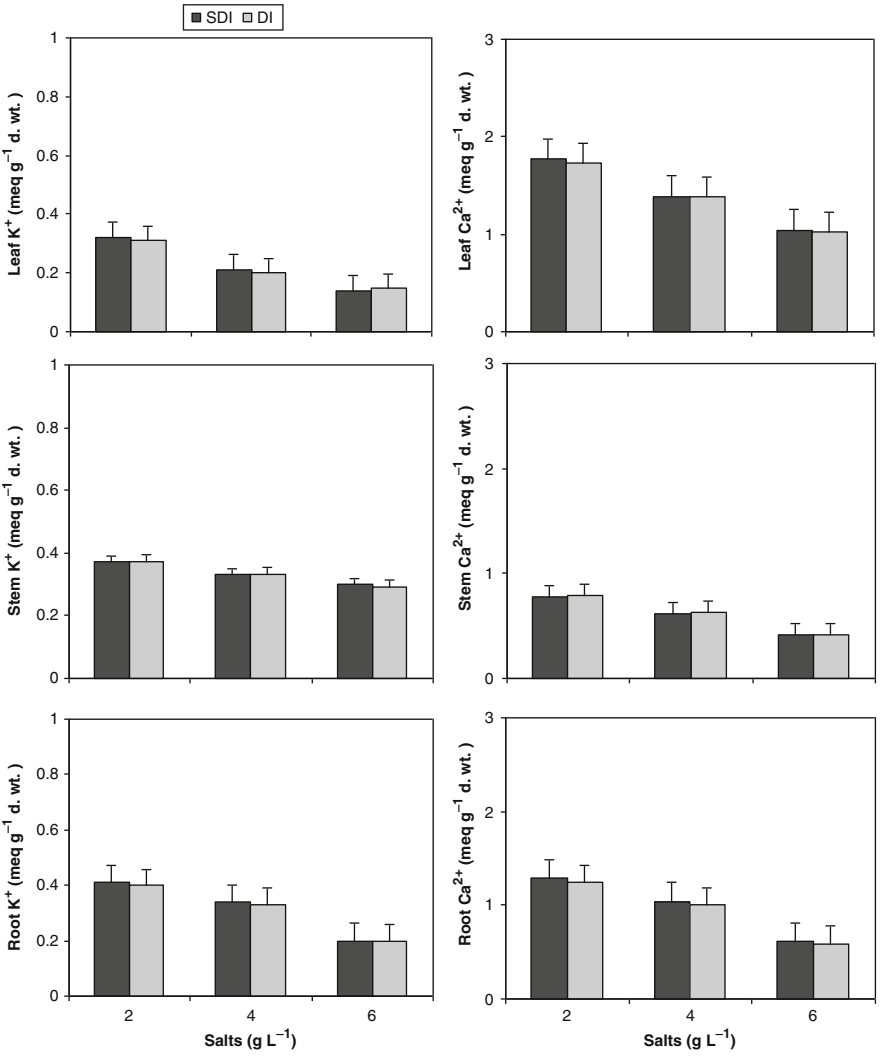

Fig. 27.7 K and Ca content in the dry matter of different organs of irrigated tomato in relation to three salt concentrations of water and two irrigation systems (DI and SDI)

affected all crop organs. Similar results were earlier observed for lentil (Lachaâl et al. 1996). The shoot/root ratio decreased progressively with the increase in salinity. The reduction resulted from the depressive effect of salt on the aerial and root parts. These results are in agreement with those observed by Cornillion et al. (1995) and Maggio et al. (2006). Engel and Marschner (1992) consider that the reduction of this ratio is an adaptive component of plants to environmental constraints; it serves to maintain a functional balance between the shoots and roots by increasing the root

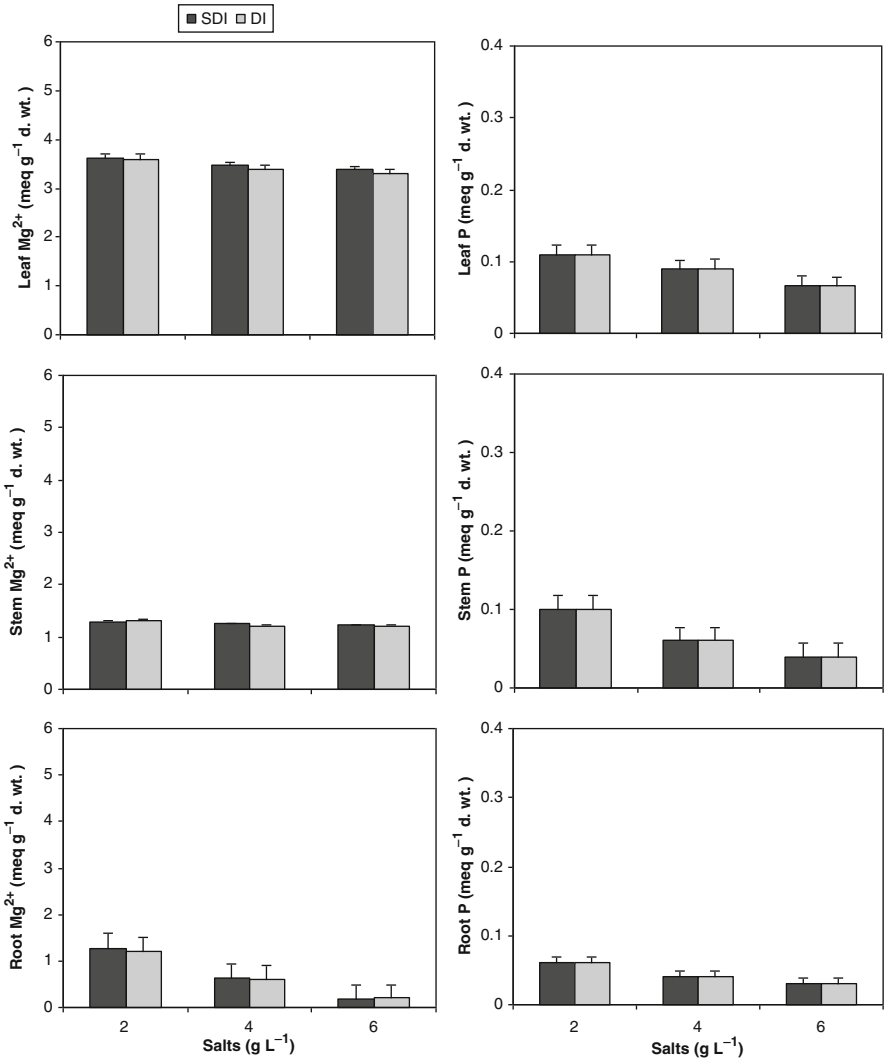

Fig. 27.8 Mg and P content in the dry matter of different organs of irrigated tomato in relation to three salt concentrations of water and two irrigation systems (DI and SDI)

length when their physiological activity is inhibited by external factors. The study of the saline stress effects for the two irrigation systems on flowering and yield of tomato shows that an increase of the irrigation water salinity decreased the agronomic characters at all crop stages (flower number, setting rate, fruits number as well as fruits size). The final consequence is a reduction of the yield. These results are in agreement with those observed for tomato by Perez-Alfocea et al. (1996), Karlberg et al. (2007) and Flowers et al. (2005). No significant difference between

DI and SDI was observed for the pepper cultivar Beldi irrigated by the water of the Mejerda (Hachicha et al. 2006) and for tomato (Incrocci et al. 2005).

The mineral nutrition study of Heinz-2274 tomato variety shows nutritional disruptions in major cations (K^+, Ca^{2+}, Mg^{2+}) and in the P anion according to the salinity for DI as well as for SDI. The significant decrease of all ions content per tissue especially under high salinity level (6 $g\,L^{-1}$) could probably be attributed to the high accumulation of toxic ions (Na^+, Cl^-) in tissue subjected to severe osmotic stress caused by high salinity level (Botia et al. 1998; Roussos et al. 2006). Results concerning the ionic contents of different organs show that Heinz-2274 excluded Na^+ from its leaves because the contents of this ion were low as compared to those of stems or roots. Such results have also been observed by Taleisnik and Grunberg (1994) and Reina-Sánchez et al. (2005) in tomato. They showed that most cultivars of tomato excluded Na^+ from leaves when they were exposed to salt stress. Like Na^+, Cl^- concentration increased in the same way with the increasing salt concentration in the irrigation water. Salt stimulates the absorption of Na^+ and Cl^-. This stimulation induces a reduction of contents in Mg^{2+}, K^+, Ca^{2+} and P (Maggio et al. 2006). Roots being the first organs in direct contact with salts, showed a reduction in Ca^{2+} (about 55-69%) for the studied variety. This significant decrease causes a reduction in Ca^{2+} content at the shoots level (Bernstein et al. 1995). Such reduction is associated with a respiration rate increase and a competitive effect with Na^+ (Cuartero and Fernández-Muñoz 1999). In the same way, a deficiency in K^+ and Mg^{2+} is also in correlation with an excessive accumulation of Na^+, suggesting a competitive effect between these ions and the toxic ion, Na^+ (Juan et al. 2005; Maggio et al. 2006). Therefore, the growth decrease observed was due to a low accumulation of K^+, Ca^{2+}, $Mg^{2+}$and P rather than to a strong accumulation of Na^+ and Cl^- in the tissues.

5 Conclusion

The experiment on tomato crop irrigated with three water qualities by DI and SDI permitted to show a significant decrease of growth parameters particularly with the more saline water (6 $g\,L^{-1}$). This significant decrease refers to leaf area (60%) and biomass of all aerial and root organs, as well as, the shoot/root ratio. The reduction of this ratio was similar for SDI and DI. The depressive effect of salt was more important on the growth of aerial parts than of roots. Concerning mineral nutrition, tomato accumulated more Na^+ and Cl^- in different crop organs in the presence of salt. This high accumulation of these ions induced a reduction in the contents of K^+, Ca^{2+} and Mg^{2+} in different organs, whereas the P content remained constant. The effect of the two irrigation systems on tomato crop was almost uniform.

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Chapter 28
Lipid and Carbohydrate Metabolism of Cowpea (*Vigna unguiculata* L. Walp) Cultivars in Relation to Temperature Stress

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Contents

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Abstract Cowpea (*Vigna unguiculata* L. Walp) is a legume adapted to subtropical to tropical conditions. It is susceptible to chilling temperatures, primarily during the early stages of germination. Twenty five cultivars were screened for germination at low (10°C), moderate (30°C), and at high (40°C) temperatures. Temperatures held at 10°C, <40°C, <30°C had a negative effect on germination percent and coefficient of velocity of germination (CVG). Three cultivars (Texas Cream 40, Black Crowder and Mississippi Purple) were chosen such as 'Texas Cream 40' was able to germinate at very high and low temperatures. 'Black Crowder' demonstrated acceptable germination at high temperatures but negatively affected at low temperature. Cultivar 'Mississippi Purple' obtained low germination percentage and CVG at all temperatures studied. Lipid, sugar compositions, and peroxidase activity were determined in whole ungerminated seed, cotyledon and embryo tissues of the cowpea cultivars. The main sugars present in cowpea seed were sucrose, raffinose, and stachyose. Sucrose contents were higher in the embryo tissue of cultivars with low percent germination, and reduced in the cultivar with higher percent germination suggesting the use of sucrose for germination. Raffinose and stachyose contents were higher in ungerminated seed. In germinated seed, raffinose and stachyose contents were found only in cotyledon tissues at 10°C. The most abundant fatty acids in cowpea seed were palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, and arachidic acid. The results showed that the long-chain fatty acids appear to be important in the cowpea seed germination process. Peroxidase activity was affected by cultivars, type of tissue and temperature. The highest peroxidase activity was found at low temperature (10°C) in embryo tissue of the cultivar with the highest germination. High peroxidase activity was related to ability of seed to germinate at low temperature. Thus, the information provided by this research will facilitate future plant physiological and genetic studies of cowpea as well as other plant species.

Keywords Carbohydrate • Lipid • Peroxidase • Temperature • Seed • Germination • Cowpea

1 Introduction

Cowpea (*Vigna unguiculata* L. Walp), an indigenous African annual legume, is commonly known as southern pea, blackeye pea, crowder pea, lubia, niebe, coupe or frijole, (Craufurd et al. 1996; Hall et al. 1997). It is a high protein (25%), fiber (6.3%) and low fat (1.9%) vegetable crop having good nutritional qualities (Ricardo 1985). It is a chilling sensitive crop (Ehlers and Hall 1997; Hall et al. 1997) and is adapted to warm weather and humid conditions (Craufurd et al. 1996; Hall et al. 1997; Terao et al. 1997; Singh et al. 2003). It is more tolerant than common beans to drought, waterlogging, infertile soils, and acid stress. Cowpea is cultivated for food (Hall et al. 1997), silage, green manure for soil improvement, livestock feed and pasture. Due to its nutritional content, versatility, adaptability, and high yield, cowpea was chosen by the U.S. National Aeronautical and Space Administration as one of the few crops to be tested for cultivation in the space station (Ehlers and Hall 1997). Furthermore, it is one of the mandated crops by the International Institute of Tropical Agriculture (Smartt and Hymowitz 1985; Quin 1997; Fery 2002). Cowpea was a major agronomic crop in the United States during the early part of the twentieth century, with production peaking at 2.4 million ha in 1937 (Fery 1990). By the early 1980s, however, annual cowpea production in the United States was estimated at 80,000 ha (Fery 1981). Cowpea has long been valued in the southern United States as a vegetable crop, and an extensive industry currently distributes fresh, canned, frozen, and dry-pea products nationwide. Additionally, cowpea has long been a popular item with home gardeners throughout the south. There is a broad range of characteristics among cowpea cultivars grown for horticultural use in the United States (Fery 1990, 2002; Islam et al. 2005, 2006).

Cowpea has been reported to have greater adaptation to high temperatures than any other crop species. Germination greater than or equal to 80% has been observed in cowpea at temperatures ranging from 10°C to 40°C (Ismail et al. 1997, 1999; Terao et al. 1997). Temperatures outside this range adversely affect germination of cowpea. Variation in germination percentage and rate of germination under stress temperatures have been observed among cowpea cultivars (Marsh 1993; Craufurd et al. 1996). Temperature plays an important role during seed germination and initial root growth. The rate of seed germination and seedling emergence is determined by temperature (Craufurd et al. 1996). As a chilling-sensitive crop, cowpea is most susceptible during germination and early seedling development. If cowpea is planted late in the spring when temperatures are still low in subtropical areas, seed germination is hindered. Good stand establishment is necessary to avoid loss of yield, thus it would be beneficial to plant a cultivar able to tolerate low temperatures.

A wide range of cellular responses occur when seeds are exposed to different temperatures, including adjustments in the level of fatty acids. Lipid contents have been related to seed vigor in some seed (Perl et al. 1987). Seed of broad bean (*Vicia faba* L.), evening primrose (*Oenothera Lamarckiana* De Varies), and carrot (*Daucus carota* L.) showed a drop of approximately 20–30% in the lipid fraction when subjected to accelerated aging; however, in cucumber (*Cucumis sativus* L.), squash

[*Praecitrullus fistulosus* (Stocks) Pang], and pea (*Pisum sativum* L.) seed, an increase in lipid content was observed. In pepper (*Capsicum annum* L.) seed, the lipid content did not change with seed vigor level, but noticeable changes in fatty acid composition were observed. In the labeling of the lipids present in pea seed, phospholipids (70%), triglycerides (20%), waxes (8%), monoglycerides (2%), and diglyceride were detected (Harwood and Stumpf 1970). Free fatty acids were seldom detected. The most common fatty acids in the phospholipids were palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids. The longer chain fatty acids were more common in the wax fraction. The endogenous fatty acids present in the seed were not always related to those synthesized during germination (Harwood and Stumpf 1970). In germinating seed, *de novo* synthesis of long chain fatty acids occurs at early stages of germination. Fatty acids including palmitic (C16:0), stearic (C18:0), oleic acid (C18:1), linoleic (C18:2), and linolenic (C18:3), have been detected in cotyledons and axes of water oak (*Quercus nigra* L.), white oak (*Quercus alba* L.), americam muskwood [*Guarea guidonia* (L.) Sleumer and *Carapa guianensis* Aubl.], silver maple (*Acer saccharinum* L.), and red buckeye (*Aesculus pavia* L.) (Connor et al. 1996, 1998, 2000; Connor and Bonner 1998, 2001; Connor and Sowa 2000). Significant changes in the amounts of individual fatty acids in the seed, and the subsequent changes in the total fatty acid content have been reported in these species.

Seed vigor of seed has also been associated with carbohydrate content. In corn (*Zea mays* L.), the decline of sucrose and raffinose contents during aging was closely correlated with the loss of ability to germinate and rate of germination (Bernal-Lugo and Leopold 1992). Furthermore, the quantity of oligosaccharides larger than sucrose ($C_{12}H_{22}O_{11}$) decreased as the period of imbibition during the germination process increased (Koster and Leopold 1988). As a chilling-sensitive crop, cowpea is affected during germination and early seedling development if planted late in the spring when temperatures are still low in subtropical areas. Good stand establishment is necessary to avoid yield loss, thus it would be beneficial to plant a cultivar able to tolerate low temperatures. The objectives for this research were to determine the carbohydrate and fatty-acid metabolism of the cowpea seed prior to and during germination and evaluate the peroxidase enzyme activity in cowpea seed and its effect on temperature tolerance.

2 Materials and Methods

2.1 Cultivars/Genotypes Used in This Study

Twenty five cowpea cultivars were evaluated for germination percentage and coefficient of velocity of germination (CVG): (1) Pinkeye Purple Hull (PPH), (2) California Black Eye (CBE), (3) Tahoro O' Adhan (TA), (4) Elite (E), (5) Big Boy Purple Hull (BBPH), (6) Texas Cream (TC), (7) Tennessee White Crowder (TWC),

(8) Cream 8 (C-8), (9) Guajirio Yoro Mami (GYM), (10) Mississippi Cream (MC), (11) C.T. Pinkeye Purple Hull (CTPPH), (12) Tetapeche Grey (TG), (13) Mayo Colina (MAC), (14) Speckled Purple Hull (SPH), (15) Mayo Colina Pinto (MCP), (16) Black Crowder (BC), (17) Whippoorwill (W), (18) Knuckle Purple Hull (KPH), (19) Corriente (C), (20) Frijol Reata (FR), (21) Mississippi Purple (MP), (22) Mississippi Pinkeye (MPE), (23) Zipper Cream (ZC), (24) Mississippi Silver (MS), and (25) Bisbee Black (BB). The seeds were obtained from Native Seed, Search, Tucson, AZ., USA. To get the seeds from the same lot the cultivars/genotypes were grown in the same year under a common set of environmental conditions, handled using the same harvesting/seed processing equipment, and stored under the same conditions. The seeds from the above lot were used in two different years experiment. Trials were carried out over 2 years using the above cultivars/genotypes grown to initially screen for germination and coefficient of variability of germination tests (Islam et al. 2005, 2006). Three cultivars (Texas Cream 40, Black Crowder and Mississippi Purple) were chosen for further study for biochemical characteristics such as Texas Cream 40 which showed ability to germinate at very high (40°C) and low (10°C) temperature; Black Crowder which had acceptable high germination at 40°C, but reduced germination at 10°C; and Mississippi Purple which exhibited lower germination at all temperatures tested (Islam et al. 2005, 2006).

2.2 Germination Test

Germination percentages of all cultivars were evaluated adopting the methods described by the Rules for Testing Seed (AOSA 1991), except for the number of seed used per replication and the inclusion of different temperatures. Seeds were germinated using the "between-paper" method, in which two sheets of germination paper (Anchor brown germination paper, Anchor Paper Co., St. Paul, Minnesota) were placed in the bottom of 48 × 50 cm trays and an additional sheet of the same paper was used to cover the seed. The paper sheets were previously saturated with 2.5 times their weight of water. Four replications of 20 seed of each cultivar were randomly assigned to trays. Each experiment repeated for four times.

2.3 Environmental Conditions

The germination tests were conducted in germination chambers each of which was set at a different temperature: 10°C (low temperature), 30°C (moderate temperature), and 40°C (high temperature). All chambers were maintained at 100% ± 1 relative humidity. Light/dark periods were maintained at 8/16 h d^{-1} at all three temperatures.

2.4 Evaluation of Germination Percentage

Seeds were counted as germinated when radicle protrusion was approximately 1 cm. The final count for germination was done 8 days after planting the seed in the trays. Our preliminary observation shows that within 8 days we had maximum germination. The germination percentage for each cultivar was calculated as the mean of the four 20-seed replications. A germination percentage over 75% was considered as reasonable for cowpea seed. Each experiment was repeated four times.

2.5 Coefficient of Velocity of Germination (CVG)

The same procedures and conditions as used in the germination study were used in CVG evaluation. The numbers of germinated seeds were evaluated every day for a period of 8 days. We have conducted a preliminary observation using the seeds of the above cowpea cultivars. This showed that no significant germination occurred after 8 days. The CVG was calculated using the Kotowski's (1926) modified method proposed by Islam et al. (2005):

$$\mathrm{CVG} = \frac{\sum \mathrm{G1} + \mathrm{G2} + \mathrm{G3} + \ldots\ldots\ldots + \mathrm{Gn}}{(\mathrm{T1} \times \mathrm{G1}) = (\mathrm{T2} \times \mathrm{G2}) + (\mathrm{T3} \times \mathrm{G3}) + \ldots\ldots + (\mathrm{Tn} \times \mathrm{Gn})}$$

where, G = number of germinated seeds and T = number of days after incubation corresponding to G.

2.6 Extraction of Carbohydrates

Seed carbohydrates were extracted and identified following the methodology suggested by Connor and Sowa (2000). To determine the initial content of carbohydrates in cowpea seed, whole dry seeds were ground using a hand mill machine and 0.5 g of the resulting flour was used for carbohydrate extraction. For the extraction from the germinating seed, after radical protrusion and before first leaf expansion, the seeds were dissected into cotyledons and axes. Tissues from each cultivar germinated at each temperature were separately dried in a freeze-dry system (Laconic Freezone 4.5 LABCONCO Corporation, Kansas City, MO). After removing all moisture, the tissue samples were finely-ground using a mortar and pestle. A 0.2–0.5 g dry tissue sample was used for each carbohydrate extraction. The tissue samples were placed in test tubes with 10 ml of 80% ethanol solution and then boiled in a water bath at 75°C for 1 h. The samples were then passed through a piece of filter paper in filter funnel. Additional ethanol solution was used to rinse the samples. The extracts were poured into evaporation flasks and

then roto-evaporated to dryness in a flask-evaporator (Buchler Instruments. Fort Lee, NJ). The evaporation flasks were rinsed with 10 ml of distilled water, and the samples were freeze-dried overnights in a stoppered vacuum flask attached to the freeze-dry apparatus. When all the moisture was removed from the flasks, the samples were dissolved in 1 ml of trimethylsilylimidiazole (TMSI), then heated in a water bath at 75°C for 30 min, and aspirated to dryness. The samples were then resuspended in 2 ml of chloroform and stored refrigerated until analysis.

2.7 Analysis of Carbohydrates

The analysis of carbohydrates was performed in a Hewlett Packard (HP) gas chromatograph (GC) equipped with a flame ionization detector and using a 125-5037 DB-5 column (30 m length × 0.53 mm I.D. and 0.5 μm film thickness) (J & W Scientific Folsom, CA). The program in the GC preset a detector and injector temperature of 230°C. An initial oven temperature of 210°C was held for 7 min and then increased 15°C min^{-1} until a temperature of 270°C was reached and held for 25 min. The flow was kept at 1.76 nn $ml^{-1.}$ A graph and numerical map with the respective percentage peak area of each sugar was obtained. The carbohydrates were identified by comparing with standards of pure sugars prepared in a similar manner to the tissue samples and injected in the GC.

2.8 Extraction of Lipids

Fatty acids in the seeds were extracted and identified through a modification of the methodology suggested by Whitaker (1986). To determine the content of fatty acids in cowpea seed, whole dry seeds were ground using a glass homogenizer and pestle. For extractions from germinating seeds, after radicle protrusion and before first leaf expansion, the seed were dissected into cotyledons and embryos. Two to five grams of each type of tissue from each cultivar germinated at each temperature were finely chopped and transferred to Pyrex culture tubes and were then covered with isopropanol using a Pasteur pipette. Tubes were then boiled in a water bath for 3–5 min, removed from the water bath, and allowed to cool at ambient temperature. The supernatant (isopropanol fraction), was transferred to another tube and then dried under nitrogen to prevent airoxidation. The crude remains of the extract were resuspended with approximately 2 ml of 2:1 chloroform: methanol. To prevent autooxidation, the supernatants were then aspirated under nitrogen atmosphere until dry. Three ml of chloroform and 1.5 ml of methanol were added to these fractions, followed by the addition of 1.5 ml of 0.85% sodium chloride. This wash procedure described by Folch et al. (1957) was used to remove non-lipid contaminants from the samples. The tubes were then sealed under nitrogen and centrifuged at 1,500 *g* for 5 min to obtain better separation. The pellet parts were

removed with a Pasteur pipette and dried under nitrogen. The residues were taken up in 1.5 ml of 2:1 chloroform: methanol. The extracts from the sample were combined. A second addition of 1.5 ml of 0.85% sodium chloride and a second centrifugation at the same speed was performed to improve separation. The pellets were then removed and dried under nitrogen. The remaining portions were taken up in 2 ml of chloroform.

2.9 Transesterification of Lipids

The individual polar fractions were dried under nitrogen at 40°C. The samples were then resuspended in 0.5 ml of chloroform followed by the addition of 0.5 ml of 0.6 N KOH in dry methanol. The tubes were sealed under nitrogen and placed on a rototorque rotator in the dark at room temperature for 2 h. After the addition of 0.5 ml of distilled water and 50 µl 6 N HCl, the fatty acid methyl esters (FAME) were recovered by extraction with 2 ml of hexane.

2.10 Analysis of Lipids

Fatty acids were identified as described by Whitaker (1986). Lipids were analyzed in a Varian 3300 gas chromatograph (Gas Chromatography, Varian Associates, Sugarland, TX) equipped with a flame ionization detector and using a Supelcowax 10 fused-silica wide-bore capillary column (30 m length × 0.53 mm i.d. and 1.0 µl film thickness) (Supelco, Inc., Bellenfonte, PA). The program used established an injector temperature of 250°C and a detector temperature of 300°C. The initial column temperature was set at 190°C and a holding time of 3 min. The column temperature was increased 3°C/min until the final temperature of 220°C was reached, and a holding time of 20 min. Data were transferred to a Varian 4290 integrator (Varian Instrument Division, Walnutcreek, CA), where they were expressed as percentage (%) area under the peak. Identification of the FAMEs was achieved by comparing them with the standards of FAME mixture reference # 625006, 625007, and 625009 (Alltech Association Inc., Deerfield, IL).

2.11 Peroxidase Extraction

Peroxidase was extracted using a slight modification of the methodology of Silva et al. (1990). Seeds were separated into cotyledons and axes and 3.0 g of each tissue were used for extraction. Each sample was ground using a pre-chilled mortar and pestle, and 3.0 ml of citrate phosphate buffer (pH 6.0) were added as a grinding

solution. This material was then placed in a centrifuge tube. An additional 3.0 ml of the buffer were used to wash the mortar and pestle and this plus a small amount of polyvinyl-polypyrrolidone (PVP) was added to the tube. The samples were centrifuged at 6,000 × *g* at 5°C for 10 min and the supernatant was saved and maintained at freezing temperature for the next step of enzyme analysis.

2.12 Peroxidase Activity

Peroxidase activity was measured using the methodology of Silva et al. (1990). The enzymatic activity of peroxidase was measured spectrophotometrically in a Spectronic Genesys 5 spectrophotometer (Spectronic Instruments Inc., Rochester, NY). One ml of crude peroxidase extract was added to a reaction mixture consisting of 2 ml of 0.05 M citrate phosphate buffer (pH 6.0) and 2 ml of 4.0×10^{-4} guaiacol. The reaction was initiated by adding 2 ml of 3% H_2O_2. The change in the absorbance at 470 nm was used to measure the enzyme activity.

2.13 Statistics

Each experiment was arranged in a completely randomized design (CRD) with five replicates of 20 seed each for each cultivar at each temperature (for germination and CVG). The data were analyzed as a combined series of CRDs. Data were subjected to analysis of variance (ANOVA) using the general linear models (GLM) procedure of SAS version 8.1 (SAS Instit., Inc. Cary, North Carolina, USA). Mean separations were done using Fisher's protected least significant difference (LSD) test.

3 Results and Discussion

3.1 Seed Germination and CVG

The germination percent and coefficient of velocity of germination (CVG) is shown in Table 28.1. The above three cowpea cultivars were chosen for biochemical evaluation based on physiological performance of the seed namely performance in the germination and CVG tests under temperature stress conditions (Islam et al. 2005, 2006). Texas Cream 40 (TC-40) performed well at 10°C, 30°C and 40°C temperatures; Black Crowder (BC) was more sensitive to chilling temperature than high temperature (Islam et al. 2005, 2006), and Mississippi Purple (MP) with low germination and CVG at all temperatures such as 10°C, 30°C and 40°C (Islam et al. 2005, 2006).

Table 28.1 Germination percentage and coefficient of velocity of germination (CVG) of three selected cowpea cultivars evaluated at different temperatures

	Germination (%)				CVG			
Temperature	10°C	30°C	40°C	LSD (0.05)	10°C	30°C	40°C	LSD (0.05)
MP	C	B	B	8.52	B**	A	A	0.014
	17.5b[a]	63.5a**	65.8a		0.110b	0.152b	0.151a	
TC-40	A*	A	A	8.98	C	A	B	0.011
	89.8b	93.4a	88.3b		0.125a	0.169a	0.155a	
BC	B	A	A	7.75	B	A	A	0.011
	27.0c	93.8a	87.5b		0.111b	0.154b	0.154a	
LSD(0.05)	5.04	9.27	10.11		0.013	0.012	0.010	

MP Mississippi Purple, *TC* Texas Cream, *BC* Black Crowder

*A–C Means within a cultivar not followed by a common capital letter are significantly ($p<0.05$) different according to Fisher's protected LSD test

**Means within a temperature not followed by a common letter are significantly ($p<0.05$) different according to Fishers's procted LSD test

[a]Analysis performed on arcsine transformed data

3.2 Sugar Compositions

Carbohydrates were determined in whole ungerminated seed and in cotyledon and embryo tissues of germinating seeds at low (10°C), moderate (30°C), and high (40°C) temperature. The main sugars present in cowpea seed were sucrose, raffinose, and stachyose. The sugar content was affected by temperature, cultivar, and types of tissue.

3.3 Sucrose Content

There was a significant temperature × cultivar × tissue interaction effect for sucrose content of cowpea seed (Table 28.2). The initial sucrose content in ungerminated seed was highest in BC, followed by TC-40, and lowest in MP. At low temperature (10°C), the higher mean sucrose content was in the embryo tissue of MP followed by BC, and the lowest sucrose content was in TC-40. In cotyledon tissue, the highest mean sucrose content was in TC-40, and the lowest in MP, and BC (Table 28.2). At moderate temperature (30°C), the highest sucrose content was in cotyledon of TC-40 (53.51 mg g^{-1}) and the lowest in MP. In embryo tissues, highest mean sucrose content was in TC-40, and the lowest in MP and BC. At high temperature (40°C), the highest mean sucrose content in cotyledon tissues was in TC-40 and MP, followed by BC. In embryo tissue there were no significant differences among cultivars for mean sucrose content. Sucrose is a primary nutrient in the cells of higher plants (Xu et al. 1989). During seed development, changes in carbohydrates occur in such a way that monosaccharide content decreases whereas oligosaccharide content increases, but during the germination process this pattern is reversed

Table 28.2 Sucrose content of whole seed, cotyledon (Cot.) and embryo (Emb.) tissues of seed of three cultivars of cowpea germinated at three different temperatures

	Temperature							
	Ambient	10°C		30°C		40°C		
Cultivars	Whole seed	Cot.	Emb.	Cot.	Emb.	Cot.	Emb.	LSD (0.05)
MP	E*	C	A	C	D	B	B	3.58
	9.9c	40.8c	67.9a	41.3b	29.6d	47.7ab	47.2ab	
TC-40	E	AB	D	A	C	AB	B	4.69
	14.8b	49.8b	26.7d	53.5a	37.3c	49.1a	46.4ab	
BC	E	C	A	BC	D	BC	B	4.16
	18.3a	39.9c	65.8a	42.9b	28.2d	43.6b	45.9ab	
LSD (0.05)	2.06	4.72		3.22		4.02		

MP Mississippi Purple, *TC*-40 Texas Cream-40, *BC* Black Crowder
a–d Means within a temperature not followed by a common lowercase letter are significantly ($p<0.05$) different according to Fisher's protected LSD test
*A–E Means within a cultivar not followed by a common capital letter are significantly ($p<0.05$) different according to Fisher's protected LSD test

(Vertucci and Farrant 1995). At low temperature, TC-40 seed had faster and higher percent germination than MP and BC (Islam et al. 2006); thus, the higher sucrose content in the cotyledon and lower sucrose contents in embryos of the above cultivar suggest faster and more efficient use of sucrose in germination process and seedling development in this cultivar. Xu et al. (1989) indicated that during germination seeds have a dual role: first as a source of sucrose produced in cotyledons and secondly as sink when the young seedling uses sucrose from development.

3.4 Raffinose Content

There was a significant temperature × cultivar interaction for raffinose content (Table 28.3). In whole ungerminated seed, the highest raffinose content mean was in BC (2.5 mg g^{-1}), and lowest in TC-40 (1.5 mg g^{-1}) and MP (1.3 mg g^{-1}). In germinating seed at low temperature (10°C), raffinose contents in cotyledon tissues were 0.67 mg.g^{-1} (MP), 0.62 mg g^{-1} (BC), and 0.35 mg g^{-1} (TC-40). No raffinose was detected in embryo tissues at low temperature or in any tissue at 30°C and 40°C.

3.5 Stachyose Content

There was a significant temperature × cultivar interaction for stachyose content (Table 28.4). The highest stachyose mean content in the whole ungerminated seed was in BC (10.4 mg g^{-1}), followed by TC-40 (7.8 mg g^{-1}), and MP (5.8 mg g^{-1}). Stachyose was also detected in cotyledon tissues of germinating seed at low

Table 28.3 Raffinose content (mg g^{-1} d. wt.) of whole seed, cotyledon (Cot.) and embryo (Emb.) tissues of seed of three cultivars of cowpea germinated at three different temperatures

	Temperature							
	Ambient	10°C		30°C		40°C		
Cultivars	Whole seed	Cot.	Emb.	Cot.	Emb.	Cot.	Emb.	LSD (0.05)
MP	A*	B	C	C	C	C	C	0.03
	1.3b**	0.67a	0.00	0.00	0.00	0.00	0.00	
TC-40	A	B	C	C	C	C	C	0.05
	1.3b	0.53b	0.00	0.00	0.00	0.00	0.00	
BC	A	B	C	C	C	C	C	0.10
	2.5a	0.62a	0.00	0.00	0.00	0.00	0.00	
LSD (0.05)	0.30	0.07		ns		ns		

MP Mississippi Purple, *TC*-40 Texas Cream-40, *BC* Black Crowder
*A–C Means within a cultivar not followed by a common capital letter are significantly ($p<0.05$) different according to Fisher's protected LSD test
** a–b Means within a temperature not followed by a common lowercase letter are significantly ($p<0.05$) different according to Fisher's protected LSD test

Table 28.4 Stachyose content (mg g^{-1} d. wt.) of whole seed, cotyledon (Cot.) and embryo (Emb.) tissues of seed of three cultivars of cowpea germinated at three temperatures

	Temperature							
	Ambient	10°C		30°C		40°C		
Cultivars	Whole seed	Cot.	Emb.	Cot.	Emb.	Cot.	Emb.	LSD (0.05)
MP	A*	B	C	C	C	C	C	0.03
	5.8c**	2.2a	0.00	0.00	0.00	0.00	0.00	
TC-40	A	B	C	C	C	C	C	0.05
	7.8b	0.79b	0.00	0.00	0.00	0.00	0.00	
BC	A	B	C	C	C	C	C	0.10
	10.4a	2.35a	0.00	0.00	0.00	0.00	0.00	
LSD (0.05)	1.51	0.27		ns		ns		

MP Mississippi Purple, *TC*-40 Texas Cream-40, *BC* Black Crowder
*A-C Means within a cultivar not followed by a common capital letter are significantly ($p<0.05$) different according to Fisher's protected LSD test
** a-b Means within a temperature not followed by a common lowercase letter are significantly ($p<0.05$) different according to Fisher's protected LSD test

temperature (10°C) in BC (2.4 mg g^{-1}), MP (2.2 mg g^{-1}), and TC-40 (0.8 mg g^{-1}). No stachyose was detected in tissues of germinating seeds at moderate or high temperatures, which suggest that larger chain sugars had been reduced to sucrose or other monosaccharides. Koster and Leopold (1988) demonstrated that raffinose as well as stachyose have an important role in dessication tolerance of pea, corn, and soybean seed, probably helping to prevent sucrose crystallization. The increase in sugar content when comparing the dry ungerminated seed to the sugar content in cotyledon tissue of germinating seed at low temperature indicated either "de novo" synthesis of or convection of other products to sucrose. Miguel and Browse (1995)

pointed out that seed storage components such as lipids, proteins and carbohydrates are converted to sucrose in order to feed the developing embryo.

Sucrose, raffinose and stachyose contents were significantly different among the cultivars studied. Black Crowder had the highest sucrose, raffinose, and stachyose contents, and this cultivar also exhibited high germination percentage. The lowest sucrose, raffinose, and stachyose contents were in Mississippi Purple, which had low germination percentage. The sucrose content in ungerminated seed was highest in BC, followed by TC-40, and lowest in MP. Sucrose is a primary nutrient in cells of higher plants (Xu et al. 1989). During seed development, changes in carbohydrates occur in such a way that monosaccharide content decreases whereas oligosaccharide content increases, but during the germination processes this pattern is reversed (Vertucci and Farrant 1995). In this study, BC seed had faster and higher percent germination than TC-40 and MP; thus, the higher sucrose content in the above cultivar suggests, faster and more efficient use of sucrose in germination process and seedling development in this cultivar. Xu et al. (1989) indicated that during germination, seeds have a dual role: first as a source of sucrose produced in cotyledons and secondly as a sink when the young seedling uses sucrose for development. The highest raffinose content was in BC (2.46 mg g^{-1}), and lowest in TC-40 (1.53 mg g^{-1}) and MP (1.25 mg g^{-1}). The highest stachyose content in the whole ungerminated seed was in BC (10.54 mg g^{-1}), followed by TC-40 (7.78 mg g^{-1}), and MP (5.77 mg g^{-1}). The increase in sugar content when comparing the dry ungerminated seed to the sugar content in cotyledon tissue of germinating seed at low temperature indicated either "*de novo*" synthesis of or convection of other products to sucrose. Miguel and Browse (1995) pointed out that seed storage components such as lipids, proteins and carbohydrates are converted to sucrose in order to feed the developing embryo.

3.6 Fatty Acid (Lipid) Contents

The main fatty acids present in cowpea seed and seedlings were palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), and arachidic acid (C20:0). The feature of the cowpea seed fatty acids composition was presented in Table 28.5. The fatty acid content was affected by temperature, cultivar, and types of tissue.

3.7 Palmitic Acid (PA)

There was a significant temperature × cultivar × tissue interaction for PA (C16:0) (Table 28.6). At low temperature (10°C), the highest mean PA content was in cotyledon tissue of TC-40, followed by MP and BC. In embryo tissue, TC-40 had higher PA content than MP. At moderate temperature (30°C) in cotyledons, the highest

Table 28.5 Fatty acid composition in cowpea seeds

Common name	Scientific name	Carbon atoms	Double bonds	Structures
Saturated				
Palmitic acid	Hexadecanoic acid	16	0	$CH_3(CH_2)_{14}COOH$
Stearic acid	Octadecanoic acid	18	0	$CH_3(CH_2)_{16}COOH$
Arachidic acid	Eicosanoic acid	20	0	$CH_3(CH_2)_{18}COOH$
Unsaturated				
Palmitoleic acid	9-Hexadecenoic acid	16	1	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
Oleic acid	9-Octadecenoic acid	18	1	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
Linoleic acid	9, 12-Octadecadienoic acid	18	2	$CH_3(CH_2)_4CH=CHCH_2CH=CH$ $CH_2(CH_2)_6COOH$
Linolenic acid	9, 12, 15-Octade-catrienoic acid	18	3	$CH_3CH_2CH=CHCH_2CH=CHCH$ $_2CH=CH(CH_2)_7COOH$

Table 28.6 Palmitic acid (PA) (C16:0) concentrations (%) of cotyledon (COT) and embryo (EMB) tissues of seeds of three cowpea cultivars germinated at different temperatures

	10°C		30°C		40°C		
Cultivar	Cot.	Emb.	Cot.	Emb.	Cot.	Emb.	LSD (0.05)
MP	B*	D	A	B	A	C	2.55
	28.04b**	21.20d	34.99a	27.00d	35.28a	25.91c	
TC-40	BC	D	A	BC	B	C	2.64
	30.99a	23.45c	36.08a	30.56bc	33.28a	28.48bc	
BC	C	D	A	BC	B	BC	2.14
	27.52b	22.43cd	32.09b	28.85cd	29.83b	29.44b	
LSD (0.05)	1.96		2.17		3.30		

MP Mississippi Purple, *TC*-40 Texas Cream-40, *BC* Black Crowder
*A–D Means within a cultivar not followed by a common capital letter are significantly ($P<0.05$) different according to Fisher's protected LSD test
**a–d Means within a temperature not followed by a common letter are significantly ($P<0.05$) different according to Fisher's protected LSD test

mean PA content was in TC-40 and MP, and the lowest in BC. In embryo tissues at 30°C, the highest PA content was in TC-40, followed by BC and MP. At high temperature (40°C), the highest mean PA content was in cotyledon tissue of MP and TC-40 followed by BC. In embryo tissue, MP was significantly lower than BC (Table 28.3). PA content was lower at low temperature for all cultivars, suggesting that it does not play an important role in seed tolerance to chilling temperature. Harwood and Stumpf (1970) pointed out that the initial production of fatty acids in germinating pea seed showed a "lag" which depended on temperature. At higher temperature, lag time was shorter. They also observed that production of fatty acids during the first 30 h of germination initiated with palmitate and stearate, followed later by oleic acid. No polyunsaturated acids were detected during this period of pea seed germination. Higher PA contents were found in cotyledons where it is deposited or synthesized.

Table 28.7 Palmitoleic acid (PAA) (C16:1) concentrations (%) of cotyledon (COT) and embryo (EMB) tissues of seeds of three cowpea cultivars germinated at different temperatures

	10°C		30°C		40°C		
Cultivar	Cot.	Emb.	Cot.	Emb.	Cot.	Emb.	LSD (0.05)
MP	A*	A	B	B	B	B	1.22
	4.33a**	3.49a	0.00	0.00	0.00	0.00	
TC-40	B	A	C	C	C	C	1.64
	3.65a	5.60a	0.00	0.00	0.00	0.00	
BC	A	A	B	B	B	B	2.20
	4.28a	5.56a	0.00	0.00	0.00	0.00	
LSD (0.05)	ns		ns		ns		

MP Mississippi Purple, *TC*-40 Texas Cream-40, *BC* Black Crowder

* A–C Means within a cultivar not followed by a common capital letter are significantly ($P<0.05$) different according to Fisher's protected LSD test

** Means within a temperature not followed by a common letter are significantly ($P<0.05$) different according to Fisher's protected LSD test

3.8 Palmitoleic Acid (PAA)

There was a significant effect of temperature on PAA (C16:1) content (Table 28.7). In germinating seed, PAA was evident only at low temperature (10°C). The highest PAA contents in cotyledons were MP (4.79%), and BC (4.28%), and the lowest was TC-40 (3.65%). There were no significant differences among cultivars in PAA content of embryo tissue. De Santis et al. (1999) reported that PAA, a component of the mitochondrial inner membrane isolated from shoots of maize seedlings, had no significant role in chilling tolerance.

3.9 Stearic Acid (SA)

There was a significant temperature × cultivar × tissue interaction for SA (C18:0) content (Table 28.8). At low temperature (10°C), the SA content means differed only between embryo tissue of MP and cotyledon tissue of BC. At moderate temperature (30°C), the highest SA content was in embryo tissue of TC-40, followed by BC and MP, whereas in cotyledon tissue, the highest mean was in BC followed by TC-40 and MP. At high temperature (40°C), TC-40 had higher SA content in embryo tissue than BC. In cotyledon tissues, BC (4.36%) and TC-40 (3.87%) showed higher SA content than MP (2.77%). The content of this fatty acid in embryos of all cultivars was higher at 30°C than at 10°C. Stearic acid is found in fatty acid composition of important oilseeds such as soybean (*Glycine max* L. Merr.), rapeseed (*Brassica napus* L.), sunflower (*Helianthus annuus* L.), palm (*Elais guinenis* L.), cotton (*Gossypium hirsutum* L.), peanut (*Arechis hypogaea* L.), and coconut (*Cocus nucifera* L.) in proportions of approximately 4% (Miguel and Browse 1995). Thus, this long-chain fatty acid appears to be important in germination process.

Table 28.8 Stearic acid (SA) (18:0) concentrations (%) of cotyledon (COT) and embryo (EMB) tissues of seeds of three cowpea cultivars germinated at different temperatures

	10°C		30°C		40°C		
Cultivar	Cot.	Emb.	Cot.	Emb.	Cot.	Emb.	LSD (0.05)
MP	CD*	B	CD	AB	D	A	0.56
	3.23ab**	4.13a	2.87c	4.49b	2.77c	4.72ab	
TC-40	BCD	D	D	A	CD	B	1.28
	4.05ab	3.77ab	3.60c	6.63a	3.87b	5.17a	
BC	D	CD	B	A	B	BC	0.61
	3.18b	3.36ab	4.60b	5.22b	4.36ab	4.19b	
LSD (0.05)	0.94		0.83		1.02		

MP Mississippi Purple, *TC*-40 Texas Cream 40, *BC* Black Crowder
*A–D Means within a cultivar not followed by a common capital letter are significantly ($P<0.05$) different according to Fisher's protected LSD test
**a–c Means within a temperature not followed by a common letter are significantly ($P<0.05$) different according to Fisher's protected LSD test

Table 28.9 Oleic acid (OA) (C18:1) concentrations (%) of cotyledon (COT) and embryo (EMB) tissues of seeds of three cowpea cultivars germinated at different temperatures

	10°C		30°C		40°C		
Cultivar	Cot.	Emb.	Cot.	Emb.	Cot.	Emb.	LSD (0.05)
MP	3.64	3.18	4.97	4.73	4.97	4.98	0.66
TC-40	3.47	2.86	3.82	6.54	6.46	7.40	2.06
BC	3.82	2.99	5.26	4.97	5.79	4.24	0.72
LSD(0.05)	0.76		0.73		2.04		

MP Mississippi Purple, *TC* Texas Cream, *BC* Black Crowder

3.10 Oleic Acid (OA)

There was a significant cultivar × tissue interaction effect for OA (18:1) content (Table 28.9). Higher OA content was observed in the cotyledon tissues of BC and MP, followed by TC-40 at low temperature (10°C). In embryo tissue, OA content was in descending order: MP, BC, and TC-40. At moderate temperature (30°C), the highest OA content was in embryo tissue of TC-40, followed by BC and MP. In cotyledon tissue, BC and MP had higher OA content, and TC-40 (3.82%) had the lowest. At 40°C, the highest mean oleic acid content in embryo tissues was observed in TC-40, followed by MP and BC. In cotyledon tissue, no significant differences were found among cultivars. For all cultivars, OA content was lower at low temperature. OA is an unsaturated fatty acid which is thought to play an important role in chilling tolerance during germination and in other physiological processes. In TC-40, the OA content, which was higher in ungerminated seed, decreased drastically when exposed to chilling temperature. This decrease was more evident in cotyledons, which could be explained by the effect of temperature on enzyme activity.

Table 28.10 Linoleic acid (LA) (C18:2) concentrations (%) of cotyledon (COT) and embryo (EMB) tissues of seeds of three cowpea cultivars germinated at different temperatures

	10°C		30°C		40°C		
Cultivar	Cot.	Emb.	Cot.	Emb.	Cot.	Emb.	LSD (0.05)
MP	B*	C	A	C	A	C	2.02
	39.46a**	37.00b	41.85a	36.24cd	41.93a	36.71b	
TC-40	A	B	A	B	A	B	2.00
	39.60a	34.91b	38.01bc	34.45d	38.55b	34.38c	
BC	A	C	AB	C	A	B	1.75
	40.04a	35.10b	39.40b	35.55d	41.38a	37.9b	
LSD(0.05)	2.15		1.99		2.25		

MP Mississippi Purple, *TC* Texas Cream, *BC* Black Crowder

* A-C Means within a cultivar not followed by a common capital letter are significantly ($P<0.05$) different according to Fisher's protected LSD test

** a-d Means within a temperature not followed by a common letter are significantly ($P<0.05$) different according to Fisher's protected LSD test

Miguel and Browse (1995) suggested that temperature controls the rate of OA production by the fatty acid synthase, which decreases as the temperature decreases, whereas "de novo" synthesis and activity of oleate desaturase is stimulated at low temperature. Results of this study agree with Miguel and Browse (1995), as OA content increased the temperature at which cowpea seed germination increased. Additionally, the higher initial content of OA in ungerminated seed of TC-40, along with the activity of oleate desaturase could help increase the content of linolenic acid. Higher OA content was observed in cotyledon tissue, emphasizing its importance at the beginning of germination.

3.11 Linoleic Acid (LA)

There was a significant temperature × cultivar interaction and a significant effect of tissue on the mean LA (18:2) content (Table 28.10). At low temperature (10°C), no significant differences in LA content were detected among cultivars, but LA was more abundant in cotyledon than in embryo tissue. At moderate temperature (30°C), the highest mean linoleic acid content in cotyledon tissue was in MP, followed by BC and TC-40, whereas in embryo tissue, no significant differences were found. At high temperature (40°C), the highest LA content in cotyledon tissue was in MP and BC, and the lowest in TC-40. There was a similar trend in the embryo tissue. This unsaturated fatty acid is thought to play an important role in seed germination at low temperature. In this experiment, the increase in LA content from dry seed (35.98%) to cotyledons of germinating seed (39.60%) at low temperature was highest in TC-40, which was the cultivar with the higher germination percentage.

Table 28.11 Linolenic acid (LIA) (C18:3) concentrations (%) of cotyledon (COT) and embryo (EMB) tissues of seed of three cowpea cultivars germinated at different temperatures

	10°C		30°C		40°C		
Cultivar	Cot.	Emb.	Cot.	Emb.	Cot.	Emb.	LSD (0.05)
MP	C*	A	D	B	D	B	1.28
	21.27b**	30.95a	15.06d	27.51a	15.06d	27.52a	
TC-40	D	A	D	C	D	B	2.15
	18.18c	29.62a	18.06c	21.75b	17.78c	24.54b	
BC	C	A	D	B	D	B	1.65
	21.18b	30.71a	18.19c	22.96b	19.26c	24.24b	
LSD(0.05)	2.43		1.44		1.70		

MP Mississippi Purple, *TC* Texas Cream, *BC* Black Crowder

* A-D Means within a cultivar not followed by a common capital letter are significantly ($P<0.05$) different according to Fisher's protected LSD test

** a-d Means within a temperature not followed by a common letter are significantly ($P<0.05$) different according to Fisher's protected LSD test

3.12 Linolenic Acid (LIA)

There was a significant temperature × cultivar × tissue interaction effect for LIA (18:3) content of cowpea seed (Table 28.11). At low temperature (10°C), the highest LIA content was in the embryo tissue of MP, BC, and TC-40, while in the cotyledon tissue, the means were MP, BC, and TC-40. The same trend was observed at moderate and high temperatures. This unsaturated fatty acid is important in maintaining membrane permeability and in chilling tolerance. In this study, the seed of TC-40 had a slightly higher initial LIA content, which could be related to its ability to germinate under cool conditions. The highest content of LIA was in the embryo tissue and it was higher at low temperature than at higher temperatures. This suggests a more important role for this fatty acid in protecting the seedlings from chilling injury than in low temperature germination tolerance acquisition.

3.13 Fatty Acids Content in Ungerminated (Whole Seed)

Figure 28.1 shows the fatty acid composition in ungerminated (whole seed) seed of three cowpea cultivars. The C18:2/C18:1 ratio was higher in BC followed by MP and TC-40. The 18-carbon unsaturated to 18-carbon saturated fatty acid ratio was lower in TC-40 than in BC and MP. The ratios of unsaturated to saturated fatty acids were higher in BC, TC-40 than in MP (Fig. 28.1). In ungerminated seed, PA (C16:0) content was highest in MP. This cultivar had a lower germination percentage at all temperatures, suggesting that PA was not essential to the germination process. PAA (C16:1) was extracted in small quantity in seed of TC-40. SA (C18:0) content was highest in the TC-40 which was the cultivar with highest germination percentage, and was lowest in MP which showed low germination. This long-chain fatty acid would appear to be important in germination process. OA (C18:1) content was highest

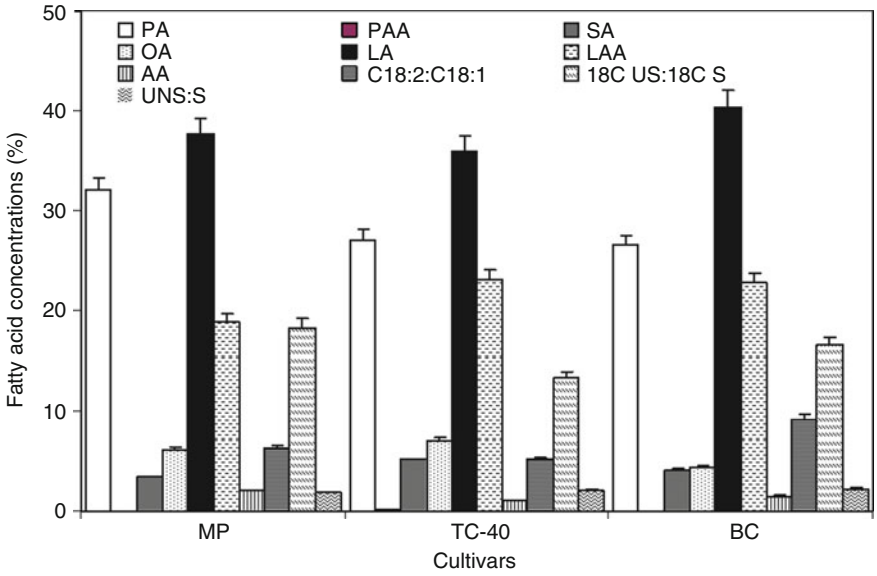

Fig. 28.1 Fatty acid concentrations (%) of ungerminated (whole seed) of three cowpea cultivars. Data are means of four individual experiments. Each experiment consists of four replications of 20 seeds each. Bars represent the standard error of the mean when absent concealed by the symbol. *PA* palmitic acid, *PAA* palmitoleic acid, *SA* stearic acid, *OA* oleic acid, *LA* linoleic acid, *LAA* linolenic acid, *AA* arachidic acid, C18:2/C18:1 polyunsaturated fatty acids: mono-unsaturated fatty acids, 18 US: 18 S 18-carbon unsaturated fatty acid: 18-carbon saturated fatty acid; *UNS: S* unsaturated: saturated, *MP* Mississippi Purple, *TC*-40 Texas Cream 40, *BC* Black Crowder

in TC-40, the cultivar with highest germination and lowest in BC, a cultivar with low germination. LA (C18:2) content was highest in the cultivars with low germination (Fig. 28.1). LAA (18:3) content was higher in ungerminated seed of TC-40 and lower in MP. There was a significant effect of temperature on the AA (C20:0) content in cowpea seeds. This large chain fatty acid was detected only in the whole seed. The highest mean arachidic acid contents were in MP (1.97%), a cultivar with low germination, and TC-40 (1.43%), and the lowest in BC (1.05%). Miguel and Browse (1995) reported that long-chain fatty acids are common in seed from the *Brassicaceae* and other species such as meadowfoam (*Limnanthes alba* Benthan) and jojoba (*Simmondsia chinensis* L.).

3.14 Ratios of the Fatty Acids in Cotyledon and Embryo Tissue

There were significant differences in the 18:2/18:1 ratios (polyunsaturated: mono-unsaturated) at moderate temperature [TC-40 (10.04 cot. and 5.28 emb.), MP (8.43 cot. and 7.74 emb.0), and BC (7.51 cot. and 7.30 emb.)], and also at high temperature (Fig. 28.2). Temperature affects not only fatty acid and triacylglycerol synthesis

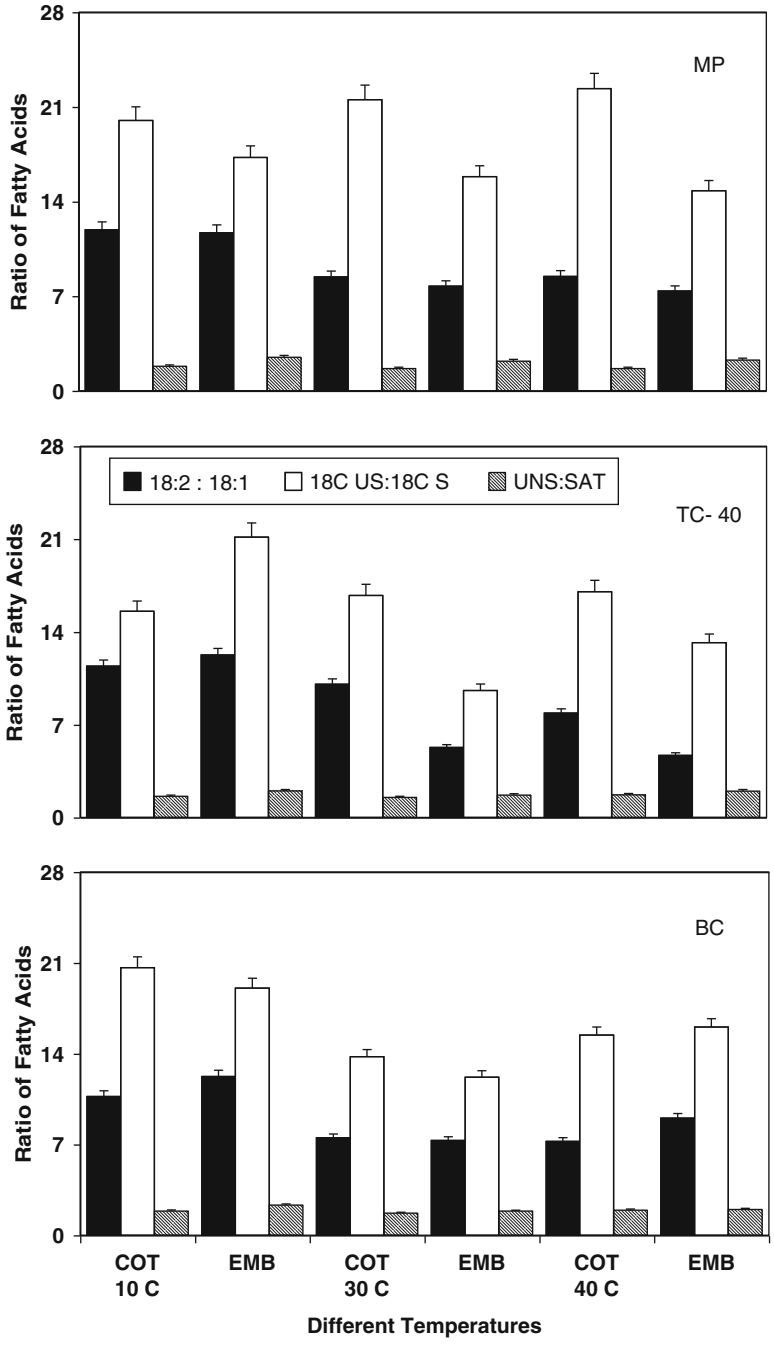

Fig. 28.2 Ratio of fatty acids of cotyledon (COT) and embryo (EMB) tissues of seeds of three cowpea cultivars germinated at different temperature. Data are means of four individual experiments. Each experiment consists of four replications of 20 seeds each. Bars represent the standard error of the mean when absent concealed by the symbol. 18:2: 18:1 polyunsaturated: mono-unsaturated, 18 US: 18 S 18-carbon unsaturated: 18-carbon saturated, *UNS: SAT* unsaturated: saturated, *MP* Mississippi Purple, *TC*-40 Texas Cream 40, *BC* Black Crowder

but also the response of oleate desaturase. Low temperature controls the 18:2/18:1 ratio of lipids by stimulating *de novo* synthesis and activity of oleate desaturase. It also controls the rate of oleate production by fatty acid synthase, which decreases as the temperature decreases. Thus 18:2 content increases as temperature decreases (Miguel and Browse 1995; Murata and Los 1997). At low temperature (10°C), the 18-carbon unsaturated to 18-carbon saturated fatty acid ratio was slightly higher in embryos of TC-40 than in embryos of BC and MP. In cotyledon tissue this ratio was higher in BC than in MP and TC-40. The 18-carbon unsaturated to 18-carbon saturated fatty acid ratio decreased when seeds were exposed to moderate temperature for embryos of TC-40 and BC. A small increase in this ratio, compared to moderate temperature, was observed at high temperature in the embryos of TC-40 and BC. BC had better germination at higher temperature than MP (Table 28.10). At low temperature (10°C), means were higher in embryo tissue than in cotyledon tissue as follows, MP, BC and TC-40. This ratio was higher in embryo tissue at low (10°C) than at moderate (30°C) temperature. Miguel and Browse (1995) reported that in membrane and storage lipids of plants grown at lower temperatures contained more polyunsaturated 18-carbon fatty acids than mono-unsaturated fatty acids. Membrane lipids contain mainly unsaturated fatty acids such as 18:3 and 16:3. When plants or plant parts are exposed to low temperature, the desaturation of fatty acids occurs mainly from 18:2 to 18:3 (Murata and Los 1997). The importance of the desaturation of fatty acid of membrane lipids in tolerance to low temperature has been demonstrated in transgenic systems. When relative levels of saturated molecular species of phosphatidylglycerol are reduced by transformation with glycerol-3-phosphate acyltransferase from a chilling resistant plant, plants become more tolerant to low temperature (Murata and Los 1997). Usually when cold temperature sensitive organisms are subjected to temperatures lower than optimal for their normal development, the degree of unsaturation of fatty acids in their membranes decreases significantly (Murata and Los 1997). The presence and composition of fatty acids in the seed affected its ability to germinate under different temperatures. The fatty acid composition of tomato seed, for example, accounts strongly for its ability to germinate at low temperature. Cold germinating genotypes (CGG) showed a higher proportion of LA and lower proportion of OA than non cold germinating genotypes (NCG). This relationship was not affected by the period of incubation at 10°C. This suggests that the genes controlling germinating ability affect the overall fatty acid composition towards increased unsaturation, even in the storage lipids (Maluf and Tigchelaar 1982). Harwood and Stumpf (1970) pointed out that the initial production of fatty acids in germinating pea seed showed a "lag" which depended on temperature. They also observed that production of fatty acids during the first 30 h of germination initiated with palmitate and stearate, followed later by oleic acid. No polyunsaturated acids were detected during this period of pea seed germination. Stearic acid is found in fatty acid composition of important oilseed such as soybean (*Glycine max* L. Merr.), rapeseed (*Brassica napus* L.), sunflower (*H. annuus* L.), palm (*Elais guinenis* L.), cotton (*G. hirsutum* L.), peanut (*A. hypogaea* L.), and coconut (*Cocus nucifera* L.) in proportions of approximately 4% (Miguel and Browse 1995). Thus, this long-chain fatty acid appears to be important in germination process.

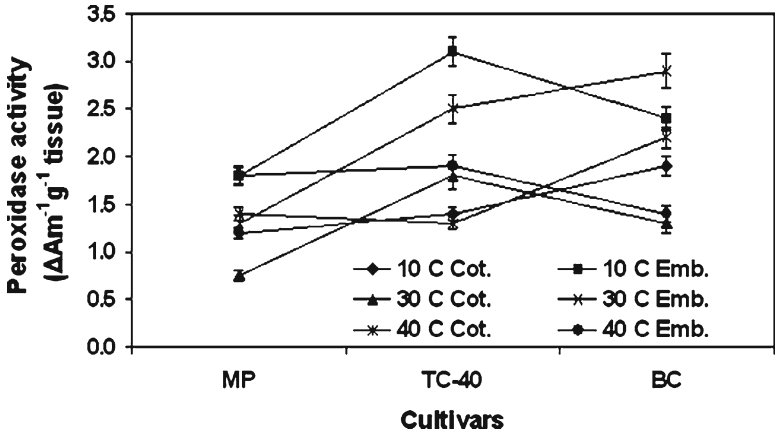

Fig. 28.3 Peroxidase activity (ΔA m^{-1} g^{-1} tissue) cotyledon (COT.) and embryo (EMB.) tissues of seed of three cultivars of cowpea germinated at three temperatures. *MP* Mississippi Purple, *TC*-40 Texas Cream 40, *BC* Black Crowder

3.15 Peroxidase Activity

There was a significant temperature × cultivar × tissue interaction for peroxidase activity in cowpea seed tissues (Table 28.4). At low temperature (10°C) which is considered a chilling temperature for cowpea cultivars, the highest peroxidase activity was in embryo tissue of TC-40 (3 ΔAm^{-1} g^{-1}), followed by embryo tissue of BC (2.7 ΔAm^{-1} g^{-1}). The lowest peroxidase activity was in cotyledon tissues of TC-40 (1.4 ΔAm^{-1} g^{-1}) and MP (1.9 ΔAm^{-1} g^{-1}). At moderate temperature (30°C) the higher peroxidase activity was in embryo tissues of BC (2.9 ΔAm^{-1} g^{-1}) and TC-40 (2.5 ΔAm^{-1} g^{-1}) and the lowest in cotyledon tissue of MP (0.8 ΔAm^{-1} g^{-1}). As at low temperature, higher peroxidase activity was observed in embryo tissue than cotyledon tissue. At high temperature (40°C) the highest peroxidase activity was in cotyledon tissue of BC (2.2 ΔAm^{-1} g^{-1}), followed by embryo tissues of TC-40 (1.9 ΔAm^{-1} g^{-1}) and MP (1.8 ΔAm^{-1} g^{-1}). The lower peroxidase activity was in cotyledon tissues of MP (1.38 ΔAm^{-1} g^{-1}) and TC-40 (1.4 ΔAm^{-1} g^{-1}), and in embryo tissues of BC (1.4 ΔAm^{-1} g^{-1}) (Fig. 28.3). Peroxidase activity in plant tissue increases as a response to stress treatment (Wagih and Coutts 1982a, b). The enzyme activity of cowpea leaves was increased by mechanical abrasion or as a response to infection by Tobacco Necrosis Virus. Chilling temperatures is thought to increase the levels of active oxygen species, which could increase the risk of chilling injury, thus increase in the activity of the active oxygen species scavenging system is desirable to increase tolerance to chilling temperature. The cowpea cultivar with the higher germination percentage, at low temperature, had higher peroxidase activity, suggesting the peroxidase acted in scavenging free radicals, which if accumulated could cause damage to cellular components and alters metabolic function. This is in agreement

with Anderson et al. (1995) who mentioned that peroxidase activity increased in chilling acclimated maize seedlings.

4 Conclusions

In conclusion, it is apparent that the main sugars present in cowpea seeds were sucrose, raffinose, and stachyose. Raffinose and stachyose are stored carbohydrates present only in dry ungerminated seed and in cotyledon tissues of germinating seeds at low temperature. No "*de novo*" synthesis of these sugars was detected. Sucrose "de novo" synthesis was found at higher temperatures. An accumulation of this sugar was evident in embryo tissues of cultivars with reduced ability to germinate at low temperature. The results also indicated that high peroxidase activity was related to ability of seeds to germinate at low temperature. The cowpea cultivars with high germination percentage showed higher sucrose, raffinose, and stachyose, as well as peroxidase activity in ungerminated seed. The differences in germination capacity may be related to over expression or inhibition of genes encoding synthesis of relevant molecules that reflect the composition differences demonstrated in this study (Khan and Ungar 1997; Castellon et al. 2003). Furthermore, the cowpea cultivars with high germination percentage showed higher palmitoleic acid (PAA), stearic acid (SA), and oleic acid (OA) contents in ungerminated seed. Cultivars with high germination percentage at low temperature showed higher 18:2/18:1 and 18-carbon unsaturated to 18-carbon saturated ratios. Furthermore, unsaturated fatty acid contents were found higher at lower temperatures. The results suggest that different cultivars obtained by genetic combination may present relevant differences in their fatty acid composition. The results also show significant changes in the amount of individual fatty acids in the cowpea seed during the germination processes. These changes could be explained by the possible translocation of fat-like substances from reserves (cotyledons) to the sink where the respiratory process is more active, or due to lipid peroxidation. The results may help in future breeding for specific constituents to enhance or reduce the fatty acid and sugar contents, and for the improvements of desired quality criteria of cowpea seeds. Thus, the information provided by this research will facilitate future genetic as well as plant biological studies of cowpea cultivars.

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Chapter 29
A Review on Barley Yellow Dwarf Virus

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Contents

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Abstract Barley yellow dwarf (BYD) is an economically important, most widely distributed, and destructive viral disease of cereals. The disease is caused by barley yellow dwarf virus (BYDV). The virus is phloem-limited pathogen and causes variable symptoms depending upon the virus isolate, crop species, time of infection and environmental conditions. In general, yellowing and reddening of leaves starting from upper tips to downward, dwarfing and reduction in size and number of ears and grains are observed in infected plants. BYDV is an isometric Luteovirus containing ssRNA genome and is transmitted in a persistent manner by more than 20 species of aphids. The disease has a serious economic impact and yield losses are variable and may range from 5% to 50%. Presently, five isolates of the virus such as PAV, MAV, RMV, RPV and SGV have been identified based on epitope profile and aphid vector specificity. The five biologically well defined isolates are sub-divided in two subgroups; subgroup 1 including PAV, MAV and SGV and subgroup 11 including RMV and RPV. Although the disease can be managed by integrated approaches such as cultural practices, use of pesticides and host plant resistance, but planting of crop varieties with tolerance or resistant is the most economical and practical approach. Development of host plant resistance through genetic engineering is another possible

option to combat the problem. Recombinant DNA technology has opened up the prospect of increasing genetic diversity in crop plants by their transformation. This will further help to increase the genetic resistance of bread wheats while using BYDV-resistant genes already identified in wild relatives of wheat and other species. Given the economic significance of the disease and its world wide occurrence, this review has been presented to update the knowledge of various aspects of BYDVs.

Keywords BYDV of cereals • Symptomatology • Properties • Serology • Transmission • Diagnosis • Control

1 Introduction

Barley yellow dwarf (BYD) is a cereal disease caused by an aphid transmitted virus belonging to the family Luteoviridae and genus Luteovirus, the members of which cause yellows disease. The virus which causes this disease is known as barley yellow dwarf virus (BYDV). Barley yellow dwarf is also called cereals yellow plague, cereal yellow dwarf, yellow dwarf and red leaf (Wiese 1987; Burnett 1990). It is distributed worldwide, infects practically all members of the cereals and grasses and possesses all the attributes of destructive plant viruses (Plumb 1983; Rochow et al. 1986). It has been reported in most areas of the world and is believed to damage cereals long before 1951, when it was first described on barley (Singh et al. 1993). The disease occurs on most cereals and numerous grasses, but is not known to affect dicotyledonous plants. The reduction in yield over large production areas is up to 25% (Wiese 1987).

BYDVs are ubiquitous across the globe where Poaceae (wild or cultivated) are grown. These viruses occur wherever cereals are grown. Incidence studies, particularly in the developing world, have been conducted only in some countries. It is evident that worldwide distribution of BYDVs is uneven. As a whole, it appears that PAV-like variants are the most common, but other strains can be predominant in particular agro-ecological zones. The distribution of BYDVs has been shown in Fig. 29.1. The disease was first identified in Netherlands by Oswald and Houston (1951), and later was confirmed in the United Kingdom (Watson and Mulligan 1957). Before the European identification of the disease, however, cereal diseases characterized by yellowing, dwarfing and decreased yields had been observed in North America, sometimes in epidemic proportions. Widespread outbreaks with significant yield losses were probably caused by BYDVs in 1907 and 1949 (Hewings and Eastman 1995). The first reports of barley yellow dwarf viruses in Australia and New Zealand were made more than 47 years ago by Smith (1955, 1957). In Hungary, BYDV was first identified and described in winter barley by Szirmai (1967). Five years later, it was observed in winter wheat during the spring of 1972 by Szunics and Szunics (1980). The virus was later recorded in maize by Milinkó et al. (1984), in rice by Pocsai et al. (1985), in Hungary by Pocsai et al. (1995).

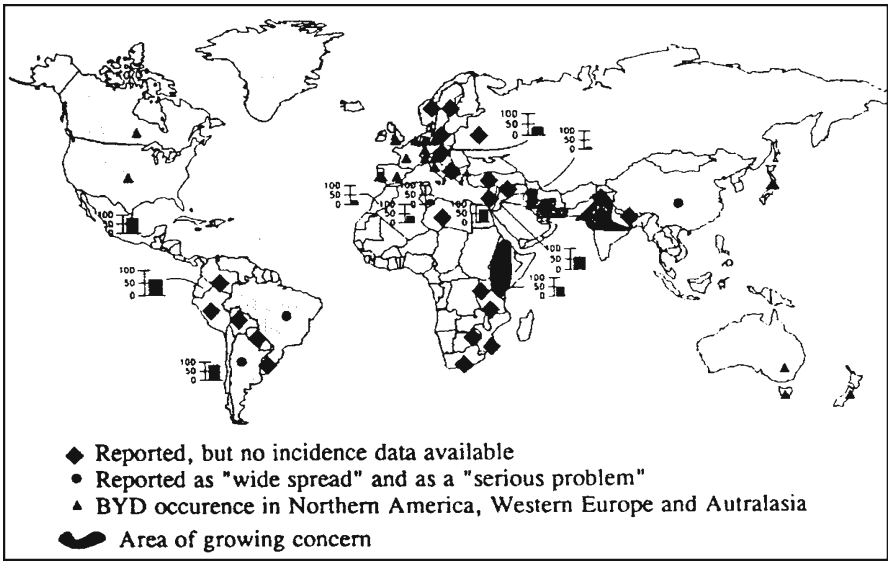

Fig. 29.1 Worldwide presence and incidence of the barley yellow dwarf disease (BYD) in bread wheats as compiled from Burnett (1990), Comeau and Makkouk (1992) and other references. For industrialized countries in Northern America, Western Europe and Australasia, only presence is displayed even if incidence data is available. For Hungry, incidence data was available only for barley

Occurrence of BYDV in some developing countries is well documented (Lister and Ranieri 1995) and was first reported on wheat and barley from India by Nagaich and Vashisth (1963). In Pakistan BYDV was first observed in 1964 near Pakistan-Afghan border on a single plant of local wheat and after that BYDV was noticed in NWFP on all commercial wheat varieties (Aslam and Iftikhar 1990). Later on, the occurrence of BYDV in various provinces of Pakistan was confirmed by several authors (Khalid et al. 1992a, b; Bashir et al. 1994, 1997). Two workshops organized by CIMMYT, Mexico are good sources of information on the distribution and importance of the disease (CIMMYT 1984; Burnett 1990). According to Plum (1983) BYD is truly a global problem.

BYDVs can have a serious impact on, and be an important limiting factor for grain production wherever cereals are grown. However, global yield losses due to the BYDVs are difficult to estimate due to the availability of insufficient information. Average yield losses attributable to natural BYDV infection can range from 11% to 33% (Lister and Ranieri 1995). In some areas the losses have been reported to reach up to 87% (Gildow and Frank 1988). Yield losses caused by BYDV ranged from 27% to 100% in different barley varieties (Pocsai and Kobza 1983). The relationship between the disease incidence and yield loss was found to be linear in wheat and oats. A 1% increase in BYD disease incidence causes yield reduction to increase from 20 to 50 kg/ha in wheat and from 30 to 60 kg/ha in oats. Hewings and

Eastman (1995) calculated that hypothetical 5% losses caused by BYDVs in the United States in 1989 would result in crop losses valued at $847.0 million for corn, $387.1 million for wheat, $48.5 million for barley, and $28.0 million for oats. The greatest yield loss is seen if infection occurs prior to stem elongation (Mann et al. 1997), and severe infections at this growth stage may kill the plants. A PAV-like virus may also cause sugarcane yellow leaf disease in Brazil, Hawaii, and Australia (Vega et al. 1997). In Denmark severe infection of BYDV was seen in 2000. Yield losses were recorded as high as 80% (Gron Viden 2000). Thus the range of economic losses caused by BYDVs may be greater and increasing than previously thought. In Pakistan although there is no specific report on the losses caused by BYDV, but the incidence of the disease has been reported from 0.5% to 5% (Aslam and Ahmad 1990; Bashir et al. 1997).

Keeping in view the world wide occurrence and economic significance of the disease, the present review is being presented to update the information on BYDV globally available, so that future strategies be developed to prevent its spread and better management based on available scientific knowledge.

2 Host Range and Symptoms

BYDV was first reported in oats (*Avena sativa),* barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) from California, USA by Oswald and Houston (1951, 1953a, b). (*Given above*) Oswald and Houston (1951) reported that wheat, oats and barley were susceptible to BYDV.

BYDVs can infect more than 150 species in the family Poeaceae (D'Arcy 1995). Wheat (*Triticum aestivum, T. durum*), barley (*Hordeum vulgare*), oats (*Avean sativa*), rice (*Oryza sativa*), rye (Secale cereale) and sorghum (*Sorghum vulgare*) are all susceptible to BYDV infection and suffer different degrees of damage, depending on interaction between virus isolate and host cultivar. The agriculturally important grasses in genera Lolium, Poa, Bromus, Festuca, Phleum, and *Cynodon* are also susceptible. Bruehl (1961) summarized many studies and produced a list of 97 susceptible species from 34 genera within the family Poaceae. Slykhuis (1967) reported that about 100 species of grasses were susceptible to BYDV. He also stated that no dicotyledonous plants were known to be susceptible. Bruehl (1961) pointed out that because of the extremely wide host range of this virus among long-lived grass species, there was very little chance of its being eradicated as a result of the elimination of the host. Nevertheless, in most areas where BYDV occurs in cereals, non-cereal grasses ply a role in the epidemiology of the disease. The extensive host range and numerous aphid species that can transmit the BYDV enable the virus to survive in many different environments (van Riessen et al. 1998) but no BYDV isolate is known that can infect dicot plants.

Barley yellow dwarf (BYD) sometimes is difficult to diagnose under field conditions and may go unrecognized in some cases. Field symptoms are easily confused with nutrient or water deficiencies. The disease resembles aster yellows MLO in

small grains (Banttari 1965). The symptoms of BYD vary with the crop cultivar, the age of the plant at the time of infection, the strain of virus, the number of aphids present and environmental conditions. It appears that virus interferes with translocation by partially plugging the phloem. It can cause sever stunting of plants, inhibition of root formation, delaying or prevention of heading and reduction in yield. The symptoms persist, or vary seasonally, or disappear soon after infection.

Symptoms of barley yellow dwarf are highly variable and can be confused with those of wheat streak mosaic, nutrient deficiency, root and crown diseases, and environmental stress. Diagnosis is even more difficult when both wheat streak mosaic and barley yellow dwarf are mixed in the same field or even in the same plant. A big stumbling block to laboratory confirmation of barley yellow dwarf is the difficulty in consistently detecting all five strains of barley yellow dwarf virus. This sometimes leads to a false negative diagnosis when, in fact, barley yellow dwarf virus is present. On the positive side, developing technologies using new serological, biochemical and biotechnological methods are expected to accurately and rapidly detect all five barley yellow dwarf virus strains. Barley yellow dwarf is diagnosed in the field by the presence of yellowish to reddish stunted plants grouped singly or in small patches among normal plants. Early infection of any of the cereal grains may result in severe stunting, excessive or reduced tillering, bright yellowing or reddening of older leaves, delayed heading or ripening, increased sterility, and fewer and lighter-weight kernels. Post-seedling infections are progressively less severe to the point where only the upper leaves, or the flag leaves, show discoloration. Mildly infected wheat may not show any discoloration. The leaves of plants infected with barley yellow dwarf virus are shorter than normal, and the flag leaf may be severely shortened. Leaves often are stiffer and more erect. Root systems are reduced and diseased plants are more easily pulled than healthy plants. Stunted plants result from the failure of the stem inter node to elongate. This leads to a "telescoped" plant where the leaves may unfurl before they have fully emerged from the sheath of the previous leaf. Infected plants are "dwarfs" and have lost their normal confirmation. Even the head, or panicle, doesn't emerge fully or properly. Patterns of barley yellow dwarf in a field either may be seen as random within the crop or as circular or angular patches which reflect the pattern of movement of the aphid vectors or carriers. Many of the infected plants ripen prematurely, after which they may be invaded by sooty molds which give a dirty appearance to the plants and may lower germination of harvested seed. Symptoms described in major hosts by (Watkins and Leslie 1997) are as under.

2.1 Wheat

Different kinds of symptoms of BYD on wheat are reported. However, the most common symptoms reported in wheat are bright yellowing of leaf tips and margins, later spreading to entire lamina, distortion of leaves, dwarfing and stunting, excess tillering and production of sterile spikes in some cases (Khetarpal et al. 1994). If winter wheat is infected in the fall, yellowing of leaves usually does not occur

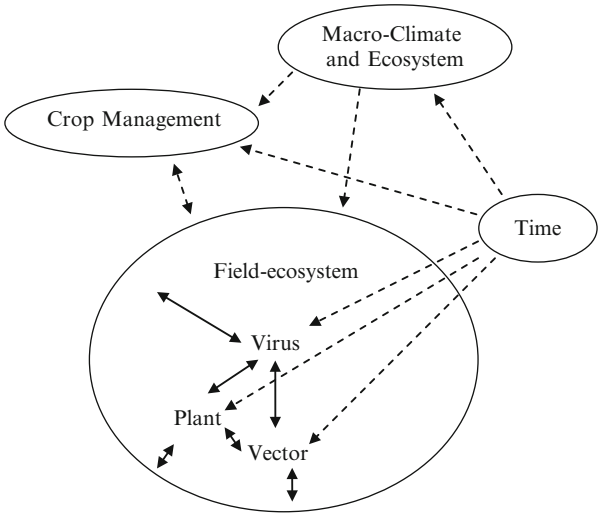

Fig. 29.2 Conceptualization of a pathosystem of a vector-transmitted plant virus

until mid-spring. In severe fall infections, some stunting and reduced tillering may develop. Barley yellow dwarf symptoms start to become obvious at about the jointing stage of growth. Barley yellow dwarf virus does not produce a distinct mosaic pattern, as do wheat streak mosaic virus or soil-borne wheat mosaic virus. The pattern of symptom expression is similar to that in barley or oats. Leaf symptoms begin as blotches near the tip, and with time, these turn various shades of yellow, red or purple. Progression of symptoms is from leaf tip to base and margin to mid-rib. Symptoms are more pronounced under cool temperatures, causing the tips of flag leaves to sometimes become reddish-purple. The yellowing in wheat is not as brilliant as it is in barley, and the reddening of leaves is not as pronounced as it is in oats. In wheat, the pale yellowing of older leaves is the more typical symptom (Fig. 29.2). The extent of yellowing, stunting and yield reduction is contingent on whether the plant is infected as a seedling or during post-seeding development. Leaves of some cultivars under some conditions remain green, but plants are stunted. Under field conditions, barley yellow dwarf first appears in small-localized patches that increase in size as more and more infected plants show symptoms. Generally the plants in the center of these patches show more severe symptoms, with the symptom intensity decreasing toward the perimeter.

2.2 Barley

In barley, a bright yellow discoloration begins at the leaf tip and rapidly progresses down the entire blade. This symptom may sometimes confuse with nitrogen deficiency. Necrotic brown flecking or spotting may accompany the yellowing. The blotchy or uneven discoloration within leaf blades is one of the most characteristic symptoms of barley yellow dwarf and helps to distinguish it from other virus

diseases and nutritional or environmental maladies. The brilliant yellowing is seen on older living leaves, and infected plants stand out among surrounding healthy plants in a field. Reddening or purpling of leaves may occur with some barley varieties. The blotchy yellowing begins near the leaf tip; as it progresses towards the leaf base, the green tissues discolor unevenly along the leaf margins, leaving a green stripe along the midrib area. When adult plants become infected, usually only the upper most leaves on the main stem turn yellow.

2.3 Oats

Symptoms vary according to the oat variety, the virus strain, the growth stage of the plant at the time of infection, and the general health of the plant. The main color change is to shades of yellow, reddish-orange, reddish-brown, or purple. First symptoms of infection are yellowish-green spots or blotches near the tips of older leaves. Eventually these blotches enlarge and coalesce, turning various shades of yellow, red and brown, entire leaf ultimately becomes a reddish-orange to brown or purple. Severely infected plants are shorter, produce lower test weight grain, and have more blasted florets.

2.4 Maize and Rice

The lower leaves show purpling and yellowing. Leaves turn yellow to orange, the discolouration begins at the tips and edges and progresses down the leaves.

3 Properties/Features of BYDV

3.1 Particle Morphology

All BYDVs have many common features. The virus particle is ~25 nm icosahedral (T = 3) virions. One major (22 kDa) and one minor (50–55 kDa) coat protein. 5.6–5.8 kb positive sense RNA genome with no 5′-cap and no poly(A) tail (Miller et al. 2002).

3.2 Physical Properties

Usually one sedimenting component in partially purified preparations; sedimentation coefficient is 115–118S (for BYDV-MAV). 1.4–1.405 g cm in CsCl (for BYDV-PAV). Density is 1.335 g cm^{-3} in Cs_2SO_4 for BYDV-PAV.

3.3 Biochemical Properties

Virion contain 28% nuclei acid (in BYDV-MAV-like isolate), 72% protein and no lipid (Paliwal 1982). Genome consists of single-stranded, linear RNA with molecular weight 2.0×10^6d. Total genome size is 5.673 kb (Gerlach et al. 1987). Genome is unipartite, largest (or only) genome part 5.6 kb (BYDV-RPV; 5.677 for BYDV-PAV). Genome nucleic acid was isolated by Brakke and Rochow (1974). Base composition is 24.6% G, 29.6% A, 23.8% C and 22% U. Poly A region is absent. Information on nucleotide sequence on various isolates of BYDV has been reviewed by Miller et al. (1987).

3.4 Features of the Genome and Proteins

Non-genomic nucleic acid found in the virions is satellite RNA (Miller et al. 1987). RNA Sub-genomic mRNA is found in infected cells (Gerlach et al. 1987). Virion protein (s) one; Mr 23500 (MAV), or 24400 (PAV), or 24450 (RPV) (Scalla and Rochow 1977; Hammond et al. 1983). Virus-coded non-virion proteins identified by genomic sequence analysis; six proteins found. Mr. 95000; probably a polymerase, read through protein. Mr of second largest 69000; possibly with a transport function. Mr. of 3rd 34000; probably a polymerase. Mr. of 4th 22000; coat protein. Mr of 5th and smaller 6000; possibly VPg; 17000 internal gene coding for coat protein (Brunt et al. 1996).

3.5 Cytopathology

Virions are found in phloem, cytoplasm, nuclei and around plasmodesmata. Viruses of different BYDV subgroups alter the nucleus in different ways. Other cellular changes are degradation of phloem tissue to which virions are confined (Gill and Chong 1975).

4 Classification and Serological Relationships

4.1 BYDV Isolates

BYDVs present challenges in classification. They are members of the luteovirus group (Randles and Rathjen 1995), which is defined as having icosahedral, T D 3, 25–30 nm virions that are non mechanically transmissible, but rather are transmitted only by aphids in a persistent, circulative manner, and are confined to the phloem

tissue in the plant. The studies by Rochow 1969 and Rochow and Muller 1971 have clarified the differences and described five variants or isolates (RPV, RMV, MAV, PAV, and SGV), that were initially classified by their biological properties and have subsequently been shown to be serologically distinct (Lister and Rochow 1979; Torrance et al. 1986). Four aphid vectors were used to distinguish the five isolates of BYDV reported from New York State: *Rhopalosiphum padi, Macrosiphum avena, Rhopalosiphum maidis* and *Schizophis graminum*. The basis for the distinction was the relative efficiency of transmission of the five isolates by these vectors and their vector specificity. The isolates and their major vectors are: RPV transmitted by *Rhopalosiphum padi*, RMV transmitted by *Rhopalosiphum maidis*, MAV transmitted by *Macrosiphum avenae* (now called *Sitobion avenae*), SGV transmitted by *Schizophis graminum* and PAV transmitted by *Rhopalosiphum padi, S. avenae* and others aphids. These are distinguishable serologically (Waterhouse et al. 1988). Laboratories worldwide use antibodies to classify local isolates into one of the above serotypes. However, aphid-transmission properties do not always correlate with serotype (Lister and Sward 1988; Creamer and Falk 1989). Symptoms can vary widely among different PAV isolates (Chay et al. 1996a, b). Thus, the simple five-serotype scheme may have been over applied.

4.2 Subgroups of Isolates

Numerous observations support division of barley yellow dwarf viruses into two viruses and even into separate genera. The former notion was first proposed based on cytopathological differences (Gill and Chong 1975) and subsequently supported by serological evidence (Waterhouse et al. 1988) and most strikingly, by differences in genome organization (Miller et al. 1995; Mayo and Ziegler-Graf 1996). The BYD viruses (BYDv) are presently sub-divided in two major sub-groups based on serological relationships (Rochow 1970a; Rochow and Duffus 1981), cytopathological ultrastructure of infected cells (Gill and Chong 1975; Randles and Rathjen 1995) and dsRNA profile obtained from infected tissue (Gildow et al. 1983). Sub-group 1 includes the strains PAV, MAV, and SGV and are related to soybean dwarf virus, whereas the strains RPV and RMV are included in Subgroup 11 and are closely related to beet western yellow virus (BWYV), potato leaf roll and carrot red leaf viruses, and more distantly to pea enation mosaic and southern bean mosaic sobemovirus (Duffus and Rochow 1978; Rochow and Duffus 1981; Martin and D'Arcy 1990). The acronyms have originally been chosen according to their principal aphid vector species (e.g. RPV for Rhopalosiphum padi virus by Rochow 1970b). These viruses are persistently transmitted by aphid vectors. The virus is not known to multiply in the insect. Newborn nymphs (young aphids) are virus-free and acquire BYDVs by feeding on infected plants. The pathosystem of BYDVs is extremely complex. Figure 29.2 may be one way of conceptualizing this complexity arising from the interaction between virus, host plant and virus vector with the environment and time.

The International Committee on the Taxonomy of Viruses (ICTV) Working Group on Luteoviruses is considering a reclassification in which Subgroup I serotypes

would be called BYDV, and members of Subgroup II would be renamed as cereal yellow dwarf virus (CYDV). This dichotomy extends to other luteoviruses at the level of gene homologies and organization (Miller et al. 1995; Mayo and Ziegler-Graf 1996). Beet western yellows luteovirus (BWYV) and potato leaf roll luteovirus (PLRV) resemble Subgroup II BYDVs. Soybean dwarf luteovirus has a Subgroup I-like organization and replicase, but the structural genes are most similar to those of Subgroup II (Rathjen et al. 1994). The chasm between Subgroups is so deep that the Subgroup II BYDVs are more similar to PLRV and BWYV in genome organization, replication genes, and cis-acting signals than they are to Subgroup I BYDVs. Conversely, other than in the structural genes, Subgroup I BYDVs are more closely related to SDV than to BYDVs in Subgroup II. Hence, the ICTV is also considering raising each subgroup category to the level of virus group or genus. The groups would all be members of the Luteoviridae family.

4.2.1 Comparison of Subgroup 1 with Subgroup II Isolates

PAV is the best studied BYDV, especially at the molecular level. It is also the most widespread (D'Arcy 1995) and usually causes the most severe symptoms. RPV is the best-studied Subgroup II BYDV, but it is much less well characterized than other Subgroup II luteoviruses (BWYV and PLRV), or PAV. Thus, PAV and RPV as representative members of each Subgroup, but often use BWYV or PLRV as additional representatives of Subgroup II. BYDV was also compared with related viruses outside the luteovirus group. The polymerase and translational frameshift signals of Subgroup I luteoviruses are more similar to those in red clover necrotic mosaic (RCNMV) and other diantho viruses than they are to those in Subgroup II luteoviruses (Xiong et al. 1993; Miller et al. 1995). Conversely, the polymerase genes of subgroup II luteoviruses are most closely related to those of sobemoviruses. Pea enation mosaic virus (PEMV), the sole member of the enamovirus group, has two RNAs, each encoding its own polymerase (Demler et al. 1996). The polymerase of RNA1 is Subgroup II-like (Demler and de Zoeten 1991) and that of RNA2 is Subgroup I-like (Demler et al. 1993). PEMV is aphid transmissible, probably by the same mechanism as luteoviruses, but it is also mechanically transmissible (Demler et al. 1996).

4.3 Current Classification

BYDV is the sole member of genus Luteovirus and the type member of the Luteoviridae family (formerly luteovirus group) (D'Arcy et al. 2000). BYDV serotypes were divided into two subgroups, which were subsequently reclassified as separate species. Currently, only BYDV-MAV (transmitted primarily by *Sitobion avenae*) and BYDV-PAV (transmitted efficiently by *S. avenae* and *Rhopalosiphum padi*) are barley yellow dwarf viruses. Former BYDV serotype RPV (transmitted primarily by *R. padi*) was given a new name, Cereal yellow dwarf virus -RPV (CYDV-RPV) and placed in

genus Polerovirus along with four non-BYDV viruses in the Luteoviridae. A third genus, *Enamovirus*, consists only of RNA-1 of the bipartite Pea enation mosaic virus (PEMV). Its organization resembles poleroviruses, but lacks open reading frame (ORF) 4. Comprehensive reports on all aspects of the Luteoviridae are available (Smith and Barker 1999).

After publication of the BYDV sequence (Miller et al. 1988), the sequences of several other luteoviruses were determined in rapid succession. These revealed a taxonomic dilemma that has continued to this day. Essentially the replication machinery of the Luteoviridae has two different evolutionary histories, whereas the proteins that form the virus particles and interact with the aphid vectors clearly have a common origin. Functional and comparative genomic analyses of BYDV and related viruses indicate that BYDV belongs to Tombusviridae family (Miller et al. 2002). The replication proteins and the RNA sequences that control replication and translation most closely resemble those of viruses in the Tombusviridae family. Yet the coat protein, movement and aphid transmission proteins clearly resemble those of the other Luteoviridae, including genus Polerovirus. Like the Tombusviridae, BYDV RNA lacks a 5′ cap or any other modification (Allen et al. 1999), and terminates at the 3′ end with the sequence CCC, preceded by a conserved stem-loop (Koev et al. 2002). In contrast, the poleroviruses, including CYDV- RPV have a protein (VPg) linked to the 5′ end and terminate in GU. In poleroviruses ORF codes for a suppressor of the post-transcriptional gene silencing defense response (Pfeffer et al. 2002). This ORF is absent in BYDV. ORF 1 of poleroviruses encodes a proteinase and the VPg which also are lacking in BYDV. All of the luteovirus-like genes of BYDV can be deleted and the remaining RNA can still replicate in protoplasts. Thus, the core of the virus, i.e. the gene expression and replication framework is more closely related to the Tombusviridae family than to other members of the Luteoviridae (Miller et al. 2002).

4.4 Gene Function and Expression

BYDV has a positive sense, 5.7 kb genomic RNA that encodes six open reading frames (ORFs) and produces three sub genomic RNAs (Kelly et al. 1994) that serve as mRNAs for downstream genes. Only ORFs 1 and 2 are translated from genomic RNA. ORF 2 is translated only as a fusion with ORF 1 via -1 ribosomal frame shifting (Di et al. 1993). ORF 2 encodes the active site of the viral RNA-dependent RNA polymerase (RdRp). The role of the ORF 1 product alone is unknown. These are the only two ORFs that are essential for RNA replication in plant cells (protoplasts) (Mohan et al. 1995). ORF 3 codes for the major coat protein (CP). ORF 4, which is imbedded in the sequence that codes for ORF 3, but in a different reading frame, codes for a protein required for systemic infection of plants (Chay et al. 1996a). ORFs 3, 4, and 5 all are translated only from subgenomic RNA1 (sgRNA1). ORF 5 is translated as a fusion with CP via in-frame read-through of the CP stop codon (Brown et al. 1996). ORF 4 is translated via

leaky ribosomal scanning (Dinesh-Kumar and Miller 1993). ORF 6 is translatable only from sgRNA2 in vitro (Wang et al. 1999), via the TE in its 5′UTR. Its role, if any, is unknown.sgRNA3, consists of the 3′-terminal 300 nucleotides of the BYDV genome. It is scarce in protoplast infections after 48 h, but accumulates to very high level in plants, a week after inoculation (Kelly et al. 1994; Koev et al. 1998) sgRNA3 has no ORFs and no known function

4.5 Translational Control

BYDV RNA undergoes a number of unusual events during translation (protein synthesis). It lacks the 5′ cap and poly (A) tail that are necessary for translation of normal host mRNAs. Normally eukaryotic mRNAs must circularize via proteins that bind the 5′cap, the poly (A) tail, and each other, prior to binding of the ribosome (Sachs et al. 1997). Instead, BYDV RNA harbors a sequence in the 3′ untranslated region (UTR) called the 3′ TE that brings about efficient translation initiation at the first AUG at the 5′ end of the genome and sub genomic RNA1 (Wang et al. 1997). The 3′ TE base pairs with a sequence in the 5′ UTR to circularize the mRNA and facilitate translation initiation (Guo et al. 2001). This is the first known case of a functional mRNA formed by base pairing between the UTRs rather than by protein-mediated circularization. It provides an example of how BYDV research has revealed fundamental new insight on the workings of the eukaryotic translation machinery. A similar structure and interaction exists in genus Necrovirus of the Tombusviridae, but not in genus Polerovirus of the Luteoviridae. The translation signals of poleroviruses, which also lack a cap and a poly (A) tail, have not been well characterized, but they bear no resemblance to those of BYDV. The BYDV polymerase is expressed via -1 ribosomal frame-shifting, i.e. in the region of overlap between ORF 1 and ORF 2, a small number of ribosomes translating ORF 1 shift back one nucleotide relative to the mRNA and resume translation in the new ORF (ORF 2). This event, which is common among retroviruses such as HIV (Dinman et al. 2002) and various other RNA viruses, is controlled by the mRNA sequence in and around the frame-shift site. The exact mechanism of frame*shifting is unknown. Because host genes are not known to use frame-shifting, disruption of frame-shifting is a promising target for antiviral research (Dinman et al. 2002). BYDV provides new insight into the mechanisms of ribosomal reading frame maintenance because its RNA has a novel structure for bringing about the frame-shift. Unlike any other known RNA, base pairing between a region four-kilo bases downstream in the 3′ UTR, and the frame-shift site is necessary for frame-shifting (Barry and Miller 2002). In contrast, for the Poleroviruses, a small pseudo knotted RNA structure is sufficient to bring about frame-shifting (Kim et al. 1999). Viruses in genus Dianthovirus (Tombusviridae) have a frame-shift structure very similar to BYDV (Kim and Lommel 1998), although it is not known if long-distance base pairing to a downstream sequence modulates diantho viral frame-shifting. However, it emphasizes yet again how BYDV in many ways is more like the Tombusviridae than other Luteoviridae.

4.6 Relationship Among Serotypes

Sequences of coat proteins of many isolates of BYDV have been determined, revealing much variation within serotypes. Based on these sequences, the most common serotype, PAV, has been unofficially subdivided into two Subgroups, A and B, which have about 90% amino acid sequence homology in the CP gene (Mastari et al. 1998). Coat proteins of PAV isolates within subgroup A have at least 96% amino acid sequence identity to each other. The complete genomes of few isolates have been determined. Completed genome of a severe isolate called PAV-129 was sequenced by its discoverer Gray (1996). This isolate is quite divergent from other completely sequenced PAV isolates. In the replicase ORFs 1 and 2, PAV-129 has only 80 and 88% sequence homology, respectively, to ORFs 1 and 2 of all other PAV isolates and MAV, which are all around 97% identical to each other. The 3′ terminus also differs significantly in sequence but retains a similar secondary structure to the other PAV isolates (Koev et al. 2002). The coat protein sequence of PAV-129, with 86% identity to Subgroup A PAV isolates, falls into Subgroup B (Miller et al. 2002), and PAV-129 is the first Subgroup B isolate for which the complete genome has been determined. If other members of Subgroup B prove to be as different from Subgroup A members throughout their genomes as PAV-129, then there is more taxonomic confusion. For example, BYDV-MAV has the most divergent coat and read-through proteins (76% and 60% homology, respectively, to all PAVs), but the remainder of its genome is more closely related to PAV Subgroup A than Subgroup A is to PAV-129. To map the severe symptom determinants of PAV-129, chimeric isolates of an infectious clone PAV6 (originally derived from the PAV-Ill and PAV-Aus isolates) (Di et al. 1993; Mohan et al. 1995) and PAV-129. A genome with the 5′ half (replication genes) derived from PAV6 and the 3′ half (structural genes) derived from PAV-129, replicated only about 10% as efficiently as full-length PAV6 (Koev et al. 2002). Given the divergence of the replication genes and cis-acting signals recognized by them, it is not surprising that replication was reduced. Despite the reduced virus accumulation, the chimera still caused more severe symptoms than PAV6. The chimera also was more efficiently aphid transmitted (32%) than full-length PAV6 (13%). Thus the 3′ half of the PAV-129 genome, which includes the CP, aphid transmission, and system movement genes, contains symptom determinants and (not surprisingly) aphid transmission efficiency determinants.

5 Transmission of BYDVs

BYDV is only transmitted by aphids. There are some reports of transmission through seed, which have not been established and confirmed (Szirmai 1979; Mills et al. 1980). Fruit fly (Oscinella frit) has been claimed as vector of BYDV but no confirmation and supporting evidence are reported.

5.1 Aphid Vectors

BYDVs are persistently transmitted by aphids to all common small grain cereals (Singh et al. 1993) and are not transmissible through seed, soil or sap. More than 20 aphid species transmit BYDVs and the most important are *Rhopalosiphum padi* L., the oat bird-cherry aphid; R. maidis (Fitch), the corn leaf aphid; *Macrosiphum avenae* Fabr., the English grain aphid; and *Shizophis graminum* (Rondani), the greenbug. In some areas, *Metopolophium (Acyrthosiphon) dirhodum* (Walker) is an important vector. In South Africa, the Russian aphid (*Diuraphis noxia*) is a reported as vector (Wiese 1987).

5.2 Aphid Transmission

BYDV isolates are transmitted in the persistent or circulative manner. The acquisition and infection feeding periods of 48 h or more are required to obtain maximum transmission rate. Once acquired, the virus is retained for many days, often the rest of the vector's life. As the virus is phloem limited, the aphids that transmit BYDV must feed to acquire virus and usually multiply on susceptible hosts. After feeding, the virions pass through at least three barriers in the aphid by specific uptake. They do not replicate in the aphid. Each BYDV serotype is transmitted efficiently by only a limited number of aphid species (Power and Gray 1995). The vector specificity of BYDVs does not always correlate with serotypes. (Lister and Sward 1988; Halbert et al. 1992; Lei et al. 1995). For example, Creamer and Falk (1989) described an RPV isolate from California (RPV-CA) that is transmitted efficiently by Sitobion avenae, which gives it the transmission phenotype of PAV. The genomes of RPV-CA and the type RPV-NY isolate exhibit sequence homology in the 30 halves but are unrelated in their 50 halves, based on the northern blot hybridization. The transmission phenotypes of BYDVs may be altered transiently by heterologous encapsidation during mixed infections (Rochow 1970a, b; Creamer and Falk 1990; Wen and Lister 1991). Aphid transmission has been discussed in detail (Wen and Lister 1991; Power and Gray 1995).

5.2.1 Role of the Readthrough Domain

Aphid transmission of luteoviruses requires that the genome be encapsidated (Chay et al. 1996a, b). BYDV virions contain 180 subunits of CP (Randles and Rathjen 1995). A few copies of CP in the virion also contain the RTD (Filichkin et al. 1994; Wang et al. 1995). BYDV mutants deficient in the RTD can form virus particles (Filichkin et al. 1994; Mohan et al. 1995) but are not aphid transmissible (Chay et al. 1996a, b). These experiments can be difficult to interpret because a portion of the RTD may also be required for efficient virus movement within the plant

(Brault et al. 1995). Chay et al. (1996a, b) avoided this problem by using a complementation experiment in which an RTD mutant that replicated better than wild-type RNA in protoplasts was heterologously encapsidated in wild-type CP and CP-RTD that were provided by a co-infecting PAV transcript containing a mutation in another gene. The RTD mutant RNA could then be transmitted by aphids to plants, in which it replicated and spread. The virus in these plants could not be transmitted by aphids. Thus the absence of aphid transmission of the RTD mutant that was observed originally was not due to an inability to replicate or spread in the plant. Similarly, mutations in ORF 5 of BWYV knocked out its ability to be transmitted by aphids to plants (Brault et al. 1995; Bruyere et al. 1997). In these experiments, infectious DNA clones of the virus were transmitted to *Nicotiana clevelandii* plants by *Agrobacterium*-mediated inoculation. Those with mutations in ORF 5 were not transmittable by aphids to other plants. BYDV virions purified from plants contain an approximately 50-kDa, C-terminally truncated form of the RTD (Cheng et al. 1994; Filichkin et al. 1994; Wang et al. 1995), suggesting that the amino terminal half of the RTD provides the aphid transmission function.

5.2.2 Interactions Within the Aphid

How do the CP and RTD interact within the aphid? After acquisition from the phloem, virions are transported to the aphid hindgut. The virus must then cross three distinct transmission barriers to complete the transmission process (Gildow 1993; Gildow and Gray 1993). The first barrier is the hindgut epithelium. The virus is transported into the hemocoel in coated vesicles (Gildow 1993). The recognition at the hindgut membrane appears to be luteovirus-specific but not serotype-specific, as most BYDVs can be acquired into hemolymph of both vectors and non-vectors, whereas unrelated viruses, such as BMV, that are not transmitted in a circulative manner are excluded. It is likely that a hindgut receptor interacts with CP domain(s) shared among most BYDVs (Gildow 1993; Chay et al. 1996a, b). The readthrough protein appears not to be required for the translocation of BYDV virions across the hindgut membrane because PAV mutants lacking the RTD still accumulate in the hemolymph (Chay et al. 1996a, b).

5.2.3 In the Hemolymph

Infectious virus can remain in the hemolymph for the life of the aphid if the aphid is not continuously feeding (Power and Gray 1995). Virions may persist by interacting with the most abundant protein in the aphid, called symbionin, that is produced by a bacterial endosymbiont. Symbionin is closely related to heat-shock proteins in the GroEL family. GroEL is a chaperonin, i.e. a protein that facilitates proper folding of other proteins. Van den Heuvel et al. (1994) reported in vitro binding of pea leaf roll virus (PLRV) virions to symbionin. In vitro, PAV virions, and RTD in particular, specifically bind symbionin (SymL) isolated from R. padi and S. avenae, but

not GroEL (Filichkin et al. 1997). PLRV was reported to bind symbionin-like molecules from both vectors and non vectors (van den Heuvel et al. 1994). Therefore, it is unlikely that symbionin determines vector specificity of luteoviruses. Van den Heuvel et al. (1994) speculated that the interaction between virions and symbionin is involved in maintaining virus integrity and thus infectivity. Symbionin binding may also permit the virus to evade the aphid immune system (Gray 1996). Filichkin et al. (1997) proposed that the interaction occurs with the N terminus of the RTD that is conserved among luteoviruses. If the purpose of the interaction is indeed to stabilize virions, localization of binding to the RTD appears to be inconsistent with the observations of Chay et al. (Chay et al. 1996a, b) who detected virions lacking RTD in hemolymph. However, the level of virus lacking RTD was possibly reduced to a level below a threshold required for aphid transmission, but still detectable by the PCR method they used (Chay et al. 1996a, b). Treatment of aphids with antibiotics to kill the endo-symbiotic bacteria and purge symbionin from the hemolymph inhibited the ability of aphids to transmit luteoviruses (van den Heuvel et al. 1994). However, this treatment made the aphids sick and thus may have inhibited their ability to transmit virus only indirectly. The biological role of symbionin remains to be demonstrated.

5.2.4 Entering the Accessory Salivary Gland

Once the virus reaches the accessory salivary gland (ASG) it must penetrate the ASG basal lamina and plasmalemma to be released into salivary canals and ducts. The virus is then excreted in the aphid saliva during the feeding. Both basal lamina and basal plasmalemma of ASG have been implicated in vector-specific transmission of BYDVs (Gildow and Gray 1993; Power and Gray 1995). The association of BYDVs with basal lamina probably requires specific interaction between the BYDV capsid and binding sites on basal lamina (Gildow and Gray 1993). PAV and MAV virions did not attach to the basal lamina of non-vectors. Similarly, BMV virions failed to accumulate in the basal lamina when injected into the aphid's hemocoel. However, RPV did concentrate in basal lamina of a non-vector but was unable to cross the ASG basal plasmalemma (Gildow and Rochow 1980). The transport of BYDVs across the ASG plasmalemma occurs in coated vesicles, probably via a receptor-mediated endocytosis (Gildow 1993). The receptor on the ASG plasmalemma as well as domains on BYDV virions that interact with the receptor are unknown, and identification of these entities is a major goal of current research. Because the RTD is not required for virions to enter the hemocoel, but is required for aphid transmission, it may be required for transport of luteoviruses across the ASG membranes (Chay et al. 1996a, b). The coat protein may be the major determinant of vector specificity. By examining exchanges of regions of the CP gene between infectious genomic clones from serotypes with different vector specificities, it was found that a portion of the CP itself seemed to harbor the vector-specificity determinant. Thus, even though the RTD is required for transmission, it may not be the component that determines the precise vector specificity that distinguishes BYDV isolates.

5.2.5 Dependent Transmission

Some times aphids are able to transmit viruses from a plant only if the plant is also infected by a second virus called helper virus. Rochow (1973) reported that R. padi does not regularly transmit MAV isolate from infected oats; it often transmits MAV together with RPV, a serologically unrelated isolate, from plants doubly infected by both isolates. Rochow and Gill (1978) reported that dependent transmission occurs with a range of BYDV isolates. Dependent transmission by R. padi was 81% from doubly infected plants with either MAV or 6407 together with RPV or 6524 isolates of BYDV. Furthermore, it is also reported that dependent transmission of five isolates of BYDV occurred in tests with aphid species (Rochow 1982). R. padi transmitted RMV, MAV and SGV isolate in the presence of RPV from the mixed infections. The PAV isolate was equally effective in enabling R. padi to transmit RMV. The RMV helped in the transmission of PRV and MAV isolates by R. maidis.

5.2.6 Effect of Temperature on Virus Transmission

Temperature affects the specificity of BYDV transmission. RPV and PAV transmission by S. graminum was higher at high temperature, while the transmission of PAV by Sitobion avenae was greater at low temperature (Rochow 1969). Epidemic of BYD is expected to occur at low temperature with optimum humidity. Both the factors are favorable for cereal's growth and aphid multiplication and migration (Mathre 1985).

6 Interaction of BYDV with Other Factors

6.1 Biotic Factors

6.1.1 Interaction with Other Diseases

Comeau and Makkouk (1988) reported that in case of certain fungal diseases, most of the damage is BYDV-dependent and the damage could be almost entirely attributed to the virus. For example, Fusarium root rot of oats disappeared almost entirely from the province of Quebec, Canada, when very susceptible oat cultivars were replaced by line with moderate tolerance to the virus. Fusarium root rot is generally not very aggressive in farmers' fields, however, in experiments between 1972 and 1974, 50–65% of the plants became infected in plots artificially inoculated with BYDV. This fungal disease is therefore an example of one that has apparently been controlled by breeding for tolerance to BYDV which was the hidden causal factor. Alternaria species are other organisms that are essentially non-aggressive in absence of BYDV.

Most cereal diseases possess some aggressiveness in the absence of BYDV. However, their is field evidence on the ecological interaction of BYDV with many fungi, for example, epidemics of Septoria avanae in Canada were considered a serious disease of oats when BYDV-sensitive cultivars were grown, from the early days until about 1976. These cultivars were replaced by new ones that were less BYDV-sensitive during the period 1977–1980. Since that time only limited damage by BYDV and Septoria has been observed. However, the new cultivars are not different from the old ones when sprayed with Septoria inoculum under controlled conditions. The only logical explanation is that BYDV most likely predisposed oats to Septoria by increasing the reproductive rate of the fungus (Pelletier et al. 1974). Similar phenomena exist in bread wheat, durum wheat, and barley, but the research data has not yet been published in most cases. In Australia, BYDV was shown to have a synergistic effect with take-all disease in reducing yield (Sward and Kollmorgen 1989). In USA and Canada, results showed that BYDV induced more root rot in winter wheat. In Quebec and Ontario, it was shown that the winter-kill of winter barley was partly explained by the level of BYDV sensitivity in more than 3 years out of 10. In Quebec, the recent cultivar OAC Elmira from Ontario show better winter-survival than all checks. No significant cold tolerance was detected in this line, but it was tolerant to BYDV, which could explain its better survival.

In winter cereals, Comeau and Makkouk (1988) observed interactions between snow mold and BYDV in eastern Canada. One of the best survivors in OAC Winter triticale in northern areas, it seems that its high BYDV tolerance enhances its survival even in the presence of high snow mold levels that kill other winter triticales and winter wheats. It was observed, ‘‘hot spots’ of BYDV coincide with ‘hot spots’ of Septoria tritici in spring durum and in winter bread wheat while similar phenomenon was also observed in recent cereal nurseries distributed by International Centre for Agricultural Research in the Dry Areas (ICARDA) (Comeau and Makkouk 1988). However, there is generally no correlation between Septoria tritici symptoms and BYDV symptoms on current cultivars which are not BYDV tolerant. Under natural Septoria epidemics it is very difficult to take symptoms score on BYDV except in cases where BYDV infection was early, severe, and uniform. Such conditions are rare except in special trials where seedling dates and other practices have been altered to increase BYDV damage. If symptoms of BYDV infection occur after the boot stage, they may be mistaken for normal plant senescence; where as invisible damage and predisposition to fungal disease may occur 10–15 days earlier. That is why samples of Septoria infected plants should always be taken for BYDV detection by ELISA. The same procedure could be applied to epidemics of other fungi suspected to develop faster on BYDV-infected plants.

Trials on the interaction of BYDV with head scab (*Fusarium roseum*) showed that when BYDV infection occurs early, there is significantly more scab by kernels in BYDV-susceptible bread wheat than in BYDV-tolerant ones. It seems more than mere coincidence that the best BYDV-tolerant wheat IAS-20, was recently used with success as a parent for scab resistance (Bekele et al. 1988) although this line is not in itself highly scab resistant (Luzzardi 1985).

In one trial in Quebec, the incidence of oat leaf rust was slightly decreased on oats artificially inoculated with BYDV (Pelletier et al. 1974). In contradiction to this result, the important leaf rust strain PC-59 was often abundant on the most BYDV-sensitive oat lines in northeastern USA (Comeau 1988b). Considering this new evidence, there is now a need for more research on rust BYDV interactions in all cereal species.

6.1.2 Effect on Root Length

Semi-dwarf cultivars usually have short roots. In normal growing conditions, it has been shown that oat plant height was correlated with root depth ($r=0505$, $p<0.001$), and weight of shoots was correlated with weight of roots ($r=0.87$, $p<0.001$) (Mackey 1988). Similar relationship was also reported in bread wheat (Mackey 1973). In preliminary trials conducted in Canada, BYDV was found to have devastating effect on root length of susceptible barley cultivars, confirming similar observations on various cereals in USA (Kainz and Hendrix 1981). Little success was achieved in Canada to create BYDV-tolerant semi-dwarf cereal cultivars. This may be related to the cumulative root-dwarfing effects due in part to the dwarfing genes and in part to the root starving effect of the virus (Comeau and Jedlinski 1990).

6.1.3 BYDV and Aphid Damage

BYDV-infected sensitive cereal plants often have higher levels of amino acids in the phloem sap because of impaired translocation (Comeau 1988a). As a result the insect reproduces much more rapidly on BYDV-infected plants than on healthy plants. In response to crowding, alates are produced and migration follows. In humid, cool, and windy days, daily migration may go beyond 100 km. Under controlled conditions, scientists from the UK showed that large numbers of virus-free aphids could cause damage by themselves. For example, 10 or more aphids per head impede grain filing, however, aphids often come from BYDV infected fields some distance away. These can cause direct aphid damage plus BYDV damage to the root system. BYDV-tolerant crops could theoretically reduce the importance of aphid migration, but this is a long-term hope. Other aphid flights may originate from virus-free grasses. BYDV-tolerant crops could at least reduce in situ aphid multiplication and prolong root activity (Comeau and Makkouk 1988).

6.2 Abiotic Factors

6.2.1 Drought

Comeau and Makkouk (1988) reported that BYDV invades roots as well as aerial part, but the damage on roots can be enormous in some lines such as barley line 85 0L303.

Not only is roots growth reduced but their ability to function is also impaired. Therefore, BYDV causes reduction of uptake of water and minerals, which in turn reduces resistance to other abiotic stresses. Part of the drought damage should be attributed to BYDV which reduces the root system. The relative importance of BYDV versus drought may vary but the "drought plus BYDV" combination is always devastating. Moderate BYDV tolerance is not enough to protect plants when drought reaches a critical level; while, plants combining enough BYDV tolerance with some true drought tolerance will continue to grow.

It is worthwhile to note that some of the best BYDV-tolerant lines appear to possess drought resistance. In triticale, Muskox 658 and Merino 274 possessed this useful dual resistance while other examples of multiple resistance in bread wheat and barley have been reported by Comeau and Makkouk (1988). The barley cultivar Biirka is very sensitive to BYDV in Quebec and yields poorly when BYDV is accompanied by lack of rain. The ICARDA barley cultivar Tadmor, known for its drought tolerance, showed better BYDV tolerance than most other lines that were devoid of the Yd2 gene in Syria (Tel Hadya) in 1987–1988. In oats, the widely grown cultivar Ogle displayed BYDV tolerance and some drought tolerance; both traits are heritable but not entirely linked (Comeau and Makkouk 1988). From all available evidence, simultaneous selection for drought and BYDV tolerance would seem to be a promising filed of research. Selection for tolerance to both stresses may favor better root systems and increased water-use efficiency.

6.2.2 Mineral Stress

Mineral deficiencies observed in BYDV-infected plants usually are the result of BYDV infection rather than the cause of damage. The damaged root system of BYDV-infected oat plants did not allow proper assimilation of N, P and K (Comeau and Barnett 1979). Some wheat plants possesses a better than average ability to extract minerals from the soil. Especially phosphorus in the case of some Brazilian wheats. Many Brazilian wheat cultivars also have roots resistant to acid soil and excess of aluminum. It is striking that a large number of the best BYDV-tolerant lines in the Quebec project were found to have resistance to acid soil (Comeau and Makkouk 1988), although initial selection was made at a soil pH of about 6.0, which is good for bread wheat. The aggressive root system of these lines may have contributed to improved BYDV tolerance by better root growth throughout the soil profile. The correlation between BYDV tolerance and vigorous root system may have the strongest practical implications.

6.3 Complex Interactions

BYDV may increase the reproductive rate of aphids by modifying plant sap and also interferes with the deposition of cell wall constituents (Harper et al. 1976), making the plants more fragile and subject to seed shattering. Triple interactions involving

virus, aphids and fungi are common; climate should be considered as the fourth component. Aphids can carry fungal spores around (Fuentes and Exconde 1969; Huang et al. 1981). Moreover, aphids move to floral parts at flowering and the sticky honey dew may act as a "spore trap" to further increase fungal attack. Plant height reduction by BYDV may be in itself a further cause of increased fungal attack, for example by Fusarium and Septoria (Tavella 1987; Couture 1982).

7 Serology and Diagnosis

Enzyme linked immunosorbent assay (ELISA) using polyclonal and monoclonal antibodies are routinely used in numerous laboratories for BYDV detection and virus titre measurement. (Bashir et al. 1997; Khalid et al. 1992a, b; Khetarpal et al. 1994). The presence of plant viruses has also been detected in plant tissue by other diagnostic techniques, including immunosorbent electron microscopy (Derrick 1973), tissue blot immunoassay (TBIA) (Makkouk and Comeau 1994) and cDNA probes using virus group-specific primers and polymerase chain reaction (PCR) (Waterhouse et al. 1986). ELISA is being replaced by TBIA due to its several advantages over ELISA. TBIA is simple, quick, sensitive and cheaper than ELISA. Researchers are increasingly preferring these new techniques. They may be advantageous compared to ELISA because of specificity, sensitivity, targeting of determined sequence etc. Mostly detection methods are based on polyclonal antisera produced in rabbits. To overcome the problems of cross reaction with polyclonal antisera, production of monoclonal antibodies is the other option. Monoclonal antibodies of BYDV isolates have been produced and tested effectively for diagnosis (Pead and Torrance 1988; Torrance et al. 1986). cDNA probes have been made to clone sequences from BYDV isolates and dot blot assay using 32P-labelled probes readily detected BYDV (Waterhouse et al. 1986; Barbara et al. 1987; Eweida and Oxelfelt 1989). However, the detection on radioactive labeling and the use of probe is restricted to some laboratories in which the safety measures are available.

7.1 Antiserum Production

Production of monoclonal antibodies of hybridoma clones were reported by Hsu et al. (1984) when mice were injected with either RPV and MAV isolates which were tested for evaluation with five previously known isolates of BYDV. The sometic cells hybridization technique was used. In experiment, five cell lines were from mice injected with PAV and eight lines were from MAV injected mice. Out of 13, seven virus specific antibodies were produced three of which related with PAV, one with MAV. Two additional MAV derived antibodies reacted with both RPV and MAV. Hu et al. (1985) reported that an antiserum against SGV was obtained from the hen's egg yolk, which provided useful immunoglobulins for indirect ELISA of SGV.

In indirect ELISA, SGV was coated with a monoclonal antibody mAB-MAV4 (1: 1,000 dilutions) that reacted with SGV and MAV but not with others.

7.2 Enzyme Linked Immunosorbent Assay (ELISA)

The application of enzyme linked immunosorbent assay (ELISA) created new interests in serological detection of viruses especially viruses having low titre in their respective hosts. It is simple, rapid and more sensitive than any other serological tests. Duffus and Rochow (1978) reported that antisera produced against MAV, RPV isolates of BYDV, when tested against four isolates of beet western yellow virus (BWYV) for virus neutralization, this antisera reduced/eliminated viruses in the normal BYDV bearing areas. Usefulness of ELISA for BYDV diagnoses has been reported by (Rochow 1979; Lister and Rochow 1979). While Rochow and Carmichall (1979) and Rochow (1982) used biological and serological tests for comparing viruses collected from small grain field samples with five characterized isolates of BYDV i.e. RPV, RMV, MAV, PAV and SGV. Of 265 isolates identified, 164 were similar to PAV, 69 resembled RPV, 20 were SGV like. The PAV and MAV like strains of BYDV in ryegrasses were detected by using ELISA techniques. The plants showing foliar symptoms were found to contain large proportions of MAV like strains of BYDV. However, there was a poor association between PAV like virus and foliar symptoms (Holmes 1985). It has been reported by Rochow et al. (1987) that four isolates of luteoviruses including BYDV were identified with a modified indirect ELISA for SGV isolate to permit simultaneous use with direct ELISA procedure.

7.3 Serologically Specific Electron Microscopy (SSEM)

Serologically specific electron microscopy (SSEM) has been used successfully for diagnoses of BYDV (Paliwal 1977). It was reported by Diaco et al. (1986) that SSEM was highly sensitive procedure and can detect very small quantity of BYDV. When SSEM procedure were performed on a mixture of BYDV and morphologically different soybean mosaic virus (SW) detected only BYDV. SSEM proved very useful in detecting a wide range of BYDV isolates, even in mixed infection.

8 Control of BYD

The most appropriate method for the control of BYD will depend on the conditions under which a crop is grown. The losses caused by BYD can be reduced by adopting the following three approaches:

1. **Crop husbandry** largely by changing sowing dates and adopting cultural methods to avoid risk of infection.
2. **Killing of aphid vectors** by the use of pesticides or preventing them feeding on susceptible hosts
3. Breeding for resistant/tolerant cultivars

The selection of method depends on many local circumstances, not the least of which is the economics of growing the susceptible crop. Although some insecticides have been reported very effective but are not economical. Breeding resistant or tolerant cultivars is the most economical and practical method for controlling BYDVs.

8.1 Cultural Methods

The usual way to control BYDV is to avoid early sowing in September to October, or very late sowing in April to May. As aphids transmit BYDV, it is important to minimize the time where young plants are exposed to active aphids populations (Plumb and Johnstone 1995) or by carefully timed planting when aphid populations are monitored (Plumb 1995). Altering the sowing time does not prevent infection by BYDV, but it will put off the time of infection so that the plants are more tolerant when they are attacked. In spring planting it is usually the latest sown crops that suffer most damage, as this increases the risks of younger, very susceptible seedlings being exposed to aphids carrying virus. In spring there is usually no conflict between sowing date, potential yield, and BYDV infection, as early sowing increases yield potential and decreases virus infection. Ensuring clean seed beds, especially for autumn-sown crops, and avoiding carryover of virus on volunteers and previous grass crop, are essential precautions to avoid virus infection. Although late planting has proven to be successful for avoiding exposure of the crop high aphid population, but potential yield might be reduced as well due to the shorter growing cycle (Plumb 1984). In general, it appears to be complex to make such recommendations to crop production in developing countries. Promoting resistant or tolerant germplasm still appears to be the most feasible method for BYD management.

8.2 Use of Pesticides

Pesticides either applied as granules or sprays have been widely used to control the aphid vectors of BYDV. Insecticidal sprays should be applied only if they become essential and are economical (Eastop 1983). Pesticides have been widely used in Europe to control BYDV. When insecticides are applied, the time of application is very critical. Spread of the disease can be controlled by aphicides (McKirdy and Jones 1996) and supply of the chemical pirimicarb prior to stem elongation has

momentary shown effective in controlling the vector (Mann et al. 1997). The fact that many grass species are alternative hosts for the vector can complicate the control of BYDV as a small population of aphids can over winter here and attack new sown crops (Hewings and Eastman 1995). Due to the acquisition time and the latent period needed before new transmission occur, BYDV is actually one of the viruses where control of the vector actually makes sense.

8.3 Cross Protection

Classical cross protection between BYDV isolates has been demonstrated (Ranieri et al. 1992). Cross-protection occurs between BYDVs of Subgroup-I but not between BYDVs of Subgroup-II or between Subgroup-I and II BYDVs (Wen et al. 1991). While similar BYDVs can cross-protect against one another, mixed infections with unrelated BYDVs, i.e. one from each subgroup, can have the opposite effect. Mixed infections of PAV and RPV give more severe symptoms, including more stunting and higher virus titer, than do infections with either virus alone (Baltenberger et al. 1987; Miller et al. 1997). This is consistent with the phenomenon observed among many luteo and luteo-like viruses (Miller et al. 1997). In all cases, the synergistic interaction involves a replicating RNA with a Subgroup I-like polymerase and one with a Subgroup II-like polymerase, suggesting a positive interaction between the two polymerases or the RNAs that they recognize. This interaction has important practical applications. First, it is unlikely that transgenic plants expressing a Subgroup-I BYDV polymerase will be resistant to a Subgroup-II BYDV. More importantly, such transgenic plants could be more susceptible than untransformed plants to a virus of the opposite subgroup if they express the synergy-conferring gene at high enough levels (Vance et al. 1995; Miller et al. 1997). Genetically engineered cross protection was evaluated as a means of controlling BYD (Vincent et al. 1992) with some success. However, it will take time until transformation techniques in wheat become routine. Until such time, use of transgenic expressing viral coat proteins or other viral genes will not be feasible.

8.4 Breeding for Resistance

Genetic resistance is generally considered as being the most practical means of reducing losses inflicted by virus. In order to breed resistant cultivars, sources of resistance must be identified and available. So far no major gene has been identified in bread wheats that confer resistance to infection by BYDVs. Qualset et al. (1990) summarized the genetics of host plant resistance to BYDV and considered some of the problems facing planting breeding in identifying and utilizing the genes that are beneficial. Most success in identifying specific genes conferring resistance to BYDV has been achieved in barley which a major gene conferring tolerance has been

identified. Schaller (1984) summarized the genetic background and the recognition of the Yd2 (Rasmusson and Scheller 1959) gene that conditions resistance, which is located on chromosome 3 (Schaller et al. 1964). In California, where most of early selection work was done, five tolerant cultivars have been released and comprise the majority of the acreage. Other tolerant cultivars of barley have been released but do not appear to contain Yd2 gene (Grafton et al. 1982). BYDV infects triticale (a wheat x rye hybrid) and can be damaging. BYDV-resistant lines have been identified (Comeau and Pierre 1990) but none are agronomically acceptable. Recent work on both spring and winter triticale and methods of evaluating its response to BYDV are described by Collin et al. 1990. No resistance to BYDV in maize has been reported. However, some tolerance to BYDV has been reported in maize hybrids in Italy (Lorenzoni et al. 1990).

8.4.1 Natural Tolerance to BYDVs

Well-characterized resistance genes to BYDVs are few. Tolerance is conditioned by one to four genes in oats (Burnett et al. 1995a, b). In general, tolerance or resistance is specific only against certain isolates of a serotype or subgroup (Gray et al. 1993). A single partially dominant gene, Bdv1, confers tolerance to BYDV in some wheat varieties (Singh et al. 1993). High levels of resistance to BYDVs (PAV and RMV) in Agropyron species were reported in early 1980. Derivatives of the hybrid between Thinopyron intermedium (Host) and hexaploid wheat were tested for resistance to BYDVs in the 1980 and providing promising, i.e. resistance. The resistance derived from the partial amphiploid TAF 46 has been transferred to hexaploid wheat through recombination during cell culture (Xin et al. 1991; Griggs 1992). Several researchers have reported desirable traits in the wild relatives of cereals (Sharma et al. 1984; Brettel et al. 1988; Ceoloni et al. 1988). These traits constitute an important genetic resources for cereal improvement (Cauderon 1979). Aegilops species are often used in interspecific hybridization with durum (*Triticum durum*) and bread wheat (*Triticum aestivum*). The gene bank of the International Centre for Agricultural Research in Dry Areas (ICARDA) and CIMMYT have a large collection of Aegilops species. The genes of resistant wild wheat grasses (Thinopyrum or Agronpyron species) have been introgressed into wheat in interspecific crosses to produce wheat lines containing a portion of the wheat grass chromosome that confers substantial resistance to BYDVs (Banks et al. 1995; Sharma et al. 1995). The best characterized resistance to BYDV is conditioned by the Yd2 gene in barley, which confers resistance only to Subgroup-I BYDVs (Baltenberger et al. 1987; Burnett et al. 1995a, b). Barley lines containing the Yd2 gene express a unique polypeptide that is tightly linked to Yd2 (Holloway and Heath 1992). It encodes a putative subunit of a vacuolar proton-translocating ATPase (Ford et al. 1996). A high-resolution map of chromosome 3H around the Yd2 locus has been generated, and cloning of the Yd2 gene is imminent (Ford et al. 1996). This will provide a major breakthrough in our understanding of BYDV-plant interactions. At present, there is only one marker known

for resistance/tolerance to BYDVs which could assist in selection. Leaf tip necrosis is a marker for tolerance to gene (Bdv1) in bread wheats (Singh et al. 1993).

8.4.2 Transgenic Resistance

The most cost-effective and environmentally desirable form of disease control is genetic resistance. However, natural resistance genes to BYDV are few (Burnett et al. 1995a, b). Yet, because of the threat of BYDV, breeders are limited to lines that have significant natural BYDV resistance or tolerance. To allow breeders to expand beyond these limitations, transgenic oats tolerant to BYDV were developed (Koev et al. 1998). Oats were transformed with a gene designed to express the 5′ half of the BYDV genome driven by a CaMV 35S promoter. The most resistant line of transgenic oats initially showed mild symptoms but then recovered and grew to maturity. In laboratory growth conditions, yield was slightly reduced compared to un inoculated controls and virus was sometimes detectable, but the yield was infinitely greater than in the inoculated non-transgenic controls which were actually killed by virus infection long before flowering. The transgene was stable inherited in a Mendelian fashion. Field trials were less promising, mainly because the only line of oats that could be transformed (genetically engineered) at the time was not agronomically useful (Somers et al. 1992). It was derived by artificial hybridization of Avena fatua with a cultivar of oat (*A. sativa*), followed by back crosses to oat. These plants (with or without a transgene) were smaller and less robust than agronomic cultivars, in the presence or absence of virus infection. Fortunately, recent improvements in technology now permit transformation of such agronomic lines as Bell. Scientists greatly through improved design of transgenes to engineer resistant plants with high efficiency. Barley plants transformed with inverted sequences of BYDV genes, causing the transcripts to form long, double-stranded hairpin RNAs were immune to BYDV infection (Wang et al. 2000). Presumably the double-stranded RNA induces the host's post-transcriptional gene silencing system (Waterhouse et al. 2001). To the best of our knowledge, no plants engineered for BYDV resistance have been released for use by growers. Unfortunately, due to the low value of oats as a profit-making enterprise, and perhaps due to concern about consumer acceptance of food derived from GMO crops, corporate interest in funding transgenic oat research has waned. Perceived risks imposed by transgenic BYDV-resistant oats drew attention in a Science magazine article about an unpublished poster presentation at a scientific conference (Kaiser 2001). The experiments alleging that pollen escape from transgenic BYDV-resistant oats could lead to "super weeds " were confined to the greenhouse and used no transgenic plants. Yet the benefits of new resistance genes, such as reduced pesticide inputs and increased yields, are clear (Miller et al. 1997).

Application of insecticides on wheat in UK and Australia to control the aphid vectors of BYDV often results in substantial yield increases (Plumb and Johnstone 1995) that are attributable to the absence of BYDV infection. For more details on

the economic costs and worldwide occurrence of BYDV diseases, and the economic and environmental costs of controlling them, we refer the reader to other reports in these proceedings, and to the book BYDV: Forty Years of Progress (D'Arcy and Burnett 1995). One legitimate concern with regard to the applicability (but not safety) of virus-derived transgenes for resistance to BYDV is the wide range of sequence variation among isolates. BYDV isolates that lack high homology to the transgene would not be hindered by transgene-induced post-transcriptional gene silencing (Miller et al. 1997; Wang et al. 2000; Waterhouse et al. 2001). Thus, virus-derived transgenes may confer resistance to only a subset of BYDV isolates in the field.

Recombinant DNA technology has opened the prospects of increasing genetic diversity in crop plants by their transformation. However, no suitable systems yet exist for regenerating small grains cereal plants. Such techniques will soon be developed and will make available genetic information to overcome the present difficulties. No information has yet made of decreasing BYDV infection through resistance to the aphid vector. Although some differences in host reaction to aphid vectors have been reported but no evidence to differentiate in infection of different cultivars (Lowe 1978).

9 Conclusion

A large number of studies have been conducted since the disease BYD has been identified. Although a lot of information are available on the biology, pathology, epidemiology and disease management, but still the problem of crop losses due to BYDV infection exists in many parts of the world. Due to continuous expansion of wheat in the absence of BYDV-resistant cultivars, it is likely to increase yield losses related to BYDV. In regions of low inputs where primary infection is extensive and use of pesticides is un-economical, the only option for disease management is through manipulation of sowing dates and host resistance or tolerance. With the emergence of more knowledge on host genotype x virus isolate interaction, the development of cultivars with resistance or tolerance to virus or vectors will be possible. The use of modern molecular techniques will help in proper diagnosis, to understand the virus-vector interaction and epidemiology of the disease. In high input agriculture, pesticides are likely to remain important but their use can be decreased with the introduction of disease resistant or tolerant cultivars.

There is a continuing need to characterize BYDV isolates. Sound knowledge of a pathogen's epidemiology and ecology and of the pathogen's interaction with the host and its environment will help for better disease management and reduce crop losses. Recombinant DNA technology be used to create genetic diversity in host plant and to utilize Yd1 resistant gene already identified in wild relatives of bread wheat and other hosts. Host resistance to aphid vectors should also be explored in wheats or wild relatives and included in breeding programme.

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