Microorganisms for Sustainability 4
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Advances in Soil Microbiology: Recent Trends and Future Prospects

Volume 2: Soil-Microbe-Plant Interaction

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Contents

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Chapter 1
Soil Microbial Diversity: An Ecophysiological Study and Role in Plant Productivity

Bighneswar Baliyarsingh, Suraja Kumar Nayak, and Bibhuti Bhusan Mishra

Abstract Soil is considered as one of the most competent ecosystems for subsistence of microorganisms. Soil microbial community structure and activity depend largely on structure and status of the soil habitat. Diverse heterotrophic microbial communities in soil along with their complex web of interaction facilitate the cycling of micro- and macro-nutrients in soil ecosystem. The demand of sustained plant productivity is achieved through managing soil fertility. The dynamic relationships between different components, living or nonliving, of agroecosystem control the richness of plants or crops. In turn, soil organic matter is influenced by the inputs from plants and also their chemistry makes each ecosystem somewhat unique in its microbial community. Though the role of soil microbiome is widely known, we still have a limited understanding of its complexity. Thus, understanding the microbial diversity will enhance our ability of increasing agricultural production.

Keywords Soil microbiology · Soil microbial habitat · Plant-soil microbial interaction · Microbial diversity · Soil fertility

1.1 Introduction

In the recent past, growing understandings on the potential of microorganisms have given much emphasis to explore and study the active microbial population inhabiting the soil. Since the beginning of the nineteenth century, soil

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microorganisms have gained importance as the driver of biochemical processes that are beneficial to ecosystem. These microorganisms carry out different processes of decomposition of organic substances, transformation of elements, and also recycling of nutrients that are essential for growth of animals, plants, and crops. Some soil-inhabiting microorganisms are, however, injurious to plant and animal life, acting as pathogens affecting the host directly or releasing toxic substances in the soil ecosystem. A better understanding of the soil microbes is thus important to interpret their impacts on agriculture and environment. Hence, the soil microbiologists are not only focusing on diversity of microbes in soil but also their interaction with the environment as well as with other organisms.

1.2 Historical Perspectives of Soil Microbiology

During the mid-nineteenth century, studies of microbiologists like Louis Pasteur, Selman Waksman, and Sergei Winogradsky led the foundations for the modern soil microbiology research. Among various discoveries by Winogradsky, the father of soil microbiology, some of the notable studies are the sulfur cycle, role of CO_2 and inorganic ions on microbial growth (chemoautotrophy), and nitrification. He was also honored by naming one of the nitrifying bacteria species as *Nitrobacter winogradskii*. Nitrogen fixation can be accomplished by nonsymbiotic bacteria which was first suggested by Berthelot in the late nineteenth century. Soil microbes bring about the mineralization of litters and make easy availability of the essential nutrients for the growth and development of plants and animals. It was also emphasized that the addition of stable manure to the soil is more effective than to direct addition of inorganic nutrients.

The fixation of nitrogen by leguminous plants is one of the most important and well-studied microbial process. Studies on soil fungus and bacteria involved in nitrification and denitrification and chemical transformations of nitrogen in soil along with in composts, laid foundation for the modern era of soil microbiology, and all these processes have direct effect on plant growth and productivity. The overall relationship of soil microbial population with that of soil fertility also gave rise to the concept of inoculating desired microorganisms to soil (Kuramae et al. 2012).

Apart from agronomic importance of soil microbes, in 1939 S. Waksman and Rene Dubos found a soil actinobacteria (formerly actinomycete) *Streptomyces* sp., with antibiotic properties. Waksman was awarded Nobel Prize in 1952 for the findings on antimicrobial properties of soil microbes. Studies on the actinobacteria of the soil by Krinsky, Conn, Waksman, and Curtis, by Melin and Hayner on mycorrhizal fungi, and on the soil protozoa by Cutler widened the purview of soil microbiology. Since their discovery, soil microbes are widely studied and applied to various realms of human endeavors (Balser et al. 2010).

1.3 Habitat of Soil Microorganism

Soil is not an inert material but a dynamic and most biodiverse ecosystem as it is home to several thousand different species of organisms/microorganisms. The population, composition, and activities of soil microbes are influenced by their soil habitat. Cultivated lands are rich in organic matters and contain much higher microbial population than sandy or eroded soils. Within this habitat, the soil microbes respire, compete for food, cooperate among themselves, and respond to the changes in their inhabiting environment.

Typically, differing sizes of soil aggregates and soil pores constitute the soil microbial habitat (Sylvia et al. 2005). The texture and soil types influence the composition and population of microbial community and vice versa. Some soil microbes secrete polysaccharides, gums, and glycoproteins, which glue soil minerals together, forming the basis for soil structure. Further, fungal hyphae and plant roots bind soil aggregates together that provide favorable environment for plant growth. Rudakov (1951) suggested that active humus play an important role of cementing the soil particles into aggregates. The soil cementing substances are mostly composed of (1) compounds of uronic acids, (2) bacterial proteins or products of their lytic activities, and (3) lysates of fungal cultures and/or colloidal protein compounds synthesized by soil bacteria.

Similarly the diversity of soil microbial community is influenced by physical parameters like temperature, humidity and seasonal variations (Lipson 2007), soil acidity or alkalinity (pH), oxygen levels, and availability of nutrients. The soil fertility can be indirectly correlated with the overall microbial biomass which depends on availability and quality of carbon, termed as soil organic carbon (SOC). The amount of SOC available within the initial 1m depth of soil is more than two to three times the carbon amount present aboveground (Brady and Weil 2002). Thus most biologically active region of the soil is believed to be the upper 20–30 cm of soil where abundance of soil microbial communities are present due to rich SOC (Fierer et al. 2007; Jobbágy and Jackson 2000; Veldkamp et al. 2003). Carbon availability often declines with depth, as does overall microbial biomass. Also many microorganisms exist in topsoil having rich carbon sources, than in the subsoil. They are especially abundant in the area close to plant roots (called the rhizosphere), where sloughed-off cells and root exudates provide carbon sources. It is established that there is decrease in fungal-to-bacterial ratios with increase in depth. Approximately 3×10^4 bacteria, 1.5×10^5 fungi, 6×10^4 algae, 1×10^4 protozoa, 5×10^5 nematodes, and 3×10^4 earthworms are estimated to be present in the soil (Pankhurst 1997).

Soil fertility is one of the significant parameters of natural or managed agroecosystem to achieve sustained productivity. To access the soil quality, changes in chemical, physical, and biological properties are monitored which are the indirect measure of productivity and diversity. Biological indicators considered to access soil quality are microbial biomass, soil respiration, soil enzyme activities, earthworm numbers, etc. The metabolic quotient (qCO_2) is the estimate of the

amount of CO_2-C released per unit of microbial biomass in time that represents the thriving status of the soil microbes. Besides, biochemical indicators such as microbial enzymes are also useful in understanding soil fertility. The biological reactions most commonly studied were metabolism of nitrogenous compounds which includes ammonification, nitrification, denitrification, and the fixation of nitrogen. Microbial indicators have advantage in forecasting early to any changes in soil quality as it is being susceptible to minor changes in ecosystem.

In addition to structural diversification of soil microbes, soil organisms are naturally active during certain period of the year. Most are active when the soil is warm and moist, like during late spring and early summer. Moreover minerals, aggregation of the soil particles, and soil porosity influence diversification of bacterial community in soil microhabitats (Certini et al. 2004; Carson et al. 2007). The uniqueness of each soil ecosystem is due to typical microbial communities performing diverse activities and multifaceted interactions with their habitat (Wixon and Balser 2009) (Fig. 1.1).

1. The soil microbes provide basic macroelements of nutrition, viz., carbon, nitrogen, and phosphorous for growth and development of plants.
2. The microorganisms show indirect way of controlling the production and regulation of growth-specific phytohormones.
3. Symbiotic association with plants or plant parts (e.g., leguminous plant roots) is maintained by very special group of microbes.
4. Not all soil-dwelling microbes show beneficial interaction; some have harmful effects on higher plants. The microbes either show competition for uptake of basic nutrients from the soil or depend on the higher plants as parasites or attacking them by producing toxic chemicals.
5. Indirect injury to plant growth is observed when various bacteriophages feed on useful bacteria.
6. On the other hand, the immediate vicinity of plant parts creates a microenvironment to the microorganisms for their growth and proliferation.
7. The plant residues and root exudates are the rich source of nutrients, consumed by the microorganisms.
8. Plants metabolites may have antagonistic effects on the growth and sustainability of microorganisms.

Various groups of bacteria, fungi, and actinobacteria either penetrate the roots of plants and live there or live in close proximity of the root system. The concept of the "rhizosphere" is introduced to assign the close association between soil microorganisms and the root systems of higher plants. The "root region" encompasses both the root surface and the rhizosphere which are the zones of high microbiological activity. Typically this type of association may be considered as midway between true symbiosis, observed between root-nodule bacteria with the leguminous plants and the phenomena of parasitism. Accumulation of manure, lowering of the concentration of certain mineral nutrients, partial desiccation of the soil, and increase in soil carbonates following root excretion are the major causes of rhizosphere effect. The tuberization and protein formation in certain seeds in plants are

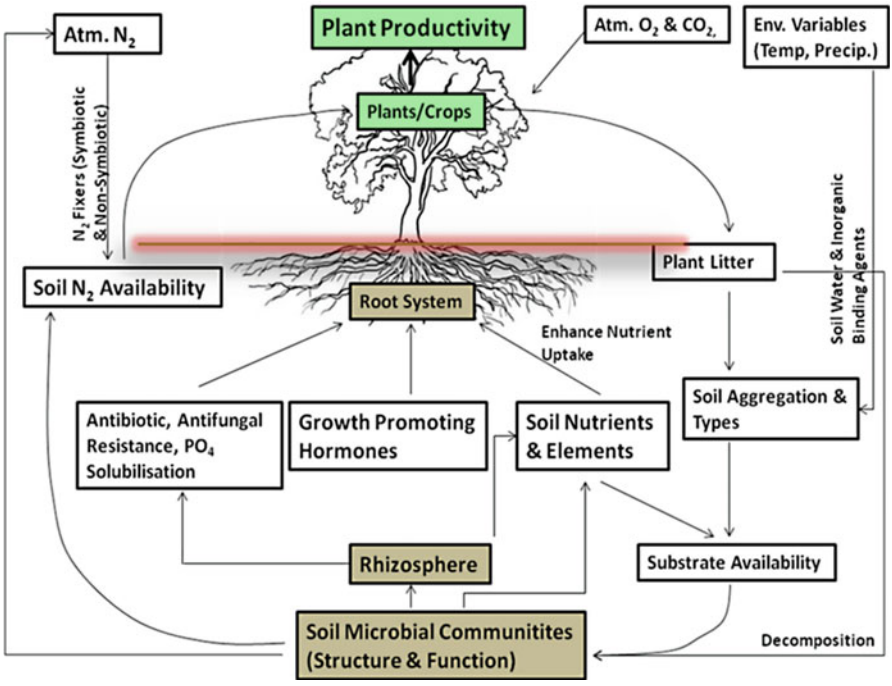

Fig. 1.1 Dynamic interactions between microbes and plants

induced by some fungi. Garrett (1951) emphasized the necessity to distinguish between rhizosphere effects and typical characteristics of living roots with that of the diseased root microenvironment.

The diversified soil microbes play pivotal roles in an array of terrestrial ecosystem functions such as nutrient recycling, sustaining plant growth, water purification, carbon storage, maintenance of soil structure, degradation of xenobiotics, nitrogen fixation, or competitor for pathogens to successfully establish desired microbial populations (Insam 2001). Similarly, the decomposition and nutrient recycling are carried out by diverse group of microbes in a cascading network of chemical process. By decomposition processes, soil microbes liberate simpler carbon compounds, nitrogen, CO_2, and minerals from dead organic material which are ultimately consumed by higher plants.

The decomposition carried out by soil microbes is either aerobic (aerobic bacteria and fungi) or anaerobic microbes. The aerobic decomposition of plant residues by the microbes produces humic acid in the soil. The abundance of humic acid and fulvic acid facilitates the gluing properties of humus. In addition, various protopectinase enzymes were produced by soil microbes like sporogenous *Bacilli*, *Bacillus polymyxa*, *B. radiobacter*, *B. mycoides*, *B. laterosporus*, and *Clostridium macerans*. Some fungi also take part in the decomposition of plant residues by producing the humic acids. *Trichoderma lignorum*, *Mucor intermedius*, and

Mortierella isabellina are few examples of fungi, mostly involved in the process of soil structure formation (Harris et al. 1966).

The solubilization of soil minerals, especially the carbonates, phosphates, and zeolites, is carried out by microorganisms with the help of CO_2; nitrous, nitric and sulfuric acids; and organic acids. Soil toxins constitute the toxins produced from soil microbes as a result of their activities. Thus the soil solution represents very dynamic part of the soil where most of the biochemical processes occur. The fouling properties of soil can be treated with heat, volatile antiseptics, or simple lime to increase productivity. Reports indicate that the germination of plant seeds and seed growth is favored by the soil microorganisms. For example, rapid evolution of CO_2 by microbial respiration creates anaerobic conditions unfavorable to oxidation and favorable for germination. Recent studies depict that microorganisms may produce hormones and/or similar substances which promotes plant growth.

1.4 Influence of Plants on Soil Microorganisms

Although, there are few experimental investigations that suggest the influence of plant diversity on soil microorganisms (Bargett and Shine 1999; Wardle et al. 1999; Stephan et al. 2000), there are reasons to expect that plant diversity can affect microbial population in the soil ecosystem. It is due to the lack of experimental procedure to accesses the direct influence of the growing plant or plant products on soil microbes. Mostly the effect of plants on soil microbes is studied indirectly by (a) measuring the numbers of microorganisms, (b) nitrifying and denitrifying capacity of the soil, and (c) oxidizing power of the soil in terms of aeration or CO_2 production. The effects of growing plants on the structure and function of soil microorganisms can be represented as:

1. The growth of soil bacteria and fungi is favored by the soluble organic and inorganic compounds like formic, oxalic, and malic acids, certain reducing and nonreducing sugars, phosphatides, and various nitrogenous compounds secreted by the plants.
2. In addition to these plants in the form of dead roots and root hairs, epidermal cells and other waste products help the growth of microorganisms.
3. Various soil minerals, including nitrates, phosphates, and potassium salts, are continuously removed by the growing plants resulting in change in soil solution and modification of microbial activities.
4. Plants excrete considerable CO_2 into the soil which increases the solubility of certain inorganic soil constituents and thereby changing the composition of the soil atmosphere.
5. Soil porosity and structure are modified and affected by the plant diversity.
6. Sometimes, plants can hinder the growth of soil microbes by removing a considerable amount of moisture from soil.

Feedback mechanisms between plant and microbial communities control the richness of plant species and productivity. For example, soil bacteria are stimulated by cowpeas, field peas, vetch, and soybean. Among the bacteria, particularly, the *Radiobacter* sp. increased in number when legumes are cultivated. In contrast, continuous production of single type of plant in a soil leaves residues that may result in a change in the chemical composition of the soil leading to microbial disequilibrium. This is evident from the fact that the pathogenic fungi population increases in soil habitat by continuous farming of wheat, flax, or clover. While the alfalfa roots have only a slight stimulating effect upon filamentous fungi, the eggplants had a significant effect (Starkey 1931). A range of stimulatory effects on soil microbes by cultivation of alfalfa, rye, and vetch plant are observed ranging least on actinobacteria, slightly on fungi, and highest toward soil bacteria.

1.5 Microbial Diversity and Soil Biological Processes

Soil is reckoned as a storehouse of microbial activity, though the space inhabited by active microorganisms is approximately less than 5% of the total occupied area. Despite their small volume, soil microorganisms are key players in the global cycling of organic matter, reworking organic residues or mineralizing them to CO_2, H_2O, N_2, P, S, and other nutrients (Table 1.1). Hence, soil microbes are playing the most vital role in plant growth regulation and contributing thereby to the interdependence of diversity – fecundity (Pradhan et al. 2014; Mitchell 2003; van der Heijden et al. 2008). Major microbiological activity is confined to the clusters of accumulated organic matter and rhizosphere. Thus, there is a growing demand to study on microbial community composition in various microhabitats in nature.

The soil as a habitat exhibits utmost variability leading to adjacent microhabitats differing widely in their physical and chemical properties. Diversified heterotrophic microbial communities residing in soil control the ecosystem carbon (C) and nitrogen (N) cycling (Fig. 1.2) through various processes that control ecosystem and represent a potential mechanistic link in between plant diversity and ecosystem function. The growth-limiting resource availability plays a key role in determining biotic community composition (Tilman 1982, 1987). The resource availability for soil microbial communities is incarcerated through organic compounds in litters which can be used for cellular energy generation (Smith and Paul 1990). Changes in plant diversity could alter the yield as well as the range of organic compounds that limit and thus control the composition as well as microbial community function.

1.5.1 Bacteria and Actinobacteria

The sustainability of soil function is the result of bacterial diversity. Endemism and clonality, which have an intrinsic spatial dimension (Grundmann and Normand

Table 1.1 Major roles of diverse soil microorganisms

Domain/phylum/ group of microorganism	Important species	Association with host	Major role of these species/ strains	References
Archaea	*Nitrososphaera viennensis*, *Methanosarcina* sp., *Methanosaeta* sp.,	No association	Contributing to ammonia oxidation in soil	Aislabie and Deslippe (2013)
			Weathering minerals as well as nutrient cycling in soil	
			Carry out N_2 fixation	
			Curtailment of atmospheric N_2 to NH_4^+	
Bacteria	*Rhizobium* sp., *Bradyrhizobium* sp., *Mesorhizobium* sp.,	Symbiotic	Fixation of atmospheric nitrogen in leguminous plant in root nodules	Giordano and Hirsch (2004)
			Present inside the host root system	
	Pseudomonas sp., *Bacillus* sp., *Klebsiella* sp., *Azotobacter* sp., *Azospirillum* sp., *Azomonas* sp.	Asymbiotic	Fixes atmospheric nitrogen usable form such as ammonia	Glick et al. (1999)
			Remain in close proximity of root system	
Actinobacteria	*Arthrobacter* sp., *Rhodococcus* sp.,	No association	Fixation of atmospheric nitrogen	Ahemad and Kibret (2014);
	Streptomyces sp., *Rubrobacter* sp., *Terrabacter* sp., *Acidimicrobium* sp., *Chloroflexi* sp., *Planctomycetes* sp., *Gemmatimonadetes* sp.		Solubilization of minerals	Bhattacharyya and Jha (2012) and Franco-Correa and Chavarro-Anzola (2014)
	Micromonospora sp., *Streptomyces* sp., *Streptosporangium* sp., *Thermobifida* sp.,	No association	Antagonistic efficiency against different fungal root pathogen	
	Frankia sp., *Nocardia* sp. *Kitasatospora* sp.,	No association	Efficient IAA producers (plant growth hormone)	
Verrucomicrobia	*Methylacidiphilum SolV*, *Methylacidiphilum Kam1*, *Verrucomicrobium spinosum*	No association	Nitrogen fixation and associative activity	Wertz et al. (2012)

Yeast	*Kluyveromyces waltii*, *Sccaromycopsis cataegensis*, *Pachytichospora transvaalensis Rhodotorula* sp., *Azotobacter* sp., *Cryptococcus* sp., *Lipomyces* sp., *Meyerozyma* sp.	Various association	Utilization of bacterial, plant root exudates for synthesis of plant protectants and other useful substances involved in plant growth	Vishniac (1995), Cho et al. (2001), Nakayan et al. (2009)
			Known producers of extracellular polymeric substances	
			Produces xylitol, phosphate solubilization and associated activities, and soil aggregation	
Cyanobacteria	*Nostoc* sp., *Anabaena* sp., *Leptolyngbya* sp.	Symbiotic and nonsymbiotic	Improved the soil C, soil N, and exopolymeric substance contents of the soil	Bano and Iqbal (2016)
			Releases amino acids and proteins, polysaccharides and carbohydrates, vitamins, and phytohormones as elicitor molecules for plant growth promotion	
			Increases resistance of plants against biotic and abiotic stresses	
Fungi	*Phoma* sp., *Trichoderma* sp., *Penicillium* sp., *Rhizoctonia* sp., *Pythium* sp.	Various association	Improve plant growth	Murali et al. (2012), Whipps (2001) and Mauchline et al. (2002)
			Induce systemic resistance (ISR) against plant pathogens	
			Enhances biomass production and promotes lateral root growth	
			Niche exclusion, antibiosis, predation, mycoparasitism, and ISR induction	

(continued)

Table 1.1 (continued)

Domain/phylum/ group of microorganism	Important species	Association with host	Major role of these species/ strains	References
AMF	*Acaulospora morrowiae*, *Glomus etunicatum*, *Rhizophagus irregularis*, *Gigaspora rosea*		Increases the soil nutrient availability	Ellouze et al. (2014) and Lovelock et al. (2004)
			Increase yield by enhancing host resource uptake by sharing	
			Improved water relations and increases antagonistism against plant pathogens	
			Phytoremediation of polluted soil	

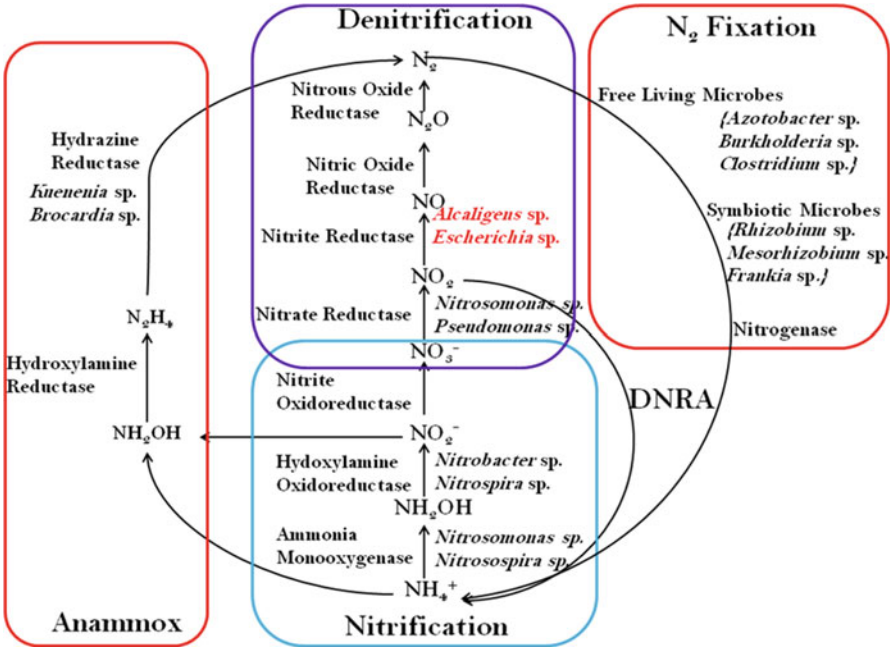

Fig. 1.2 Soil microbes involved in biological nitrogen cycles

2000), may have significant implications in soil function. Soil texture influencing bacterial community structure, even if the change is minor and with unchanged mineral composition of soil, probably results in the scattered arrangement of bacteria (Hattori and Hattori 1976). Bacterial diversity and community structure are influenced by a range of physicochemical properties of the soil matrix, including pore size, particle size, and the availability of water and carbon (Carson et al. 2009). At low pore connectivity, the maximal bacterial diversity is probably mediated by lots of interacting factors which favors the coexistence and decreases the competitive interactions (Carson et al. 2010).

Actinobacteria, being a slow grower in the soil, may colonize roots slowly but successfully. Mostly bacteria and actinobacteria bring rhizospheric change through altering the composition of small molecules like sugars, amino acids, fatty acids, flavonoids, and strigolactones leading to significant modification in the plant (Badri and Vivanco 2009) phenology. In turn, microbes inhabiting the rhizosphere release phytohormones, small molecules, or volatile compounds and regulate plant growth and root morphogenesis (Castro-Sowinski et al. 2007).

1.5.2 Fungi

Several advanced, independent soil microbiological methods for charactering the microbial biomass and soil microbial community structure uncovered that living and dead soil bacteria were associated with the clay fraction, whereas lower eukaryotes as fungi and their exoenzymes involved in the degradation of complex organic compounds were found in association with soil particulate organic matter. The plant species richness is also positively correlated with arbuscular mycorrhizal fungi (AMF) richness (Klironomos et al. 2000). AMF contribute positively to the plant productivity due to diverse relationship with increase in soil microbial diversity leading to an increase in per capita plant productivity (van der Heijden et al. 2008). The soil health is benefited by extensive mycelia networks of AMF with the glomalin secretion leading to the improvement of structural stability and quality of soil. At the instance of resource competition, AMF increases the yield by improving the resource uptake as phosphorous (P) along with carbon (C) and nitrogen (N) (Koide 1991). From an agroecological point of view, the ecological function imparted by AMF signifies the important impact these groups of symbiotic organisms have on the agricultural productivity and sustainability (Ellouze et al. 2014).

1.5.3 Archaea

There is abundant presence of archaea in soil, predominantly by the members of the phylum *Crenarchaeota*, mostly found and isolated below the topsoil. In general, these are grouped in the class of anaerobic soil microbes. Some members from *Euryarchaeota* phylum especially methanogens are active in the absence of O_2 created due to water logging (Angel et al. 2012). *Methanosaeta*, *Methanocella*, and *Methanosarcina* sp. comprising methanogens are widely found in soil microhabitat. Being strict anaerobes, they break down the organic molecules to CH_4 and CO_2 and participate in anaerobic food chain. Although, the pathways used by these methanogens for production of CH_4 from methylated compounds vary that include either reduction of CO_2 and methanol, cleavage of acetate, or reduction of methylated compounds from methane (Singh et al. 2017). Both *Methanosarcina* sp. and *Methanosaeta* sp. reduce acetate to produce methane. The oxidizing archaea like ammonia-oxidizing archaea (AOA) are more prominent at the subsoils (Di et al. 2010). Under high ammonia substrate concentrations, AOA dominates soil microbial community.

1.5.4 Cyanobacteria

In many agricultural fields, cyanobacteria are one of the vital inhabitants, where they efficiently contribute toward biological nitrogen fixation, help "P" solubilization, and improve crop productivity as well as soil fertility. The nitrogen-fixing cyanobacteria are helpful in crop production with saving partially or entirely mineralized nitrogen. Novel association between agriculturally important plants, N_2-fixing cyanobacteria, and cereal plants is now immerging (Spiller et al. 1993). Cyanobacteria, as inoculants in rice production, have enhanced soil fertility and improved soil structure, besides enhancing crop yields. Highest populations of *Anabaena* sp. (1×10^6 g^{-1} soil) and *Nostoc* sp. (9.1×10^4 g^{-1} soil) were recorded in paddy field soil, when supplimented with nitrogen. *Calothrix* sp., *Hapalosiphon* sp., and *Westiellopsis* sp. were among the other isolates from the rhizosphere region. Out of the nonheterocystous cyanobacteria from the rhizosphere, *Oscillatoria* sp. and *Phormidium* sp. were prevalent, but not in all rice cultivars, *Scytonema* sp. was found to be common in saline soil (El-Ayouty et al. 2012). Ninety-eight percent of Indian soils require inorganic phosphorus (chemical or biological) through phosphorus fertilization. Due to the low solubility and chemical fixation of phosphorus present in soil, only 0.1% is available to the crop plants. The soil microorganisms act as a biological combat system by solubilizing the insoluble inorganic phosphorous to its simplest form and making it available to the plants. The cyanobacteria involved in phosphate solubilization (PS) are *Anabaena* sp., *Calothrix* sp., *Nostoc* sp., *Scytonema* sp., and *Tolypothrix ceylonica*.

1.5.5 Yeast

Some selective variety of yeasts promote plant growth by means of pathogen suppression, phytohormone production, phosphate solubilization, N and S oxidation, siderophore production, and stimulation of mycorrhizal root colonization. Strains have been identified as biofertilizer yeasts (Eman et al. 2008). In vitro nitrification can be achieved through selective members of *Candida* sp., *Geotrichum* sp., *Rhodotorula* sp., *Saccharomyces* sp., and *Williopsis* sp. *Saccharomyces* sp. and *Williopsis* sp. were able to oxidize elemental sulfur in vitro for production of phosphate (PO_4^{3-}), sulfate (SO_4^{2-}), and tetrathionate ($S_4O_6^{2-}$) (Al-Falih and Wainwright 1995). Among yeasts, *Rhodotorula minuta*, *Saccharomyces cerevisiae*, and *Torula thermophila* cause inorganic phosphate solubilization. Other phosphate-solubilizing yeasts are *Hansenula* sp., *Klocker*a sp., *Rhodotorula* sp., and *Debaryomyces* sp., and the most efficient was *D. hansenii*. *Schwanniomyces occidentalis* exhibited PS activity on Mussoorie rock phosphate (Gaur 1990). Yeasts also synthesize antimicrobials and other metabolites required for plant growth, from polysaccharides and amino acids released by bacteria and root exudates. Yeasts are rich source of tryptophan a

precursor of indole acetic acid (IAA) that stimulates cell elongation and division (Agamy et al. 2013).

1.6 Evaluation of Soil Fertility by Measuring Microbiological Activities

Various methods have been developed by researchers worldwide to determine the soil fertility on the basis of its microbiological activity.

1. A general mechanism of assessment of soil fertility is by adding moistened soil samples with 60 or 70% of water-holding capacity, with specific nutrient solution, followed by incubation at 20–30 °C for 7–30 days and observes biological changes.
2. The biological reactions involving metabolism of nitrogenous compounds are analyzed to evaluate the soil fertility. The common biological reactions like ammonification, nitrification, denitrification, and nitrogen fixation are used in soil fertility analysis.
3. The abundance of certain organisms in the soil and presence of other organisms can be examined by various microbiological culture methods. Measuring the changes in concentration of certain important plant nutrients in the soil with respect to microbial activity and abundance provides idea about soil fertility.

The major difficulty involved in the use of such methods is the dependency of soil microbial reaction on many variables in a natural scenario. This comprises of different intrinsic soil conditions, weather condition effects, and soil management.

1.7 Conclusion

Soil microbiology is a multidisciplinary branch of science which deals with the study of microorganisms associated with soil and their interaction. The soil, being the natural habitat for the survival and growth of microorganisms, has always given great emphasis to study its physicochemical and biological characteristic features. It is very important to understand the relationship of microorganisms with respect to higher plants as well as to exploit the ecto- and endo-microbial association for a sustainable soil microbial ecosystem. Soil microbiology defines a brief idea about microbes nurtured by nature and the chemical properties of the soil depending upon the geographical limits and climatic changes. It should make an exciting study in the field of agriculture and environmental sciences to create an intricate road map by extracting knowledge behind the complex microbiological activities, interacted with the environmental cues that would dramatically improve plant productivity and thus increase socioeconomical values.

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Chapter 2
Microbial Diversity and Soil Health in Tropical Agroecosystems

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Abstract Microbial diversity is one important factor which controls agroecosystem productivity and quality. Microbial diversity is critical to ecosystem functioning due to its specificity in processes for which microbes are responsible. The presence of diverse soil microbes, bacteria, fungi, and archaea plays a critical role in cycling of major elements (C, N, P) which helps to maintain good soil health. These microbes help in maintaining soil structure, reduce susceptibility to pests and diseases, and eliminate hazardous substances from soil. In this review we addressed two significant questions concerning soil health: (1) how microbial diversity and community structure most effectively describe soil health and can be used as indicators and (2) how can soil health assessed by such indicators be improved or maintained? A summary of available techniques to characterize microbial community structure and diversity is provided, and information pertaining to strategies that can improve microbial diversity in relation to soil health by adopting suitable agricultural practices to sustain soil and crop productivity is furnished. These techniques include those for structural profiling, functional profiling, and other tools being used to assess microbial community diversity and their management through agricultural practices for improving the quality of soil and enhancing the crop productivity. Healthy soil supports high microbial diversity, activity, fertility, nutrient cycling, and disease-suppressive abilities. There is a considerable interest in understanding the nutrient cycles that regulates C, N, and P (carbon, nitrogen, phosphorus) exchange between the soil and atmosphere and how this exchange responds into tropical agroecosystem functioning under diverse edapho-climatic conditions.

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2.1 Introduction

The tropical areas are characterized by hot and humid climate almost throughout the year (Teygeler et al. 2001) with relatively low seasonal fluctuations in temperature and rainfall (Bonebrake and Mastrandrea 2010) and highly prone to soil erosion. Tropical ecosystems are generally known for their highest biological diversity on earth (Gibson et al. 2011). Soil microbial diversity plays a crucial role in maintaining soil health and is considered as key biological, chemical, and physical indicators required for enhancing soil fertility. Microbial diversity is an excellent indicator of soil health (Nielsen and Winding 2002) and collectively helps nutrient cycling and other ecosystem services. However, the impact of microbial diversity on the stability of ecosystem functioning is still debatable (Harrison et al. 1968). Besides nutrient cycling, the soil microbial diversity may also suppress soil-borne diseases (Kennedy and Smith 1995; Van Elsas et al. 2000) and is considered as an indicator of soil health (Visser and Parkinson 1992). In this chapter we provide glimpses of microbial diversity and changes under agroecosystems, techniques, and tools being used to assess microbial communities and diversity and the key influencing factors affecting diversity. Microbial diversity is then correlated with crop and soil agricultural management practices for improving the quality of the soil and sustained crop productivity in agroecosystems.

2.2 Microbial Diversity in Relation to Tropical Agroecosystems

Tropical ecosystems are ranked topmost in terms of biological diversity and are often termed as the house of the highest biological diversity on earth (Gibson et al. 2011; Mandic-Mulec et al. 2015). Microbial biodiversity has been indicative of richness of species which include plants, animals, and microorganisms, whereas microbial diversity is the part that includes protozoa, fungi, fauna, and bacteria. Different types of microorganisms present in soil are assigned different roles by the nature like some are active in nutrient cycling, while others in the suppression of diseases and used as biocontrol agents. In an agroecosystem nutrient cycling is very diverse from unmanaged ecosystem because of agricultural practices such as crop rotation, nutrient management, and application of fertilizers and pesticides.

Tropical agroecosystem is a man-made ecosystem, i.e., distinct from natural ecosystem. Different management practices and biocontrol treatments affect soil

microbial community. In case of unmanaged ecosystem, wide losses and gain of nutrients occur (Tivy 1987). Many studies have shown that agricultural and management practices such as agroforestry, organic farming (Bainard et al. 2012), etc. pose least soil perturbation. Reduced tillage (Capelle et al. 2012) and crop rotation (Altieri 1999) exert positive implications on the community structure, composition, abundance, and richness of specific group of organism (e.g. AMF, earthworms) and on soil microbial diversity.

Many authors have described the effect of agricultural practices on AMF functioning. Spore density was identified as the most crucial parameter for assessing tillage-induced changes on AMF. A clear demarcation between no-tillage and conventional tillage was observed due to differences in spore density (Castillo et al. 2006). No-tillage system involves no mechanical disruption of soil as a consequence of which AMF hyphal network remains intact. In case of no-tillage, the availability of AMF infectious propagules increases due to no disturbance of topsoil layers and new roots grow by making way through routes created by previously grown plants (Kabir 2005). The less detrimental effect of tillage has also been observed in case of enzyme activities (which imply better microbial activity), where significant increase in the activities of enzymes like amylase, cellulose, aryl sulfatase, and acid and alkaline phosphatase were observed in case of no-tillage particularly in the surface layer (0–5 cm) (Balota et al. 2004). Soil type was found to play important role in shaping microbial communities as compared to the effect exerted by plant roots; however the authors could not make any assumption about the role of rhizosphere effect in the same study. Higher concentration of PLFAs and soil microbial biomass carbon has been observed in case of no-tillage as compared to conventional tillage particularly for the topsoil layer (Feng et al. 2003).

A brief inference dealing with microbial community changes under different agrosystems has been given in Table 2.1.

2.3 Tools Used for Assessing Microbial Community and Diversity in Soil

2.3.1 Structural Profiling Technique

Structural diversity can be defined as the number of species, genes, and communities in an ecosystem (Avidano et al. 2005). Species richness and species evenness describe the structural diversity of a community (Ovreas 2000). Analysis of fatty acid methyl ester/phospholipid-derived fatty acid (FAME/PLFA) profile in soil is being used to detect structural profiling of soil from a specific agroecosystem.

Table 2.1 The influence of different systems/conditions on soil microbial communities in agroecosystem

Organism	System/condition	References
Arbuscular mycorrhizal fungi (*Acaulospora*, *Scutellospora*)	Organic farming	Oehl et al. (2003)
Arbuscular mycorrhizal fungi (*Scutellospora*)	No-tillage	Castillo et al. (2006)
Arbuscular mycorrhizal fungi (*Acaulospora*)	Wheat under conventional tillage	
Arbuscular mycorrhizal fungi spores (*Acaulospora scrobiculata*, *Scutellospora verrucosa*)	Crop rotation (maize–crotalaria Rotation)	Mathimaran et al. (2007)
Arbuscular mycorrhizal fungi	Conventional/no-tillage presence or absence of N-fertilization having sole spring wheat	Schalamuk et al. (2006)
Arbuscular mycorrhizal fungi *Glomus occultum*	No-tillage	Douds et al. (1995)
Arbuscular mycorrhizal fungi (*Glomus* spp., *Glomus etunicatum*)	Conventional tillage	
Actinobacteria and Chloroflexi	Mulberry–soybean intercropping (salt–alkali stress)	Li et al. (2016)
Acidobacteria and Proteobacteria	Monoculture of mulberry and soybean (salt–alkali stress)	
Burkholderia, *Pseudomonas*, and *Arthrobacter*	Mulberry–soybean intercropping (salt–alkali stress)	
AMF Biomass of bacteria and fungi	Perennial grasses and restored prairie	Jesus et al. (2016)
Nitrogen-fixing bacteria, lignin-decomposing bacteria	Tree-based intercropping Two coffee species (arabica and robusta) evergreen ecological conditions	Bagyaraj et al. (2015)
Starch-hydrolyzing bacteria and pectin-utilizing bacteria	Tree-based intercropping Two coffee species (arabica and robusta) evergreen deciduous conditions	

2.3.1.1 Fatty Acid Methyl Ester Analysis/Phospholipid-Derived Fatty Acid (FAME/PLFA) Profiling

FAME/PLFA profiling in soil is a culture independent technique. Analyzing PLFA in soil can help in distinguishing the microbial communities or groups of microorganisms based on fatty acid profiling (Ibekwe and Kennedy 1998). PLFA technique estimates both microbial community structure and biomass size (Kaur et al. 2005). Characterization of bacterial species based on fatty acid profiling (Purcaro et al. 2010) has become necessary to substantiate the results of 16S rRNA gene sequences and hence is being used as one of the biochemical methods to validate the identity of new taxa of unknown microbial cultures. PLFA can work as

signatures for specific organisms e.g., Gram-negative or Gram-positive bacteria, methanotrophic bacteria, fungi, mycorrhiza, and actinomycetes (Zelles 1999). To interpret changes in the microbial community structure, simpler methods to determine the fatty acid composition of the microorganisms have been developed such as microbial identification system (MIDI). High-throughput method that uses much smaller solvent volumes than the traditional protocols has been developed recently. The method involved a 96-well solid phase extraction plates and may be useful for laboratories handling large numbers of samples (Buyer and Sasser 2012).

FAME profiles also are of great concern to microbial community structure which can be used to assess the impact of soil storage and community structure. However, that may change due to the temperature at which soils are stored (Petersen and Klug 1994). Butler et al. (2012) reported significant changes in the fungal: bacterial PLFA ratio in soils treated with triclosan. Buyer et al. (2010), while assessing the impact of management practices on PLFA biomarker fatty acids in tomato agroecosystem, observed that cover cropping increased the absolute amount of all microbial groups, but Gram-positive bacteria decreased in proportion under cover crops. The higher soil temperatures under certain treatments also increased the proportion of Gram-positive bacteria. They concluded that the imposed treatments were much more significant than soil temperature, moisture, pH, and texture in controlling microbial biomass and community structure.

Chaudhary et al. (2012) assessed PLFA microbial communities associated with the rhizosphere and bulk soils planted to switchgrass or *Jatropha* where they found that switchgrass soil contained higher abundance of Gram-positive (i14:0, i15:0, a15:0), Gram-negative (16:1ω5c, 16:1ω7c, 18:1ω5c), and saturated (14:0, 15:0) PLFAs compared to *Jatropha* soil. On the contrary, *Jatropha* had a higher abundance of fungal (18:2ω6,9c), 18:1ω9c, 20:1ω9c, and 18:0 PLFAs compared to switchgrass soil. PLFA and neutral lipid fatty acid (NLFA) provide a new and promising tool for the estimation of live AM fungal biomass in soil and roots (Olsson 1999; Olsson and Johansen 2000; Sharma and Buyer 2015) and for ectomycorrhizal fungi where 18:2ω 6,9 dominates among the fatty acids and can be used as an indicator for these fungi in soil in experimental systems (Olsson 1999) (Table 2.2). Soil FAME were found to be most responsible for differentiation among cropping systems including 12:0, 16:1 ω5c, 16:1 ω7c, 18:1 ω9c, and 18:2ω6c fatty acids.

2.3.1.2 Molecular Genetic Profiling

The genetic diversity of microbial community can be assessed by measuring the heterogeneity of the DNA from the entire microbial community targeting 16S rDNA gene, the conserved sequence of eubacterial communities, by using (1) RFLP (restriction fragment length polymorphism), (2) T-RFLP (terminal-restriction fragment length polymorphism), (3) polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and PCR-temperature

Table 2.2 Fatty acid biomarker groups

Biomarker	Fatty acids	References
Gram-positive bacteria	Iso and anteiso branched fatty acids	Zelles (1999)
Gram-negative bacteria	Monounsaturated fatty acid, cyclopropyl 17:0 and 19:0	Zelles (1999)
Actinomycetes	10-methyl 16:0 and 10-methyl 18:0	Frostegard and Baath (1996)
Fungi	18:2 ω6cis	Ringelberg et al. (1997)
Protozoa	20:3 ω6cis and 20:43 ω6cis	
AMF	16:1 ω5cis	Olsson (1999)

gradient gel electrophoresis (PCR-TGGE), (4) single-strand conformation polymorphism (SSCP), and (5) amplified ribosomal DNA restriction analysis (ARDRA) (Sharma et al. 2010).

2.3.2 Functional Profiling Techniques

The functional diversity analysis of microbial community is based on different techniques like catabolic profiling (substrate utilization), CLPP (community-level physiological profiling), and substrate-induced respiration. Nutrient cycling performed by different groups of bacteria represents one of the important components of microbial functional diversity. While the whole bacterial community represents the specific carbon utilization pattern, measurement of enzyme activity profile reveals the diversity (Nielsen and Winding 2002). Carbon substrate utilization by different microbial community is one of the approaches for examining the functional and metabolic diversity.

2.3.2.1 Catabolic Profiling Based on Substrate Utilization

A simple approach to measure functional diversity is to examine the number of different carbon (C)-substrates utilized by the microbial community. Zabaloy and Gómez (2005) carried out a diversity analysis of 76 rhizobial strains isolated from Argentine agricultural soils using C- utilization patterns. Similarly in India Sharma et al. (2010) showed a remarkable diversity among soybean rhizobia based on 15 carbon sources utilization pattern where isolates were matching with reference strains. Such analysis suggests the occurrence of metabolically distinct types of rhizobia, besides commonly known taxonomic identity (*B. japonicum*, *B. elkanii*, and *S. fredii*) (Sharma et al. 2010). Besides substrate utilization pattern based on C, the community-level physiological profiling by BIOLOG® plate method

(Garland and Mills 1991) and in situ substrate-induced respiration (SIR) (Degens and Harris 1997; Campbell et al. 2003) are equally important tools to assess the communities.

2.3.2.2 Community-Level Physiological Profiling (CLPP)

CLPP method is based upon the Biological plate method using a range of 95 carbon substrates which applies for microbial community function and functional adaptation over space and time (Garland and Mills 1991). Difference between the communities can be compared and classified based on sole carbon source utilization pattern (CSUP) using BIOLOG® system in the late 1980s. CLPP involves inoculating mixed microbial community into BIOLOG®-GN (for Gram-negative bacteria), GP (Gram-positive bacteria), or 31 (ECO plates) with single carbon sources in addition to tetrazolium dye. The utilization of carbon source indicates respiration-dependent reduction of the tetrazolium dye and purple color formation that can be quantified and monitored over time. Being a culture-dependent technique, a wide variety of culture media have been used which maximizes the recovery of diverse microbial groups.

2.3.2.3 Substrate-Induced Respiration (SIR)

Anderson and Domsch (1973) introduced SIR method for measuring the total, fungal, and bacterial biomass in a short time (less than 6 h). SIR method involves activation of the microbial population in soil by the addition of a readily decomposable respiratory substrate (usually glucose), and the resulting initial maximal respiration is used for estimation of microbial biomass (Nakamoto and Wakahara 2004). West and Sparling (1986) proposed the method of SIR for biomass estimation using individual flask/bottles with soil sample added with various carbon substrates followed by measurement of CO_2 produced in 4 h in each flask. It can be measured by using GC, infrared spectroscopy, or some other suitable assays. It measures the small fraction of the microbial community that can grow within the microtiter plate well. MicroRep™ (Campbell et al. 2003) was designed for a "whole soil" analysis for distinguishing the fungal and bacterial population.

2.4 Microbial Diversity Index

Species diversity at a community level is measured by mathematical diversity index. Diversity index provide more information about species richness and evenness. The number of species per sample is called richness, and evenness is a measure of relative abundance of different species in an area. Species diversity index relates the number of species and the relative importance of individual

species. Species diversity is measured by Simpson index which is dominant index as it gives more weight to common or dominant species. The most widely used method of diversity is the Shannon–Weaver index (Shannon and Weaver 1963) which is sensitive to sample size, especially small size.

2.4.1 Simpson Diversity Index

Simpson index was first introduced by Edward H. Simpson in 1949 and was redescribed by Orris Herfindahl in 1950 where the degrees of concentration between the individuals are classified into index categories (Simpson 1949). Simpson diversity index refers to closely related indices such as Simpson index (D), Simpson index of diversity ($1-D$), and Simpson reciprocal index ($1/D$). We can calculate Simpson index for two individual random samples which belong to the same species. It is denoted by D, the value of which lies between 0 and 1. 0 represents infinite diversity and 1 represents no diversity; the bigger the value of D, the lower the diversity. D is often subtracted from 1.

$$D = \sum (n/N)^2 \text{ or } D = \sum n(n-1)/N(N-1)$$

n = The total number of organism of particular species
N = The total number of organism of all species
D = Simpson index

Inverse Simpson index and Gini–Simpson index (Hill 1973; Jost 2006) are called as Simpson index in the ecological literature.

$$\text{Inverse Simpson index} = 1/D.$$

Gini–Simpson index measures the probability of two individuals belonging to the same species. But this aspect of compositional complexity does not seem to occupy the same place with biologically meaningful concept of diversity.

$$\text{Gini-Simpson index} = 1 - D.$$

2.4.2 Shannon Diversity Index

It is sensitive to both species richness and relative abundance and is very sensitive to sample size especially for small sample. The Shannon entropy quantifies the

uncertainty (entropy or degree of surprise) associated with this prediction. It is most often calculated as follows:

$$\text{Shannon-Weaver index of diversity } (H) = C/N\left(N\log N\backslash - \sum n_i \text{v}\log n_i\right)$$

where

$C = 2.3$, $N =$ number of individuals, $n_i =$ number of individual ith species

$$\text{Species richness } (d) = d = S - 1/\log N$$

where

$S =$numbers of species
$N =$numbers of individuals,

$$\text{Species evenness } (e) = e = \bar{H}/\log S$$

where

$\bar{H} =$Shannon-weaver diversity index
$S =$number of species

2.5 Soil Health and Its Indicators

Soil health is defined as the continued capacity of soil to function as a vital living system by recognizing that it contains biological elements which are key to ecosystem function within land-use boundaries (Larson and Pierce 1994; Doran and Zeiss 2000; Karlen et al. 2001). Soil quality and soil health are interchangeable terms (Karlen et al. 2001), although it is important to define that soil quality defines soil function (Karlen et al. 2003), whereas soil health describes a finite nonrenewable and dynamic living resource (Doran and Zeiss 2000). Soil health concept directly includes the interaction between soil and plant and create healthy environment. Soil health is maintained by major soil health indicators that support the soil for crop productivity. A healthy soil is a stable soil that supports high microbial diversity with high levels of internal nutrient cycling (Elliott and Lynch 1994). These functions are able to sustain biological productivity of soil, maintain the quality of surrounding air and water environments, as well as promote plant, animal, and human health (Doran et al. 1996). Soil health is measured by a complex set of indicators i.e., biological, chemical, and physical. These indicators help to assess the soil condition and quality status capable of performing various functions required to support the plant growth under a particular set of conditions. In general soil health is dependent on the maintenance of four major functions: (C) carbon transformations, nutrient cycles, soil structure maintenance, and the regulation of pests and diseases.

2.5.1 Biological Indicators

Biological indicators of soil health that are commonly measured include soil organic matter, respiration, microbial biomass (total bacteria and fungi), and mineralizable nitrogen. Soil organic matter plays a key role in soil function determining soil quality, water holding capacity, and susceptibility of soil to degradation (Giller et al. 1997) (Table 2.1). In addition, since soil organic matter plays a key role in soil function and may serve as a source or sink to atmospheric CO_2 (Lal 1997), an increase in the soil C content is indicated by a higher microbial biomass and elevated respiration (Sparling et al. 2003). Giller et al. (1997) measured soil health by respiration, enzyme activity, and microbial biomass (total bacteria and fungi) (Sparling et al. 2003).

2.5.2 Soil Physical Indicators

Soil physical indicators describe the soil texture, bulk density, aggregation, stability, and water holding capacity (Hillel 1982) (Table 2.3). Higher bulk density like compact soil layer restricts the root growth and movement of root toward the gravity and inhibits the movement of air and water through the soil (Enriqueta-Arias et al. 2005). Tillage decreases the aggregate stability and bulk density temporally and reduces the mycorrhizal biomass (Sharma et al. 2012). Aggregate stability is an indicator of organic matter content, biological activity, and nutrient cycling in soil. All these properties are influenced by the addition of organic matter into the soil. Soil aggregation is influenced by the mycorrhiza which produces glycoprotein (glomalin) that bind the soil particle together and are thus highly correlated with aggregate stability (Woignier et al. 2014).

Table 2.3 Commonly used indicator of soil health (Brevik 2009)

Chemical indicator	Physical indicator	Biological indicator
pH	Texture	Microbial biomass C and N
Soil organic matter	Bulk density	Earthworm
Total carbon	Penetration resistance	Population
Total nitrogen	Aggregate stability	Nematode population
Cation exchange capacity	Water holding Capacity	Arthropod population
		Mycorrhiza fungi
Major and minor nutrient	Infiltration rate	Respiration rate
Electrical conductivity	Depth to hardpan	Soil enzyme activities
Heavy metals and other	Depth to water table	Pollutant
plant toxins	Porosity	Detoxification
Extractable N, P, and K	Erosive potential	Decomposition
	Aeration	Potential mineralization N

2.5.3 Chemical Indicators

Chemical indicators specify the capacity of soil to supply mineral nutrients which depends on the soil chemical nutrient profile. For example soil pH, a measure of hydrogen-ion concentration in solution measures acidity or alkalinity of soil solution. Soil pH affects the activity of microorganisms involved in nutrient cycling and also solubility of nutrient that is used for plant productivity. Ion exchange capacity mostly affects soil cation exchange capacity (CEC) binding to negative charge organic matter, clay, and soil colloid. Amount if ions (dissolved salts) in solution are the indicator of electrical conductivity in soil–water mixture (Arias et al. 2005).

2.6 Strategies to Improve Soil Health by Managing Microbial Community and Diversity

2.6.1 Crop and Soil Management Practices

Key crop and soil management practices like crop rotation/crop sequences, intercropping, and tillage practices influence the microbial diversity and functioning in the soil ecosystem processes. Leguminous crops included in the crop rotation generally tend to increase soil fertility and crop yield. Disease suppression can be influenced by cropping and management practices, like monocropping of cauliflower suppressed *R. solani* infestation assessed in a long-term field trial (Huber and Watson 1970). In semiarid areas, continuous cropping reduces the level of soil organic matter and microbial biomass in soil (Campbell et al. 2000). Rotating crops with nonhost or less susceptible plants have caused a decline in the specific pathogenic populations due to their natural mortality and the antagonistic activities of other microorganisms (Kurle et al. 2001). Sharma et al. (2012) showed the importance of including maize in rotation with soybean under conventional reduced tillage helped in enhancing soybean yield, AM inoculum load, and organic carbon. Long-term sugarcane growing under intensive cropping system with intercrops of pulse crops and incorporation of their labile C substrates improved N mineralization. The buildup of the C pool and microbial biomass C in the case of cereals, mustard, and potato intercropping would promote long-term stability. In an irrigated maize (*Zea mays*)–wheat (*Triticum aestivum*) system nontraditional mulching (*Sesbania*, *Jatropha*, and *Brassica*) increased yields by $>10\%$ and net returns by $>12\%$ compared with no-mulch and improved water potential and soil biological properties (Jat et al. 2015).

Besides crop sequences, tillage is also important factor influencing the diversity. Adoption of conservation tillage increases the soil aggregation (Lal 2004) and mycorrhizal biomass and population in soil (Sharma et al. 2012; Douds et al. 1995). Tillage can disrupt the soil network formed by AMF filaments and colonized root systems left by previous AM crops, affecting the potential colonization of

subsequent crops. The AMF hyphae can remain viable for long and wait for colonization with newly germinated plants and provide plants to acquire more nutrients and water. Sharma et al. (2012) evaluated the impact of tillage practices and crop sequences on AM fungal propagules and soil enzyme activities in a 10-year long-term field trial in Vertisols of soybean–wheat–maize (S–W–M) cropping system where S–M–W or S–W–M–W rotations under reduced-reduced tillage system showed higher soil dehydrogenase activity and fluorescein diacetate hydrolytic activity compared to other combinations. The inclusion of maize in the rotation irrespective of tillage systems showed comparatively higher mycorrhizal and higher phosphatase activities and organic carbon and maintained higher soybean yield.

2.6.2 *Microbial Inoculations*

Microbial inoculants are used to improve crop productivity in terms of managing nitrogen and phosphorus economy (Adhya et al. 2015). PGPR (plant growth-promoting rhizobacteria) have multifunctional activity known for survival of plants and their growth. Some bacteria and fungi are being used as microbial inoculants like *Rhizobium*, *Azotobacter*, and mycorrhizal fungi (Table 2.4). These are used for enhancing N and P nutrition in plants which are the most limiting crop nutrients. The occurrence of rhizobia (species of *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*,

Azorhizobium, *Allorhizobium*, and *Sinorhizobium*) plays a symbiotic role with the leguminous plant as inoculants for sustainable growth of pulses. For the improvement of root biomass in chickpea, co-inoculation of *Rhizobia* with *Bacillus megaterium* at flowering stage (Aparna et al. 2014; Wani et al. 2007) was found to be useful. Arbuscular mycorrhiza (AMF) is used as inoculum for P uptake and stabilization of soil particles eventually to improve upon soil structure. Using

Table 2.4 List of crops and strain for bio-inoculation

Crops	Application of fertilizer
Cereals	
Rice	Blue-green algae, *Azolla*
Others	*Azotobacter*
Pulses	*Rhizobium*, P-solubilizing bacteria
Oilseed	*Rhizobium*
Legumes	*Rhizobium*
Nonlegume	*Azotobacter*, P-solubilizing bacteria
Forage and fodder	
Legumes	*Azospirillum*, P-solubilizing bacteria
Others	*Rhizobium*, P-solubilizing bacteria
Soybean	*Bradyrhizobium japonicum*

Source: Organic farming (http://agritech.tnau.ac.in/org_farm)

microbial inoculants to improve crop productivity especially in terms of N- and P-economy has been explored for quite some time (Adhya et al. 2015). A rhizobacterial strain *Pseudomonas putida* GAP-P45 improved plant biomass, relative water content, and leaf water potential in maize plants exposed to drought stress (Sandhya et al. 2010). The possible mechanism by which PGPR stimulate plant growth through nitrogen fixation, solubilization, and mineralization of various nutrients in plant parts produces various plant growth hormones and ACC deaminase, antagonism against plant pathogens, and ability to trigger tolerance to various abiotic stresses (Glick et al. 2007).

AMF hyphae stabilizes soil particles into aggregates, both by enmeshing them and releasing a glue-like substance called glomalin, which holds them together and stabilizes them. Among soil biological indicators, AM-derived glomalin is comparatively new and is considered as one of analytical based potential bioindicators contributing toward enhanced ecosystem productivity as a result of improved soil aeration, drainage, and microbial activity (Lovelock et al. 2004) and for assessing the sustainability of long-term cropping systems.

2.7 Future Perspectives and Conclusion

In the tropical agroecosystem, high temperature and humidity affect crop production. Microbial diversity is the key element required for maintaining soil health. The crop and soil management practices of an agroecosystem need to be customized to harbor favorable microbial community essentially needed to perform soil processes and sustain crop and soil productivity. Microbial diversity maintains soil health by suppressing the population of disease-causing organisms; improves nutrient cycling, etc. and can be enhanced by soil amendments and management practices such as conservation tillage, composting/organic amendments, manuring and fertilizers, crop rotation/crop sequences, etc. Identifying sustainable soil management practices through analyzing soil biological, chemical, and physical indicators of management practices being followed in an agroecosystem will help in optimizing a favorable system needed for sustaining the crop and soil productivity of a particular ecosystem.

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Chapter 3
Plant Growth-Promoting Microbes (PGPM) as Potential Microbial Bio-Agents for Eco-Friendly Agriculture

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Abstract The use of chemical fertilizers generates environmental and public health problems. The exploitation of beneficial microbes as a biofertilizer has become of foremost importance in agriculture sector for their impending role in sustainable crop production. Eco-friendly approaches inspire a wide range of application of plant growth-promoting rhizobacteria (PGPRs), endo- and ectomycorrhizal fungi, cyanobacteria and many other useful microorganisms for improved nutrient uptake, plant growth and plant tolerance to abiotic and biotic stress. PGPR use different mechanisms like biofertilization including biological fixation of atmospheric nitrogen, phosphate solubilization, siderophore production and exopolysaccharides production; phytostimulation including production of indole acetic acid, gibberellins, cytokinins and ethylene; and biocontrol including stimulation of systemic resistance, competition for iron, nutrient and space, production of antibiotics, lytic enzymes, hydrogen cyanide and volatile compounds. In scrutiny of the recent advances in PGPR biotechnology, this chapter describes the rhizospheric PGPRs and the different mechanisms used by PGPR to promote the plant's growth and health.

Keywords Plant growth-promoting rhizobacteria (PGPRs) · Chemical fertilizer · Eco-friendly agriculture · Biofertilizer · Mycorrhizal fungi

3.1 Introduction

Organic farming has emerged globally as an important agricultural activity in view of the demand for safe and healthy food and long-term sustainability of the agroecosystem. Soil health and soil food web are being deteriorated by overapplication of agrochemicals particularly the inorganic nitrogenous fertilizers.

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The agricultural soils are facing problems like altered micro-ecological niches with depleted microbial diversity in terms of both species richness and species number and as such call for their replenishment. Beyond good agronomic and horticultural practices, growers often rely heavily on chemical fertilizers and pesticides. Such inputs to agriculture have contributed significantly to the spectacular improvements in crop productivity over the past 100 years (Junaid et al. 2013). In addition, additional chemicals are also being used in product security.

Keeping in view the phenomenon of sustainable agriculture or enhanced productivity using environmentally benign technology, inoculation with the free-living diazotroph, PSB or PGPR not only would be beneficial in terms of obtaining better yield but also would improve the soil microbial ecology and soil food web by establishment of inoculated biofertilizer for the benefit to the standing as well as subsequent crops. Besides, the long-term use of biofertilizers is economical, eco-friendly, more resourceful and available to small farmers. Biofertilizers being essential constituents of organic farming are the preparations containing live or latent cells of efficient strains of nitrogen-fixing, phosphate solubilizing or cellulolytic microorganisms, used for application to seed, soil or composting areas with the intention of increasing number of such microorganisms and to hasten those microbial processes which enhance the availability of nutrients to plants. Biofertilizers play a very significant role in improving soil fertility by fixing atmospheric nitrogen (N), solubilize insoluble soil phosphates and produce plant growth substances in the soil (Venkatashwarlu 2008).

3.2 Potential Characteristics of Biofertilizers and Bioagents

Rhizosphere, a narrow zone of soil adjoining plant roots, can comprise up to 10^{11} microbial cells per gramme of root and above 30,000 prokaryotic species that improve plant productivity (Venkatashwarlu 2008). Microbial composition of rhizosphere varies according to the plant species, stages of plant development and also soil type (Broeckling et al. 2008). *Proteobacteria* and *Actinobacteria* are the microorganisms most habitually found in the rhizosphere of numerous plant species. Collective rhizospheric microbial community enveloping plant roots is larger compared to that of the plant and is referred to as microbiome, whose interactions determine crop health by providing numerous services to plants, viz. organic matter decomposition, nutrient acquisition, water absorption, nutrient recycling and biocontrol. The agriculturally constructive microbial populations cover plant growth-promoting rhizobacteria (PGPRs), N_2-fixing microorganisms, mycorrhiza, plant disease suppressive beneficial bacteria and biodegrading microbes. Biofertilizers are a supplementary component to soil and crop management traditions, viz. crop rotation, organic adjustments, tillage maintenance, recycling of crop residue, soil fertility renewal and the biocontrol of pathogens and insect pests, which process can

be significantly useful in maintaining the sustainability of various crop productions. Potential characteristics of biofertilizers and bioagents including nitrogen fixation, phosphate solubilization, zinc solubilization, plant growth hormone production, siderophore production, etc. are discussed later in the chapter.

3.2.1 Exploitation of Biofertilizer for the Betterment of the Nutrient Profile of Crops

Rhizosphere microbial communities have become a subject of great interest in sustainable agriculture and biosafety programme. A major focus in the coming decades would be the safe and eco-friendly methods to exploit the beneficial microorganisms in sustainable crop production. Soil microbes like *Pseudomonas*, *Bacillus*, *Micrococcus*, *Flavobacterium*, *Fusarium*, *Sclerotium*, *Aspergillus* and *Penicillium* have been reported to be active in the phosphorus solubilization process. Several fungi like *Aspergillus fumigates* and *A. niger* were isolated from decaying cassava peels that are found to convert cassava wastes to phosphate biofertilizers. *Burkholderia vietnamiensis* produces gluconic acid, which solubilizes insoluble phosphate. *Enterobacter* and *Burkholderia* isolated from the rhizosphere were found to produce siderophores and indole compounds. Potassium solubilizing microorganisms such as *Aspergillus*, *Bacillus* and *Clostridium* are found to be efficient in potassium solubilization and mobilization. Mycorrhizal mutualistic symbiosis with plant roots provides the nutrients to plants, which leads to enhance plant growth and development, and defends plants from pathogen attack. Mycorrhizal hyphae lead to absorption of phosphate from outside to internal cortical mycelia and thus transfer phosphate to cortical root cells. Nitrogen-fixing cyanobacteria such as *Aulosira*, *Tolypothrix*, *Scytonema*, *Nostoc*, *Anabaena* and *Plectonema* are commonly used as biofertilizers. Besides nitrogen fixation, growth-promoting substances and vitamins liberated by these algae increase the root growth and yield of plants. Bacterial genera such as *Acetobacter*, *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Herbaspirillum* and *Rhizobium* have also been reported as effective maize PGPR. Inoculation with *Azotobacter* and *Azospirillum* on field-grown maize significantly increased the plant biomass by 30.7%. Similarly, the co-inoculation of *Bacillus megaterium*, *Azotobacter chroococcum* and *Bacillus mucilaginous* significantly increased maize biomass and height equivalent to half of the chemical fertilizer inputs. Many similar effective N_2-fixing PGPR inoculation results have been reported from maize plants under low fertilizer-N (ca. 48 kg N ha^{-1}) condition, with strains such as *Bacillus* spp., *Klebsiella* spp., *Azospirillum* spp., *Azotobacter* spp. and *Pantoea* spp. Researchers attributed the increase in plant-N uptake and dry biomass of inoculated plants to PGP abilities such as BNF, phosphate solubilization and root promoting phytohormone production, namely, indole-3-acetic acid (IAA), cytokinin and gibberellins (Saini et al. 2015).

3.3 Plant Growth-Promoting Bacteria

3.3.1 Nitrogen-Fixing Bacteria: Symbiotic, Associative, Free-Living

Biological nitrogen fixation (BNF) can contribute to the replenishment of soil N and reduce the need for industrial N fertilizers (Saikia and Jain, 2007; Lanier et al. 2005). The interaction of rhizobia with roots of leguminous plants results in the establishment of effective N_2-fixing symbiosis. In this process, rhizobia reduce atmospheric N to ammonia using enzyme nitrogenase and supply this essential nutrient to the host plant cells. It is an energetically unfavourable reaction, carried out by prokaryotic microorganisms including bacteria, cyanobacteria and actinomycetes, in symbiotic or non-symbiotic association with plants (Giller 2001). Although most *Rhizobium* isolates can nodulate more than one host plant species, several different bacterial species are often isolated from a single legume (Young and Haukka 1996). The exchange of chemical signals between compatible strains of *Rhizobium* and legumes has been named as molecular dialogue (Cooper 2007), which serves to initiate nodule development. In the legume rhizosphere, the rhizobia become affected by the chemotactic and growth-promoting compounds.

Azotobacter spp. is an obligate aerobe although it can grow under low O_2 concentration. The ecological distribution of this bacterium is a complex subject and is related to diverse factors which determine the presence or absence of this organism in a specific soil. Bacteria of the genus *Azospirillum* are a well-known example of so-called associative N fixers which are widespread in the soils of tropical, subtropical and temperate regions. These bacteria develop close relationships with the roots of various wild and agricultural plants (Shridhar 2012).

Cyanobacteria or blue-green algae are a diverse group of prokaryotes that often form complex associations with bacteria and green algae in structures known as cyanobacterial mats. They are the major N_2 fixers in freshwater and marine systems. In large areas of the world's oceans, cyanobacteria provide an important source of nitrogen to the marine ecosystem. Much of our understanding of the ecology and biogeochemistry of oceanic diazotrophy has been derived from studies on the filamentous cyanobacterium *Trichodesmium* spp., a cosmopolitan cyanobacterium in tropical marine systems, including oligotrophic regions throughout the Atlantic and Pacific Oceans (Matthew et al. 2008). Cyanobacteria also grow and fix nitrogen in many terrestrial environments, from rainforests to deserts and are able to survive in extreme environments because of unique adaptations like resistance to desiccation. Because of the ability to fix atmospheric nitrogen, cyanobacterial mats have been used as biofertilizer in modern agriculture. One of the commonest examples includes *Nostoc* and *Anabaena* which have the ability to fix atmospheric nitrogen through symbiosis with fern named *Azolla*. Therefore, it is considered an important potential source of nitrogen for wetland rice. The contribution of nitrogen from *Azolla* spp. to wetland rice plants has been found to be maximum when incorporated into the soil as green manure.

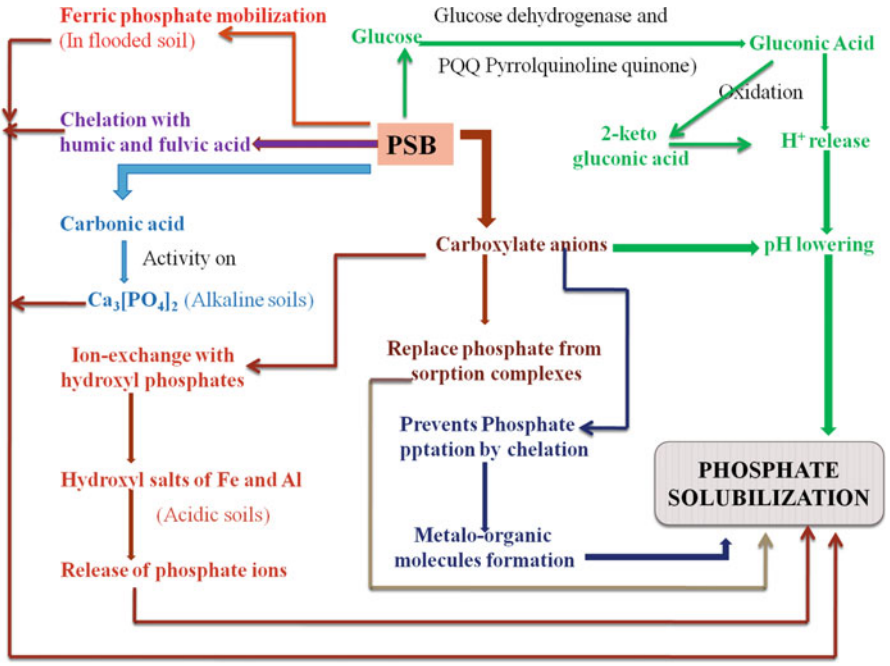

Fig. 3.1 Various organic/inorganic substances produced by PSB responsible for phosphate solubilization in soils

3.3.2 *Phosphate Solubilizing Bacteria*

Phosphorus (P) is an essential plant nutrient with low availability in many agricultural soils. Thus application of phosphatic fertilizers is a must to make up for the P lost due to the fixation of P by the soil constituents and P run-off from P-loaded soils (Vikram and Hamzehzarghani 2008). Phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{2+}, depending on particular properties of a soil. On the other hand, much of this P is in mineral form and is only slowly available to plants. The role of microorganisms solubilizing inorganic phosphates in soil by acidification, chelation, exchange reactions and production of gluconic acid (Chen et al. 2006) and making them available to plants is well known (Fig. 3.1). Phosphate solubilizing bacteria (PSB) act principally by production of organic acids, acid phosphatases and proton transfer. Another mechanism of phosphate solubilization is by phytase production, since organic P can constitute between 30 and 50% of the total P of the soil, a high proportion of which corresponds to phytate. Phytase-producing rhizobacteria have been reported to belong to genera *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Serratia* and *Staphylococcus*. Further, Jorquera et al. (2008) isolated PSB from the rhizosphere of five cultivated plants (*Lolium perenne*, *Trifolium repens*, *Triticum aestivum*, *Avena sativa* and *Lupinus luteus*), which

presented more than one mechanism for utilizing insoluble form of P. Recent reports revealed that optimum P solubilization takes place in the presence of NaCl concentration from 0 to 1.25%, but higher concentrations increase time of P solubilization from 48 to 72 h (Deshwal and Kumar 2013). The major limitation today, for use of these organisms, is the lack of consistency in mobilizing P under field conditions. This is likely due to competition with the native microflora and environmental factors that either limit the population size or activity of the PSB. Microbial biomass assimilates soluble P and prevents it from absorption or fixation.

3.3.3 Zinc Solubilizing Bacteria

Zinc is an essential micronutrient that plays a vital role in various metabolic processes in plants, and its deficiency adversely affects the growth and development of crop plants. The crop and soil management practices mine large amounts of zinc from the native pool of the soil. Additionally, the total zinc content is substantially high although it exists in fixed forms such as smithsonite ($ZnCO_3$), sphalerite (ZnS), zincite (ZnO), franklinite ($ZnFe_2O_4$), willemite (Zn_2SiO_4) and hopeite ($Zn_3(PO_4)_2{\cdot}4H_2O$), which are sparingly soluble. Consequently, large inputs of zinc fertilizers are required to be added to the soil to meet the zinc needs of crops. However, exogenous application of zinc sulphate also gets transformed into different unavailable forms like Zn(OH) and $Zn(OH_2)$ at pH of 7.7 and 9.1, $ZnCO_3$ in calcium-rich alkali soils and $Zn(PO_3)_4$ in near neutral to alkali soils of high P application and gets accumulated in the soil (Gandhi et al. 2014).

Most of the zinc-deficient plants exhibit low levels of auxin such as indole-3-acetic acid (IAA) because Zn plays an essential role in the biosynthesis of IAA. In the absence of IAA, plant growth is stunted. Bacteria such as *Stenotrophomonas maltophilia*, *Mycobacterium brisbanense*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa* and *Xanthomonas retroflexus* isolated from the rhizosphere have the capability to synthesize IAA in vitro in the presence or absence of physiological precursors, mainly tryptophan (Sunithakumari et al. 2016). This necessitates a system that releases essential quantity of zinc from the unavailable state for good growth.

Organic acids secreted by the microflora increase soil Zn availability by sequestering cations and by reducing rhizospheric pH. 5-ketogluconic acid was the major organic acid produced in the intermediary of solubilization by *Gluconobacter diazotrophicus*, while in *Pseudomonas* it was 2-ketogluconic acid that mediated solubilization process. Gluconic acid and ketogluconates are sugar acids having multiple conformations which chelate the metal cations apart from solubilization suggesting that the solubilization process might be a direct consequence of increased hydrogen ion activity in the solution. The solubilization process was not accompanied by a reduction in the pH of the medium when ZnO or $ZnCO_3$ was supplemented, and this might be partly because of their intrinsic buffering potential. Also, ZnO may act as an excellent buffer consuming 2 mol of protons per mole for

solubilization. Thus gluconic acid production appears to be related to phosphorylative and direct oxidative pathways of glucose metabolism involving enzymes pyrroloquinoline quinone (PQQ)-dependent glucose and gluconate dehydrogenases. Hence isolation and identification of such bacteria are an eco-friendly approach to eradicate zinc deficiency in plants (Saravanan et al. 2007).

3.3.4 Plant Growth-Enhancing/Hormone-Producing Bacteria

3.3.4.1 Auxins

Although plants are able to synthesize IAA themselves, the microorganisms that are the inhabitants of the rhizosphere also contribute to plant's auxin pool. Auxins synthesized by the plant and the microorganisms differ only in the biosynthetic pathway (Fig. 3.2), depending on the plant and/or microorganism. Major IAA-producing bacteria belong to *Aeromonas*, *Bacillus*, *Azotobacter*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Microbacterium*, *Sphingomonas*, *Mycobacterium*, *Kocuria varians* and *Rhizobium*, with some exceptions. Auxin regulates the expression of different genes in *Rhizobium*-legume interactions that are involved in plant signal processing and attachment to plant roots.

3.3.4.2 Gibberellins

Gibberellins are tetracyclic diterpenoid acids that are involved in a number of developmental and physiological processes in plants (Crozier et al. 2000). Apart from *Gibberella fujikuroi*, *Azospirillum* sp. and *Rhizobium* sp., production of gibberellin-like substances has also been claimed in numerous bacterial genera (Bottini et al. 2004) like *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae* (Bastián et al. 1998) and *Bacillus* sp. (Gutiérrez-Mañero et al. 2001), *Pseudomonas monteilii* (Pandya and Desai 2014) as well as *Actinomycetes* (Patil and Patil 2012).

3.3.4.3 Cytokinins

Cytokinins are adenine derivatives. Studies with the slime mould *Dictyostelium discoideum* revealed that 5′-AMP is a direct precursor of isopentenyl adenosine 5′-phosphate ([9R-5′P]iP). Zeatin is the major representative of the group cytokinins.

Many rhizosphere bacteria can produce cytokinins in pure culture, e.g. *Agrobacterium*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Erwinia*, *Pantoea agglomerans*, *Pseudomonas*, *Rhodospirillum rubrum*, *Serratia* and *Xanthomonas*.

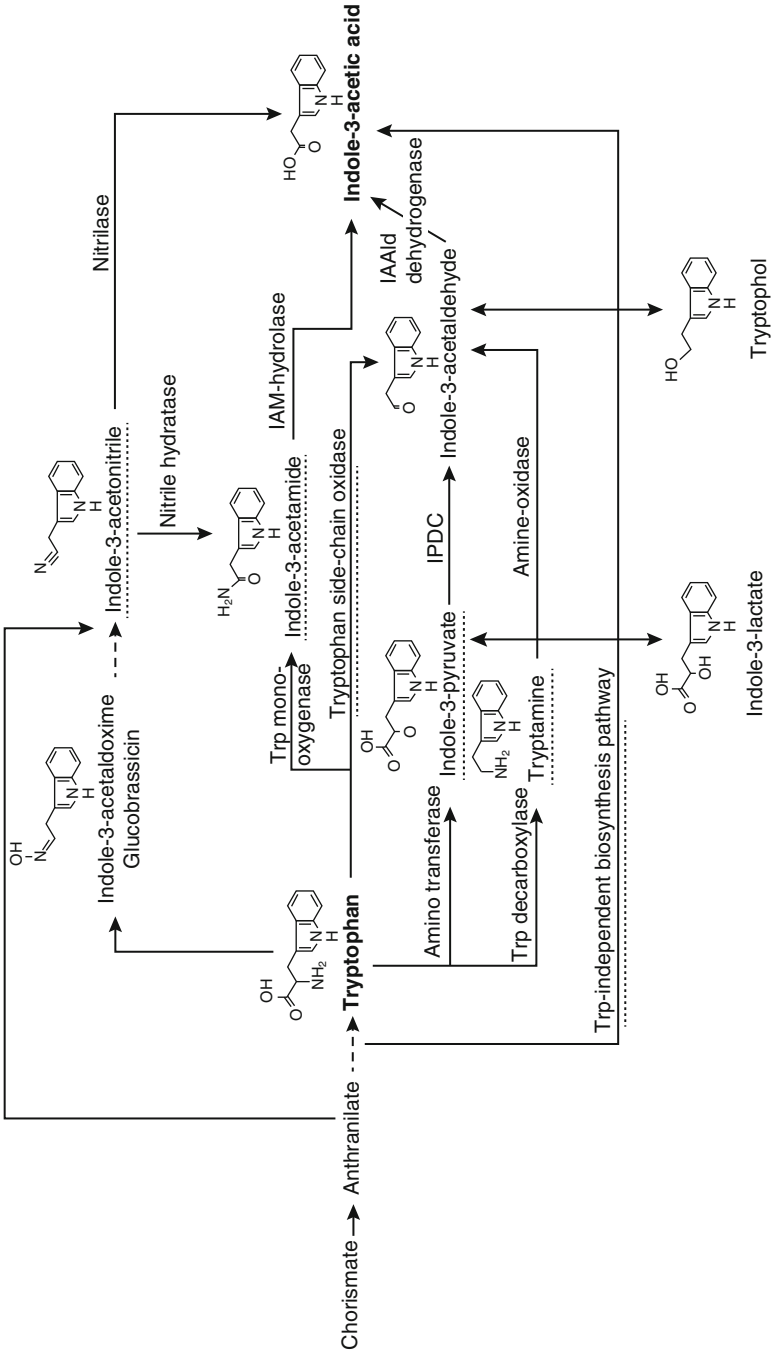

Fig. 3.2 IAA production strategies adopted by different organisms

The cytokinins produced by rhizospheric bacteria becomes part of the plant cytokinin pool and thus influencing plant growth and development.

Concerning the mechanism of action of cytokinins, one speculates that cytokinin produced by rhizosphere bacteria becomes part of the plant cytokinin pool and thus influences plant growth and development. Cytokinins, by affecting cell division, growth, nutrient translocation, retardation of senescence and plant defence, undoubtedly play an important role in the growth-defence trade-off. Großkinsky et al. (2016) has indicated towards the ability of *Pseudomonas fluorescens* G20–18 to efficiently control *Pseudomonas syringae* infection in *Arabidopsis*, allowing maintenance of tissue integrity and ultimately biomass yield using mutant analysis. While cytokinin-deficient loss-of-function mutants of G20–18 exhibited impaired biocontrol, functional complementation with cytokinin-mediated biocontrol was correlated with differential cytokinin levels.

The ability to produce auxins and cytokinins is a virulence factor for the pathogen *Agrobacterium tumefaciens* which produces crown galls. This bacterium can transfer the genes for production of auxins and cytokinins to the plant and incorporate these genes in the plant's DNA. Another bacterium from this genus, *A. rhizogenes*, modifies cytokinin metabolism, resulting in the appearance of masses of roots—instead of callus—from the infection site (Lugtenberg et al. 2013).

3.3.4.4 ACC-Deaminase Activity

Under different types of environmental stress, such as cold, drought, flooding, infections with pathogens and presence of heavy metals, plants respond by synthesizing 1-aminocyclopropane-1-carboxylate (ACC), which is a precursor for ethylene (Glick et al. 2007), a growth hormone. It has been reported that certain PGPRs also have ACC-deaminase activity that changes ACC into alpha-keto-butyrate and ammonia and thereby lower the level of ethylene in the plant. Rhizobacteria with ACC-deaminase activity belong to genera *Achromobacter*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Pseudomonas* and *Rhizobium*. More specifically, the soilborne fluorescent pseudomonads have gained particular attention throughout the global scene because of their catabolic versatility, excellent root colonizing ability and their capacity to produce a wide variety of enzymes and metabolites that favour the plant to withstand varied biotic and abiotic stress conditions. Glick et al. (1999) suggested a model explaining how ACC deaminase containing PGPR can lower plant ethylene levels and in turn stimulate plant growth. According to this model, PGPRs attach either to seed surface or roots of developing plant, in response to tryptophan and other amino acids produced by the seeds, and thus synthesize the auxin (IAA). Together with the plant produced IAA, the bacterial IAA stimulates synthesis of ACC synthase, which is responsible for the rapid transformation of S-adenosyl-L-methionine into ACC. Besides, plants inoculated with PGPR having ACC deaminase are more resistant to the injurious effects of the stress ethylene that is produced as a result of stressed environments (Saini et al. 2015).

3.4 Fungi and Their Potential as Biofertilizers

3.4.1 Mycorrhizal Fungi

The term "mycorrhiza" was coined by Albert Bernhard Frank (TAC 1989) to describe the symbiotic association of plant roots and fungi. Mycorrhiza literally meaning "fungus root" results from mutualism between roots of higher plants and certain fungi. Though the word "mycorrhiza" was coined in 1885, mycorrhizal fungi appear to have coevolved with plants for over 400 million years to become part of the root system as evidenced by fossil mycorrhiza found in carbonaceous deposits. These fungi in soil are ubiquitous throughout the world and form symbiotic relationships with the roots of most terrestrial plants. In natural ecosystems, it is exceptional for a plant not to possess a mycorrhizal root system. Therefore, it could be said that mycorrhizal association is very common or almost universal phenomenon in plant kingdom (Bagyaraj 2014).

3.4.2 Types of Mycorrhizae

3.4.2.1 Ectomycorrhiza

Ectomycorrhiza are most common among temperate forest tree species in the families Pinaceae, Salicaceae, Betulaceae, Fagaceae and Tiliaceae, as well as in some members of Rosaceae, Leguminosae, Myrtaceae and Juglandaceae. Numerous fungi have been identified as forming ectomycorrhiza. Most of them are basidiomycetous fungi belonging to the genera *Boletus*, *Suillus*, *Russula*, *Hebeloma*, *Tricholoma*, *Laccaria*, *Rhizopogon*, *Scleroderma*, *Alpova*, *Pisolithus*, etc. Some ascomycetous fungi also form ectomycorrhiza such as *Tuber* and *Cenococcum*. Thus, they are mostly fungi-forming mushrooms, puffballs or truffles. The mycorrhizal association helps in the uptake of nutrients from soil, protects roots against invasion by pathogens and also decomposes organic matter. These fungi can be cultured in the laboratory on suitable media and used for inoculating forest nurseries.

3.4.2.2 Ericoid Mycorrhiza

Ericoid mycorrhizal fungi usually colonize plants belonging to the families Ericaceae, Empetraceae and Epacridaceae, which are commonly referred to as heath plants, e.g. azalea, rhododendron, blueberry, cranberry, etc. These plants occur in temperate regions of the world. The fungus that forms mycorrhizal association is *Hymenoscyphus ericae*, earlier called as *Pezizella ericeae*, which is an apothecium-forming ascomycete. Most ericaceous species characteristically

grow on nutrient-poor, acidic soil where ammonium predominates over nitrate. Ammonium ions are relatively immobile in soil. Thus, ericoid mycorrhizal fungi help in the uptake of both N and P. In India, azaleas and rhododendrons are grown in Himachal Pradesh and North Eastern regions. The possibility of using the ericoid mycorrhizal fungus for enhancing the productivity of these plants can be an area for future research (Smith and Read 2008).

3.4.2.3 Orchid Mycorrhiza

Orchids belong to the family Orchidaceae. This family has nearly 30,000 species. Orchid seeds contain very limited reserves in the form of starch or lipid. At the time of germination, seeds absorb water and swell slightly, and the seed coat breaks exposing the epidermal hair, and this structure is referred to as the "protocorm". The protocorm has to be infected by the mycorrhizal fungus to develop into a plant. Protocorms wait up to 6 months to be infected by the mycorrhizal fungus. If not infected by the fungus, it dies. Orchids are thus obligatorily dependent on mycorrhizal fungi. The fungi involved were initially identified as *Rhizoctonia solani*, *R. repens* and other *Rhizoctonia* spp. Later, their perfect stage was discovered, and they belonged to the genera *Thanatephorus*, *Ceratobasidium*, *Sebacina* and *Tulasnella*. Extensive studies have been made in the orchid mycorrhizal fungus *Tulasnella calospora*. This fungus can be cultured in the laboratory. If the host is not available, it can survive as a saprophyte (Krishnamurthy and Senthilkumar 2005).

3.4.2.4 Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal (AM) fungi are ubiquitous in soil habitats and form beneficial symbiosis with the roots of angiosperms and other plants. The AM fungi belong to the family *Endogonaceae*, of the order *Muccorales*, class *Zygomycetes*. The AM-forming genera of the family include *Acaulospora*, *Entrophospora*, *Gigaspora*, *Glomus*, *Sclerocystis* and *Scutellospora*. A wide range of AM fungi is found in India. Bakshi (1974) was the first to give an account of 14 spore types: *Glomus macrocarpum* Tul and Tul var., *G.geosporum*, *G.mosseae*, *Glomus* sp., *Sclerocystis coremioides*, *Sclerocystis* sp., *Gigaspora*, *Calospora*, *Acaulospora* sp., *Endogone gigantea*, *E. microcarpum*, *Endogone 1*, *Endogone 2* and *Endogone 3*. Gerdemann and Bakshi (1976) reported two new species, viz. *Glomus multicauli* and *Sclerocystis sinuosa*. In general, the distribution of AM spores in rhizosphere soil is governed by edaphic and certain climatic factors. pH is the only edaphic factor which determines the abundance of AM fungi. However, pH did not influence the mycorrhizal spore density and frequency. High soil P and N content caused a reduction in infection and number of AM spores as well as decreasing the dependency of the plant on the fungal association.

Vesicular arbuscular mycorrhizas (VAM) are produced by aseptate mycelial fungi and are so-called because of the two characteristic structures, vesicles and arbuscules, found in roots with the type of infection. They are by far the commonest of all mycorrhizas and are found in Bryophytes, Pteridophytes, Gymnosperms, excluding the Pinaceae, and in virtually all families of Angiosperms. They are of general occurrence in the Gramineae, Palmae, Rosaceae and Leguminosae, which all include many crop plants. Indeed most crop plants, including herbs, shrubs and some trees, possess this type of mycorrhiza. Associations resembling modern-day VAM were present very early in the evolution of land plants. VAM may colonize the roots of a host plant either intracellularly or extracellularly; this filamentous network promotes bidirectional movement of nutrients where carbon flows to the fungus and inorganic nutrients move to the plant, thereby providing a critical linkage between the plant root and rhizosphere. VAM fungi help plants to capture nutrients such as phosphorus and micronutrients from the soil. It is believed that the development of the VAM symbiosis played a crucial role in the initial colonization of the land by plants and in evolution of the vascular plants. It has been said that it is quicker to list the plants that do not form mycorrhizae than those that do.

3.5 Biofertilizers in Relevance to Environmental Stress Tolerance by Plants

3.5.1 Genetics

It is important to combine genome manipulation techniques with microbiological interference, as it will help in improving potential of microbial assets. The role of microorganisms in inducing genes related to plant defence response has been well documented. *Medicago trunculata* showed induction of various defence-related genes with mycorrhizal colonization (Liu et al. 2007). Expression of ENOD11 and many defence-related genes and root remodelling genes gets upregulated during entry. Subsequently, this allows the formation of a pre-penetration apparatus or PPA (Bucher et al. 2009). Many disease resistance genes that work via jasmonate/ethylene signalling as well as osmotic regulation via proline synthesis genes were differentially expressed with UFLA285 induction (Baharlouei et al. 2011). Various differentially expressed genes were identified which include metallothionein-like protein type 1, a NOD26-like membrane integral protein, ZmNIP2–1, a thionin family protein, an oryzain gamma chain precursor, stress-associated protein 1 (OsISAP1), probenazole-inducible protein PBZ1 and auxin- and ethylene-responsive genes (Brusamarello-Santos et al. 2012). The expression of the defence-related proteins PBZ1 and thionins was found to get repressed in the rice with *H. seropedicae* association, suggesting the modulation of plant defence responses during colonization (Brusamarello-Santos et al. 2012) (Table 3.1).

Table 3.1 List of plant growth-promoting microbes of interest

Bio-inoculants	Plant sp.	Conditions	Effects produced	References
Bacillus sp.	*Wedelia Trilobata*	Aseptic	The phenotypic growth (root RCR, shoot diameter and shoot length), clonal growth and biomass (above ground mass and total mass) increased greatly	Dai et al. (2016)
Twenty-three strains (11 rhizospheric and 12 root endophytes) isolated from tomato root region	*Arabidopsis Thaliana*	Plate	A total of 73% isolates were able to produce organic acids, 89% IAA, 83% ACC deaminase and 87% siderophores. The most striking result was remarkable increases in the formation of root hairs for most of the inoculated plants	Abbamondi et al. (2016)
Rhizobium daejeonense, *Acinetobacter calcoaceticus* and *Pseudomonas mosselii*	*Agave americana* L.	Plant inoculation assay	All of the strains were able to synthesize IAA and solubilize phosphate and had nitrogenase activity. Significant effect on plant growth and the sugar content was also witnessed	De La Torre-Ruiz et al. (2016)
Endophytic bacteria from leaf, stem, root and rhizosphere of sugarcane	*Zea mays* L.	Greenhouse	Isolates were observed to solubilize phosphate; fix nitrogen; produce IAA, HCN, chitinase, ammonia, cellulase and pectinase; and promote plant growth	Rodrigues et al. (2016)
Streptomyces sp.	*Triticum Aestivum*	Pot	Production of IAA, siderophore, ACC deaminase, ammonia and hydrogen cyanide, solubilization of mineral phosphate, significant increases in shoot and root length, plant fresh weight, plant dry weight, number of leaves and number of roots	Anwar et al. (2016)

(continued)

Table 3.1 (continued)

Bio-inoculants	Plant sp.	Conditions	Effects produced	References
Streptomyces sp., SAI and VAI	*Cicer arietinum* L.	Field	Production of siderophore, cellulase, lipase, protease, chitinase, hydrocyanic acid, indole acetic acid and 1,3-glucanase. An increase in nodule number, shoot weight and yield, enhanced total N, available P and organic C was also observed under field conditions	Sreevidyaa et al. (2016)
Trichoderma longibrachiatum	*Triticum aestivum*	Laboratory	The relative water content in the leaves and roots, chlorophyll content and root activity were significantly increased, and the accumulation of proline content in leaves was markedly accelerated with the plant growth parameters. The antioxidant enzymes, superoxide dismutase, peroxidase and catalase, were increased by 29, 39 and 19%, respectively, under salt stress	Zhang et al. (2016)
Eighty-one isolates associated with *Firmicutes* and *Proteobacteria* phyla	*Langsdorffia hypogaea* Mart.	Laboratory	Of the total isolates, 62, 86 and 93% produced, respectively, siderophores and IAA and were able to fix N_2. In addition, 27 and 20% of isolates inhibited the growth of enteropathogens and phytopathogens, respectively	Felestrino et al. (2017)
Bacillus aquimaris DY-3	*Zea mays* L.	Pot	Tolerance to salinity was achieved. Chlorophyll content, leaf relative water content, accumulation of proline, soluble sugar and	Li and Zhang (2017)

(continued)

Table 3.1 (continued)

Bio-inoculants	Plant sp.	Conditions	Effects produced	References
			total phenolic compound and activities of superoxide dismutase, catalase, peroxidase and ascorbate peroxidase were enhanced, while lipid peroxidation levels and Na^+ content decreased	
Azotobacter vinelandii, *Pantoea agglomerans*, *P. putida*	*Onobrychis sativa* L.	Pot	Increased root and shoot lengths, shoot dry weight, nutrient uptake and mitigation of drought stress were observed	Delshadi et al. (2017)

3.5.2 Multiple Inoculations

Application of PGPR for improvement of crops has been investigated for many years, with recent attention focused on co-inoculation of PGPR with different growth attributes for growth promotion and stress tolerance. Populations of bacteria have functional roles within communities that permit their survival. Distinct microbial populations in rhizosphere frequently interact with each other. Syntrophic relationships between different organisms have been demonstrated in several microbial ecosystems. Bacteria live in consortia bound to surfaces as in biofilms, flocs or granules. Under these conditions the bacteria are positioned in a heterogeneous environment. It is increasingly apparent that in nature, bacteria function less as individuals and more as coherent groups that are able to inhabit multiple ecological niches. When these strains are made into an inoculum consortium, each of the constituent strains of the consortium not only outcompete others for rhizospheric establishments but complement functionally for plant growth promotion. Pandey and Maheswari (2007) described the relationship between two distantly related isolates, *Burkholderia* sp. MSSP and *Sinorhizobium meliloti* PP3. They discovered that in combination both the strains promote growth of host plants because of increased indole-3-acetic acid (IAA) production and phosphate solubilization than single inoculation under laboratory conditions. About 25% increase in mean growth rate was recorded for *S. meliloti* PP3 when grown in mixed-species, two-species culture with respect to single-species culture. This interaction also indicates that in soil, association with *Burkholderia* sp. MSSP favours *S. meliloti* PP3 as an adaptation of high rate of reproduction—a well-known strategy that enables organisms to successfully survive and maintain themselves in communities. Seneviratne (2003) has mentioned that co-inoculation and coculture of microbes have been observed to perform the tasks better than the individual microbes.

3.6 Constraints in Biofertilizer Technology

In spite of a significant growth of biofertilizer industry over the last 50 years, they are still far from their actual potential. Restricted nutrient mobilization potential compared to their chemical counterparts and slow impact on crop growth are the major constraints. Incoherent responses in the field under varied agroecological niches and cropping systems have also contributed to their low recognition by farmers. Besides these, there are some technological, biological, field and marketing constraints, which restrict the fast growth of biofertilizer industry. Some of the major constraints and limitations of the industry are as follows:

1. Crop-specific local strain development.
2. Vulnerability of strains to high chemical fertilizer use.
3. Declining interest in scientific community on development of biofertilizer technologies.
4. Deficiency in technology in respect to carrier suitability and product formulations.
5. Lack of automation in product handling.
6. Liquid inoculants are coming up as solution, but the technology is still immature and not available in public domain.
7. Distribution channels through government agencies are not effective which are leading to cut throat competition among bidders, resulting in low-cost poor-quality inoculant production.

3.7 Current Scenario and Future Prospects

Biofertilizers being important components of organic farming play imperative role in maintaining extended term soil fertility and sustainability by fixing atmospheric nitrogen, mobilizing fixed macro- and micronutrients or converting insoluble P and Zn in the soil into forms available to plants, thereby increasing their efficiency and accessibility (Mishra et al. 2013). In the context of both the cost and environmental impact of chemical fertilizers, excessive dependence on chemical fertilizers is not so viable a strategy in the long run. In this context, organic manures would be the practical option for farmers to increase productivity per unit area.

Ecological stresses are becoming a foremost problem and productivity is declining at an unprecedented rate. Excessive dependence on chemical fertilizers has not only adversely affected the quality of human consumption and environment but also disturb the ecological balance. Biofertilizers can help solve the problem of feeding an increasing global population at a time when agriculture is facing various environmental stresses. It is important to realize the useful aspects of biofertilizers and implement its application to modern agricultural practices. New technology developed using the powerful tool of molecular biotechnology can enhance the biological pathways of production of phytohormones and other growth-promoting

secondary metabolites. Identified and transferred to the useful PGPRs, these technologies can help provide liberation from environmental stresses. However, the lack of attentiveness regarding enhanced protocols of biofertilizer application to the field is one of the few reasons why many useful PGPRs are still beyond the knowledge of ecologists and agriculturists. Nonetheless, the recent progresses in technologies related to microbial science, plant-pathogen interactions and genomics will help to standardize the requisite protocols. The success of the science related to biofertilizers depends on inventions of pioneering strategies related to the functions of PGPRs and their proper application to the field of agriculture. The major challenge in this area of research lies in the fact that along with the identification of various strains of PGPRs and their properties, it is essential to scrutinize the actual mechanism of implementation of PGPRs for their efficacy towards exploitation in sustainable agriculture.

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Chapter 4
Plant Growth-Promoting Rhizobacteria for Abiotic Stress Alleviation in Crops

Sangeeta Paul, Ajinath S. Dukare, Bandeppa, B. S. Manjunatha, and K. Annapurna

Abstract Environmental stresses pose a major constraint to agricultural productivity. Studies have indicated an immense potential of plant growth-promoting rhizobacteria (PGPRs) in the mitigation of different abiotic stresses in crops such as temperature, salinity, drought, heavy metal toxicity, etc. Improved plant growth and yield; and enhanced tolerance to various abiotic stresses due to inoculation with PGPRs have been noted in different plants. PGPRs mitigate environmental stress in plants through an array of mechanisms which include phytohormone production, induced systemic tolerance through modulation of physiological responses, osmotic adjustments through production of osmolytes, ACC deaminase activity, exopolysaccharide production, improvement in soil physicochemical properties, production of volatile organic carbon compounds, and induction of stress-responsive genes. Screening, selection, and use of abiotic stress-tolerant PGPRs as bioinoculants are a promising strategies for enhancing crop productivity under stressed environments.

Keywords Plant growth-promoting rhizobacteria · Abiotic stress alleviation · Induced systemic tolerance · Hormonal homeostasis · ACC deaminase activity

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4.1 Introduction

Various abiotic stresses like drought, salinity, nutrient imbalances (including deficiencies and mineral toxicities), and extremes of temperature hinder the growth, development, and yield of agricultural crops. Climate changes with erratic rainfalls and prolonged drought have multiple effects on abiotic stress consequences to the crop and seriously threaten sustainable agricultural production (Selvakumar et al. 2012). Water availability is one of the major components limiting plant productivity, and drought stress may lead to yield losses of more than 50% for major crops in the world (Boyer 1982). Drought stress also leads to salinity stress (Munns 2002) which in turn is associated with soil alkalization stress. Salinity stress is another major abiotic stress impacting crop growth and productivity. Alkalinity and salinity along with waterlogging are major agricultural productivity constraints which affect about 7.3 million hectares of arable land in India alone. Nutrient stress conditions are produced in saline-alkaline soils due to unavailability of and/or disruption in the uptake of plant nutrient from soil (Maheshwari et al. 2012). Both global warming and water scarcity can increase the effect of high-temperature stress, which in turn poses grave risk to agricultural production.

The exposure of crop plants to various abiotic stresses leads to a series of morphological, cellular, physiological, and molecular changes. These changes adversely affect crop growth and development leading to reduction in crop yield (Wang et al. 2001). Most of the abiotic stresses are associated with the production of osmotic imbalance and dehydration in crop plants. Generally, all types of abiotic stresses lead to nearly similar alterations in the plants' physiological and biochemical status. At cellular level, the primary effects of all abiotic stress are enhanced accumulation of reactive oxygen species (ROS) due to change in ion imbalance and osmotic stress. This leads to disruption of various cellular structures and denaturation of macromolecules such as protein, DNA, carbohydrates, and lipid. All these changes cause significant reduction and inhibition of photosynthetic activity and alteration in cellular hormone levels. Thus, overall metabolic dysfunction leads to reduced growth and fertility, premature senescence, and low yield of crops. The overall constraints caused by some of the abiotic stresses at cellular, biochemical, and physiological level of plants are summarized in Table 4.1.

Various strategies are being employed to improve crop production under abiotic stresses. Recent research focusing on the role of microbes in improving agricultural productivity has indicated the ability of the plant growth-promoting rhizobacteria (PGPRs) to help mitigate abiotic stresses in crop plants (Dimkpa et al. 2009a; Rejeb et al. 2014). In fact, microorganisms can play a significant role in abiotic stress alleviation in plants. These bacteria colonize the rhizospheric and endorhizospheric zones of plants and promote plant growth. Microorganisms can modulate physiological responses of the higher plants to various abiotic stresses such as drought, high temperature, salinity, frost injury, and heavy metal toxicity. Production of exopolysaccharides and biofilm formation by rhizospheric microorganisms can influence soil physicochemical properties. These microbes enhance tolerance of

Table 4.1 Constraints caused by various abiotic stresses at cellular, biochemical, and physiological levels in plants

Abiotic stressor	Impact caused at cellular, biochemical, and physiological levels in plants
High-temperature/heat stress	Water deficiency in cell due to higher rate of evaporation; may lead to plant death due to increased turnover of cellular enzyme activity
Low-temperature/cold stress	Slow down rate of cellular biochemical reactions; formation of intracellular ice crystals and disruption of cell membrane; inhibition of ATP synthesis; oxidative damage due to production of singlet oxygen (O) molecule; restricted supply of $NADP^+$ and thus limiting photosynthetic light reaction and CO_2 fixation activities
Drought/low-moisture stress	Decline in the photosynthetic activity due to decreased water uptake by plant; stomata closure and alteration in cell wall plasticity; oxidative damage due to enhanced production of reactive oxygen species (ROS)
Flooding/excessive moisture stress	Interference in mitochondrial respiration due to generation of anoxic conditions near root zone; reduction in uptake and upward transport of plant nutrient through root tissue; oxygen deficiency at cellular and tissue level
Salinity stress	Nutrient imbalance/deficiencies due to disruption in nutrient uptake and allocation; disruption in photosynthetic activity, enhanced rate of photorespiration and increased production of ROS
Heavy metal/toxic metal stress	Cellular injury due to oxidative damage; alteration in water balance and stomatal opening; nutrient deficiency in plant cell, damage to cell membrane integrity and decline in cellular enzyme activity and stunted plant growth
Climate change stress	Occurrences of drought, salinity, and flooding due to increased level of CO_2 and temperature; negative impact on photosynthetic apertures and thus on process of carbon assimilations; oxidative damage; delayed flowering and reduction in crop yields

the plants to abiotic stresses, and the term induced systemic tolerance (IST) has been proposed for such changes in plants induced by the microbes at physiological, biochemical, and molecular levels. Use of these microorganisms for alleviation of abiotic stresses in plants is a new and promising technology under stressed environments.

4.2 Abiotic Stress Mitigation by PGPRs

Besides improving plant growth under unstressed soil conditions, PGPRs have also been reported to impart tolerance to abiotic stresses such as extremes of temperature, salinity, alkaline and acidic pH, drought, and heavy metal toxicity (Dimkpa et al. 2009a). Indigenous microbes isolated from stressed environment are acclimatized to adverse conditions. Efficient strains can be selected from such stress-tolerant microbes possessing multiple PGPR traits as potential bioinoculants for stressed environments. Bacterial genera including *Achromobacter*, *Azospirillum*, *Bacillus*, *Bradyrhizobium*, *Brevibacterium*, *Burkholderia*, *Enterobacter*, *Methylobacterium*,

Microbacterium, *Mycobacterium*, *Pantoea*, *Paenibacillus*, *Phyllobacterium*, *Pseudomonas*, *Rhizobium*, *Zhihengliuela*, etc. have been reported to impart tolerance to different abiotic stresses in various plants (Table 4.2).

Higher-temperature stress leads to hormonal imbalances and water deficiency in plant cell due to high evaporation rate and cellular death, thus affecting crop growth. Under such stressful conditions, extreme thermotolerant microbes have been used for conferring high-temperature tolerance to crops. PGPRs belonging to genera *Burkholderia phytofirmans*, *Pseudomonas putida*, and *Pseudomonas* spp. have shown promising potential in alleviation of high-temperature stress in different crops (Srivastava et al. 2008; Ali et al. 2009).

Low temperature slows down cellular metabolism and may even impact and disrupt cellular membrane due to formation of intracellular ice crystals (Mishra et al. 2012). Various cold-tolerant PGPRs have been reported to alleviate the constraint caused by cold stress in crop plants (Barka et al. 2006; Cheng et al. 2007). Selvakumar et al. (2012) have demonstrated the plant growth-promoting activities of several novel bacterial species such as *Pantoea dispersa*, *Pseudomonas fragi*, *Pseudomonas lurida*, *Serratia marcescens*, and *Exiguobacterium acetylicum* in alleviation of cold-temperature stress. Similarly, Cheng et al. (2007) have also reported growth promotion in canola at low temperature by cold-tolerant *P. putida* UW4.

Halotolerant PGPRs belonging to the genera *Brevibacterium epidermidis*, *Micrococcus yunnanensis*, *Bacillus aryabhattai*, *Chrysopogon aucheri*, *Azospirillum brasilense*, *Brevibacterium iodinum*, *Bacillus licheniformis*, *Zhihengliuela alba*, *Pseudomonas putida*, *Staphylococcus kloosii*, and *Kocuria erythromyxa* were reported to enhance tolerance of different plants such as tomato, soybean, barley, wheat, pepper, lettuce, cotton, chickpea, etc. to salt stress (Mayak et al. 2004b; Yildirim and Taylor 2005; Han and Lee 2005; Yasmin and Bano 2011; Yao et al. 2010). Improved growth, root length, plant biomass, plant height, seed germination, and seedling vigor were observed in canola, red pepper, wheat, cotton, radish, and barley (Siddikee et al. 2010, 2011; Nabti et al. 2007; Egamberdieva 2009; Ashraf 2004; Yildirim et al. 2008; Omar et al. 2009). Salt-sensitive wheat cultivar showed better response to inoculation (Al-Karaki 2001).

PGPRs when used as inoculants promoted plant growth and mitigated drought stress (Ruiz-Sanchez et al. 2011; Bandeppa et al. 2015). *Pseudomonas chlororaphis* and *Phyllobacterium brassicacearum* were able to induce drought tolerance in *Arabidopsis thaliana* (Cho et al. 2008; Bresson et al. 2013). Cucumber plants inoculated with a consortium of three PGPR strains belonging to *Bacillus cereus*, *Bacillus subtilis*, and *Serratia* spp. showed enhanced tolerance to drought (Wang et al. 2012). *Bacillus licheniformis* K11 strain enhanced drought tolerance in pepper (Lim and Kim 2013).

Anthropogenic activities lead to an accumulation of heavy metals such as cadmium, chromium, copper, lead, mercury, nickel, and zinc in the ecosystem resulting in heavy metal stresses in plants. Some PGPRs have the potential for transformation of such heavy metals in the crop rhizosphere and thereby mitigating the toxic effects of heavy metals on those plants (Belimov et al. 2004; Narasimhan

Table 4.2 Microorganisms reported for alleviating abiotic stress constraints in crop plants

Abiotic stressor	PGPR/microbial inoculants	Crop plant	Citation
Drought stress	*Pseudomonas* spp.	Maize	Sandhya et al. (2010)
	Pseudomonas spp.	Asparagus	Liddycoat et al. (2009)
	Pseudomonas spp.	Pea	Arshad et al. (2008)
	Pseudomonas mendocina	Lettuce	Kohler et al. (2008)
	Bacillus spp.	Lettuce	Arkipova et al. (2007)
	Bradyrhizobium elkanii	Flat crown	Swaine et al. (2007)
	Bacillus megaterium and *Glomus* spp.	*Trifolium*	Marulanda et al. (2007)
	Achromobacter piechaudii	Tomato, Pepper	Mayak et al. (2004a)
	Azospirillum	Wheat	Creus et al. (2004)
	A. brasilense	Maize	Casanovas et al. (2002)
Salt stress	*Pseudomonas pseudoalcaligenes*	Rice	Jha et al. (2010)
	Bacillus pumilus		
	Azospirillum brasilense	Barley	Omar et al. (2009)
	Bacillus subtilis	*Arabidopsis thaliana*	Zhang et al. (2008)
	Pseudomonas syringae	Maize	Nadeem et al. (2007)
	Pseudomonas fluorescens		
	Enterobacter aerogenes		
	P. fluorescens	Groundnut	Saravanakumar and Samiyappan (2007)
	Azospirillum	Lettuce	Barassi et al. (2006)
	Aeromonas hydrophila/ caviae	Wheat	Ashraf (2004)
	Bacillus insolitus		
	Bacillus sp.		
	Azospirillum	Maize	Hamdia et al. (2004)
	A. brasilense	Chickpeas, Faba beans	Hamaoui et al. (2001)
Extreme temperature stress	*B. phytofirmans*	Potato	Bensalim et al. (1998)
	Pseudomonas fluorescens	Wheat	Egamberdiyeva and Hoflich (2003)
	Pantoea agglomerans		
	Mycobacterium spp.		
	Burkholderia phytofirmans	Grapevine	Barka et al. (2006)
	P. putida	Canola	Cheng et al. (2007)
Flooding	*Enterobacter cloacae*	Tomato	Grichko and Glick (2001)
	Pseudomonas putida		

(continued)

Table 4.2 (continued)

Abiotic stressor	PGPR/microbial inoculants	Crop plant	Citation
Heavy metal stress	*Bacillus subtilis*	Rice	Asch and Padham (2005)
	Bacillus megaterium		
	Bacillus spp.		Terré et al. (2007)
	Methylobacterium oryzae	Tomato	Madhaiyan et al. (2007)
	Burkholderia spp.		
	Pseudomonas fluorescens	Rapeseed	Sheng et al. (2008)
	Microbacterium spp.		
	Bacillus subtilis	Oat	Pishchik et al. (2009)
	Pantoea agglomerans		
Nutrient deficiency	*Bacillus polymyxa*	Maize	Egamberdiyeva (2007); Adesemoye et al. (2008)
	Mycobacterium phlei		
	Pseudomonas alcaligenes		
	Bacillus spp.		
	Bacillus spp.	Maize	Oliveira et al. (2009)
	Burkholderia spp.		
	Streptomyces platensis		
	Azotobacter chroocoocum	Maize	Yazdani et al. (2009)
	Azospirillum brasilense		
	Pseudomonas putida		
	Bacillus lentus		
	Azospirillum spp.	Chickpea	Rokhzadi and Toashih (2011)
	Azotobacter chroococcum		
	Mesorhizobium ciceri		
	Pseudomonas fluorescens		

et al. 2003; Huang et al. 2005). Soil microbes such as AM fungi and PGPR absorb heavy metals in their tissues at very high rate and can alleviate the stress. Various PGPRs have been reported as inoculant for alleviation of heavy metal stress in crop plants. These rhizobacteria reduce the bioavailability of toxic concentration of heavy metal in rhizosphere which is detrimental to plant growth. Many root zone bacteria produce metal-chelating substances, such as iron-chelating siderophores, which in turn have shown to influence plant uptake of various hazardous heavy metals like zinc, copper, and iron (Carrillo-Castaneda et al. 2003, 2005; Egamberdiyeva 2007; Dimkpa et al. 2009b).

4.3 Mechanisms of Abiotic Stress Alleviation

PGPRs enhance plant growth under stressed environments by an array of mechanisms like phytohormone production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, induction of systemic tolerance in plants through modulation of physiological status of the plant, improved soil aggregation, biofilm formation, enhanced nutrient uptake, production of volatile organic compounds (VOCs), etc. The detailed mechanisms of PGPRs involved in alleviation of abiotic stress are enlisted in Table 4.3.

4.3.1 Phytohormone Production

Auxins produced by many bacteria such as *Azospirillum*, *Acetobacter diazotrophicus*, *Alcaligenes faecalis*, *Bradyrhizobium japonicum*, *Enterobacter cloacae*, *Pseudomonas*, *Rhizobium*, and *Xanthomonas* promotes plant growth (Patten and Glick 1996). IAA produced by these PGPRs plays an important role in imparting drought and salinity stress tolerance to plants by modification of root architecture (Verbon and Liberman 2016). This results in an increase in root biomass, root surface area, and volume, thereby increasing the uptake of nutrients and water (Mantelin and Touraine 2004), which helps plants to cope with water deficit (Egamberdieva and Kucharova 2009).

In addition, production of abscisic acid and gibberellic acid by rhizobacteria was also reported to enhance abiotic stress tolerance in the inoculated plants. *A. lipoferum* producing ABA and gibberellins alleviated drought stress in maize plants (Cohen et al. 2009). ABA is a stress hormone which accumulates under moisture and salinity stress. Its biosynthesis is triggered by cellular dehydration. ABA regulates transpiration rate by controlling stomatal closure and stress signal transduction pathways (Yamaguchi-Shinozaki and Shinozaki 1994). Reduced water loss from the plant due to increased ABA concentration in plant tissues leads to increased tolerance to stress (Albacete et al. 2008). Naz et al. (2009) reported improved growth of soybean seedlings exposed to saline stress on inoculation with ABA-producing PGPRs. In contrast, Yao et al. (2010) reported a decrease in salinity-induced accumulation of ABA along with improvement in seedling biomass in *P. putida*-inoculated cotton plants. Similar decrease in ABA accumulation was observed in PGPR-inoculated *Arabidopsis* and cucumber plants exposed to drought stress (Kang et al. 2014a, b). Enhanced ABA content, leading to decreased loss of water through leaf transpiration, was noted in *P. brassicacearum* strain STM196-inoculated *Arabidopsis* plants which improved their osmotic stress tolerance (Bresson et al. 2013). Cytokinin-producing *B. subtilis* conferred drought stress tolerance and enhanced shoot growth in *Platycladus orientalis* (Liu et al. 2013). Similar observations were made in lettuce on inoculation with cytokinin-producing *B. subtilis* (Arkipova et al. 2007)

Table 4.3 Mechanisms of PGPRs involved in amelioration of various abiotic stress tolerances in crop plants

Mechanism of action	Abiotic stress	PGPRs involved	Crop plants	References
Phytohormone production	Drought	*Bacillus megaterium* and *Glomus* sp.	*Trifolium*	Marulanda et al. (2007)
	Osmotic stress	*Azospirillum brasilense* Sp245	*Arabidopsis*	Cohen et al. (2008)
	Drought	*Azospirillum lipoferum*	Maize	Cohen et al. (2009)
	Salt stress	*Pseudomonas aurantiaca Pseudomonas extremorientalis*	Wheat	Egamberdieva (2009)
	Drought	*B. subtilis*	*Platycladus orientalis*	Liu et al. (2013)
	Drought	*Pseudomonas putida* H-2–3	Soybean	Sang-Mo et al. (2014)
Enhanced activity of ACC deaminase	Moisture stress	*A. piechaudi*	Tomato	Mayak et al. (2004a)
	Drought	*Variovorax paradoxus*	Pea	Dodd et al. (2004)
	Low temperature	*Burkholderia phytofirmans PsJN*	Grapevine	Barka et al. (2006)
	Low temperature	*P. putida*	Canola	Cheng et al. (2007)
	Salinity stress	*Pseudomonas fluorescens*	Groundnut	Saravanakumar and Samiyappan (2007)
	Saline stress	*Brevibacterium iodinum*, *Bacillus licheniformis* and *Zhihengliuela alba*	Red pepper	Siddikee et al. (2011)
	Drought	*Bacillus licheniformis* K11	Pepper	Lim and Kim (2013)
Production and intracellular accumulation of osmolyte	Osmotic stress	*A. brasilense Az39*	Rice	Cassan et al. (2009)
	Salt	*Rhizobium* and *Pseudomonas*	Maize	Bano and Fatima (2009)
	Drought	*A. brasilense*	Maize	Rodriguez-Salazar et al. (2009)
	Drought	*P. fluorescens*	Maize	Ansary et al. (2012)
	Drought	*Bacillus subtilis* GB03	*Arabidopsis*	Zhang et al. (2010)
	Salt stress	*Pseudomonas pseudoalcaligenes*	Rice	Jha et al. (2011)

(continued)

Table 4.3 (continued)

Mechanism of action	Abiotic stress	PGPRs involved	Crop plants	References
	Drought stress	*Bacillus* spp.	Maize	Vardharajula et al. (2011)
	Abiotic stresses	*Bacillus* spp.	Potato	Gururani et al. (2013)
Exopolysaccharide (EPS) production	Drought	*Pantoea agglomerans*	Wheat	Amellal et al. (1998)
	Drought	*Rhizobium* sp.	Sunflower	Alami et al. (2000)
	Drought	*Pseudomonas putida* P45	Sunflower	Sandhya et al. (2009)
	Salt	EPS-producing bacteria	Maize	Awad et al. (2012)
Induction of antioxidative enzymes and improved antioxidant status	Saline	*Bradyrhizobium japonicum* and *Serratia proteamaculans*	Soybean	Han and Lee (2005)
	Salinity	*Piriformospora indica*	Barley	Waller et al. (2008)
	Drought	*Pseudomonas mendocina* and *Glomus intraradices*	Lettuce	Kohler et al. (2008)
	Salt	*P. mendocina*	Lettuce	Kohler et al. (2009)
	Salt	*Sinorhizobium meliloti*	*Medicago*	Bianco and Defez (2009)
Ion homeostasis	Salt	*Azospirillum*	Maize	Hamdia et al. (2004)
	Salt	*Bacillus subtilis*	*Arabidopsis*	Zhang et al. (2008)
	Osmotic stress	*A. brasilense* and *Pantoea dispersa*	Pepper	Del-Amor and Cuadra-Crespo (2012)
Improved nutrient and water uptake	Drought	*Azospirillum* sp.	Wheat	Creus et al. (2004); Pereyra et al. (2012)
	Drought, salinity	AM Fungi	Sorghum	Cho et al. (2006)
	Abiotic stress	*Pseudomonas monteilii*, *Cronobacter dublinensis*, and *Bacillus* spp.	Basil	Singh et al. (2013)

(continued)

Table 4.3 (continued)

Mechanism of action	Abiotic stress	PGPRs involved	Crop plants	References
Induction of stress reliever genes and proteins	Drought	*Paenibacillus polymyxa*	*A. thaliana*	Timmusk and Wagner (1999)
	Drought	Bacterial endophytes	Sugarcane	Vinagre et al. (2006)
	Drought	*Paraphaeosphaeria quadriseptata*	*Arabidopsis*	McLellan et al. (2007)
	Heat stress	*Pseudomonas* sp. AMK-P6	Sorghum	Ali et al. (2009)
	Drought	*Bacillus licheniformis* K11	Pepper	Lim and Kim (2013)
Production of volatiles	Drought	*Bacillus subtilis*	*Arabidopsis*	Zhang et al. (2007)
	Salt	*Bacillus subtilis*	*Arabidopsis*	Zhang et al. (2008)

4.3.2 ACC Deaminase Activity

Plant synthesizes the gaseous hormone ethylene (C_2H_4) in its tissues from the precursor 1-aminocyclopropane-1-carboxylic acid (ACC). Under stress conditions there is increase in production of ethylene which has an adverse effect on plant growth. There is stunting of root and shoot growth due to increased biosynthesis of ethylene during drought stress. Many PGPR strains have been noted to possess the enzyme ACC deaminase (Glick et al. 2007), which can cleave the plant ethylene precursor ACC to ammonia and α-ketobutyrate (Shaharoona et al. 2006; Govindasamy et al. 2008). The presence of ACC deaminase activity has been demonstrated in a wide range of soil microorganisms (Jacobson et al. 1994; Glick 1995; Ghosh et al. 2003; Sessitsch et al. 2005; Madhaiyan et al. 2007). These PGPRs metabolize ACC, thereby lowering the ethylene level and reducing its negative effect on the plant. ACC deaminase-producing bacteria colonizing the root surfaces influence ethylene signaling and modify sensitivity of root and leaf growth to soil drying (Bashan and Holguin 1998). Thus, the deleterious effects of ethylene are reduced by the removal of ACC, thereby mitigating plant stress and promoting plant growth (Glick et al. 2007).

Bacterial ACC deaminase activity is relatively common in soil. ACC deaminase activity/genes have been reported in a wide range of bacterial isolates including *Agrobacterium*, *Achromobacter*, *Burkholderia*, *Azospirillum*, *Enterobacter*, *Pseudomonas*, *Rhizobium*, and *Ralstonia* (Blaha et al. 2006; Govindasamy et al. 2015). Inoculation with ACC deaminase-containing rhizobacteria induces longer roots in host plants and thus helps them in the uptake of water from deeper soil layers under moisture stress conditions (Zahir et al. 2008). Enhanced production of ethylene in

plant tissues under high- and chilling-temperature stress conditions has been reported by several researchers (Wang 1987; Strzelczyk et al. 1994). PGPRs that harbor ACC deaminase activity also enhance survival of plant seedlings under metal stress. Several authors have demonstrated increase in root length of plants inoculated with ACC deaminase-containing PGPRs under heavy metal stress conditions (Govindasamy et al. 2009, 2011; Jiang et al. 2008; Zhang et al. 2011; Gontia-Mishra et al. 2014).

4.3.3 Osmolyte Production and Accumulation

As a response to water deficit, plants increase the synthesis of osmolytes, thus increasing osmotic potential within cells (Farooq et al. 2009) to alleviate cell turgidity loss. Plants' adaptability to various abiotic stresses enhances biosynthesis and accumulation of quaternary compounds and other osmolytes like proline, glycine betaine, and trehalose, (Sakamoto and Murata 2000; Chen et al. 2007; Chen and Murata 2011; Hare et al. 1998).

Increased production of osmolytes by PGPRs, under drought stress conditions, is also suggested to improve survival of plants (Vanderlinde et al. 2010). PGPRs exude osmolytes in response to drought and salinity stress, which probably acts synergistically with plant-produced osmolytes and stimulate plant growth (Paul and Nair 2008; Sharifi et al. 2007). Under severe stress conditions, the beneficial effects of osmolyte-producing bacteria were enhanced in rice; differences between shoot and root dry weight and tiller number were more prominent in inoculated rice plants as compared to non-inoculated controls (Yuwono et al. 2005).

Plants inoculated with *Burkholderia* exhibited increase in proline synthesis in osmotically stressed plants (Barka et al. 2006). Increased proline content in plants inoculated with *Bacillus* strains under drought stress was attributed to the upregulation of gene for *P5CS*, which is involved in the biosynthesis of proline and inhibition of expression of the gene for *ProDH*, which is involved in proline metabolism (Yoshiba et al. 1997). *A. thaliana* transgenic plants with introduced *B. subtilis ProBA* genes showed higher proline production and an increase in their osmotic stress tolerance (Chen et al. 2007).

There is also accumulation of amino acids due to hydrolysis of proteins, which occurs in response to changes, contributing to osmotic adjustments (Iqbal et al. 2011; Krasensky and Jonak 2012). Also hydrolysis of starch under drought stress (Enebak et al. 1998) leads to accumulation of soluble sugars that contribute to cellular osmotic adjustment. There were simultaneous starch depletion and higher sugar contents in grapevine leaves during drought stress (Patakas and Noitsakis 2001). PGPRs have been reported to improve drought stress tolerance by increasing the accumulation of amino acids and soluble sugars, in inoculated plants. On exposure to drought stress, *A. lipoferum* inoculation enhanced accumulation of

free amino acids and soluble sugars and also improved maize growth (Bano et al. 2013). Maize seedlings inoculated with *Bacillus* strains had higher sugar content due to starch hydrolysis, thus imparting resistance to plants during drought stress (Nayer and Heideri 2008).

Adverse impact of drought stress on plant growth has been postulated to be due to a decline in sugar content, and their enhanced accumulation in the inoculated plants probably led to an increase in their drought tolerance (Sandhya et al. 2010). *A. brasilense* producing trehalose increased drought tolerance and plant growth in maize (Rodriguez-Salazar et al. 2009).

4.3.4 Induction of Antioxidative Enzymes and Improved Antioxidant Status

Accumulation of reactive oxygen species (ROS), a by-product of regular cellular metabolism (Lee et al. 2001), is usually aggravated under adverse environmental conditions (Breusegem et al. 2001). However, ROS also acts as a signal for the activation of stress response and defense pathways (Pitzschke et al. 2006). There is induction of antioxidative enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase and accumulation of nonenzymatic antioxidants like cysteine, glutathione, and ascorbic acid (Price et al. 1990; Mittler 2002; Del Rio et al. 2003). High activities of antioxidative enzymes are linked with oxidative stress tolerance in plants (Stajner et al. 1997). Enhanced activity of different antioxidative enzymes and greater accumulation of antioxidants have been reported in response to drought stress in PGPR-inoculated plants, thereby mitigating adverse effect of drought stress (Han and Lee 2005; Chakraborty et al. 2013).

Inoculation with PGPR strains under abiotic stresses also results in decreased antioxidative enzyme activities suggesting reduced stress impacts. Maize plants were inoculated with five drought-tolerant plant growth-promoting *Pseudomonas* species, namely, *P. entomophila*, *P. stutzeri*, *P. putida*, *P. syringae*, and *P. montelli*. When exposed to drought stress, inoculated plants exhibited significantly lower activity of antioxidative enzymes (Sandhya et al. 2010), indicating a lowering of stress in the inoculated plants. Reduced activity of the antioxidant enzymes APX and GPX in *Bacillus* spp.-inoculated maize plants indicated increased tolerance to drought stress (Vardharajula et al. 2011). Similarly, exposure of two barley cultivars to salt stress led to a considerable increase in the activities of catalase and peroxidase enzymes as compared to plants inoculated with *A. brasilense* (Omar et al. 2009).

4.3.5 Exopolysaccharide Production

The water potential of rhizospheric soil is a key parameter that determines the availability of water, oxygen, and nutrients to plants and microorganisms. Availability and retention of water by soil are determined to a very large extent by its physicochemical properties and structure. These properties are disturbed under drought and salinity stress, making soil unsuitable for growth of plants.

Bacteria produce exopolysaccharides (EPS) which play an important role in influencing soil structure. Production of these exopolysaccharides is reportedly enhanced under salinity and water stress. Microbial polysaccharides can bind soil particles to form micro- (<250 μm diameter) and macroaggregates (>250 μm diameter; Oades 1993). EPS play an important role in increasing the water-binding capacity of soil and help regulate supply of nutrients and water to roots (Roberson and Firestone 1992). EPS also help the microorganisms to irreversibly attach and colonize the roots due to involvement of a network of fibrillar material that permanently connects the bacteria to the root surface. Bashan et al. (2004) reported the role of polysaccharides producing *Azospirillum* in soil aggregation. The EPS released into soil as capsular and slime materials by soil microbes can be adsorbed by clay surfaces due to cation bridges, hydrogen bonding, van der Waals forces, and anion adsorption mechanisms, thus forming a protective capsule around soil aggregates (Tisdall and Oades 1982). Bacterial exopolysaccharides bind cations such as Na^+ and reduce their availability for uptake by plant and, thus, help alleviate salt stress in plants (Geddie and Sutherland 1993). Ashraf (2004) observed that inoculation with EPS-producing rhizobacteria reduced uptake of Na^+ by wheat plants and increased root and shoot biomass. Extracellular biofilm production by rhizobacteria is another strategy which helps in improving abiotic stress tolerance by binding and making availability of water molecules in plant rhizosphere (Timmusk and Nevo 2011).

4.3.6 Ion Homeostasis

Excessive accumulation of Na^+ and Cl^- can induce salt stress in plants, leading to toxicity and causing substantial damage to the plants. Soil biota has been reported to help maintain Na^+ homeostasis in inoculated plants exposed to salinity stress. In beneficial interactions, these microbes help regulate the expression/activity of plant root ion transporters or reduce their foliar accumulation or may even increase K^+/Na^+ ratios (Zuccarini and Okurowska 2008). Selective increase in P, K^+, and Ca^{2+} uptake has been reported in PGPR-inoculated maize and tomato plants exposed to salt stress, resulting in enhanced K^+/Na^+ ratios (Mayak et al. 2004b; Nadeem et al. 2007).

4.3.7 Nutrient Uptake

Adverse effects of abiotic stresses on plants are exacerbated by impaired mineral status. PGPRs help in nutrient acquisition by the plant through various mechanisms and thus help in reducing the negative impacts of environmental stresses. The uptake of phosphorus, an important plant nutrient, is affected in plants exposed to drought and saline stress. PGPR-mediated enhanced phosphorus uptake has been associated with the ability to solubilize P and increase its uptake (Gyaneshwar et al. 2002). PGPRs promote root development and alter root architecture by the production of phytohormones such as indole acetic acid (IAA) resulting in increased root surface area and numbers of root tips, thus providing new sites for nutrient uptake (Kloepper et al. 2007). PGPR-inoculated wheat plants given 75% of the recommended dose of fertilizers (RDF) gave yields equivalent to full RDF indicating supplementation of the nutrient requirement of the crop by PGPR (Shaharoona et al. 2008). Similar observations were also recorded in tomato plants (Hernandez and Chailloux 2004).

4.3.8 Other Mechanisms

PGPRs can modulate plant's physiological response to water deprivation and salinity stress. *A. thaliana* inoculated with *P. brassicacearum* strain STM196 showed changes in transpiration rate and also reproductive delay which improved plant's resistance to drought (Bresson et al. 2013). An increase in photosynthetic rate was observed on inoculation with *P. fluorescens* and *Azospirillum* in *Pinus halepensis* and rice, respectively (Rincon et al. 2008; Ruiz-Sanchez et al. 2011). Creus et al. (2004) reported that inoculation with *Azospirillum* resulted in increase in plant water content, relative water content (RWC), leaf water potential, and apoplastic water fraction in wheat exposed to water deficit. *Staphylococcus kloosii*- and *Kocuria erythromyxa*-inoculated radish plants showed improvement in photosynthetic pigment content and RWC and decreased electrolyte leakage on exposure to saline conditions (Yildirim et al. 2008). A volatile organic compound 2,3-butanediol produced by *Pseudomonas chlororaphis* induced stomatal closure in the inoculated plants and also imparted stress tolerance in the plants by interfering in ethylene, ABA, salicylic acid, and jasmonic acid signaling pathways (Cho et al. 2008). This volatile was found to induce nitric oxide (NO) production in the plant affecting plant's survival under drought stress (Cho et al. 2013).

4.4 Induction of Stress-Responsive Genes

Although not many reports are available on priming of stress-responsive plant genes, inoculation of PGPRs has been reported to modify plant response at gene level under stress conditions, since these are capable of eliciting drastic physiological changes that modulate the growth and development of the plant. ABA-dependent and ABA-independent pathways are known to mediate gene expression in plants during water stress. The ABA-dependent pathways are thought to mediate gene expression through an ABRE-element and b-ZIP transcription factors (Busk and Pages 1998), while the other pathway is through a MYC and MYB elements and related transcription factors (Yamaguchi-Shinozaki and Shinozaki 1993).

Environmental stresses increase the risk of cellular damage due to protein aggregation and denaturation. There is upregulation of heat shock proteins (HSPs), also known as chaperones such as GroEL, GroES, DnaJ, DnaK, ClpB, ClpA, ClpX, small heat shock proteins (sHSP), and proteases (Munchbach et al. 1999). These proteins are primarily involved in stabilization of new proteins and proper refolding of denatured proteins. Plant small heat shock proteins (sHSPs) are involved in preventing irreversible aggregation of denatured proteins and facilitating their refolding during stress (Sarkar et al. 2009).

4.5 Conclusions

Environmental stresses have an adverse impact on agricultural productivity. Plant growth-promoting rhizobacteria help increase abiotic stress tolerance of plants and reduce their negative effect on plant growth. The complex and dynamic interactions between the plants and PGPRs can lead to production of biomolecules, alter cellular physiology and hormonal homeostasis, or induce expression of stress-responsive genes. Use of PGPRs selected from stressed environments is a promising cost- and time-effective strategy for mitigation of abiotic stresses in crops. Based on the leads available, future efforts should be concentrated to evaluate the promising PGPRs under field conditions in a diverse range of crops.

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Chapter 5
Phosphate-Solubilizing Microorganisms in Sustainable Agriculture: Genetic Mechanism and Application

A. Pradhan, A. Pahari, S. Mohapatra, and Bibhuti Bhusan Mishra

Abstract Phosphorus (P) is the second important nutrient in terms of plant requirement and uptake. Though it is present in the soil in both organic and inorganic forms, its accessibility is constrained as it occurs mostly in insoluble forms. Additional requirement of P to satisfy nutritional requirements of the crop is usually supplemented as chemical P fertilizer. A number of soil microorganisms named phosphate-solubilizing microorganisms (PSMs) have been tested for solubilizing/mineralizing insoluble soil P, releasing in soluble form and making it available for plant uptake. PSMs are environment-friendly and deliver P to plants in a more sustainable manner. The present chapter focuses on the biochemical, molecular, and genetic mechanisms of P release by different PSMs. Phosphorus solubilization through diffusion of strong organic acids produced in the periplasm of the organism, into the adjacent soil environment, is one of the important mechanisms for P solubilization and is genetically controlled. The use of PSM is a promising approach to develop and fulfill P demand of the growing crop without causing any environmental hazard.

Keywords Phosphate-solubilizing microorganisms (PSMs) · P-solubilizing bacteria · Organic acid · Genetics of P solubilization · Biofertilizer

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5.1 Introduction

Organized agriculture involving domestication of plants and animals was developed around 12,000 years ago, as per the first historically verifiable revolution in agriculture. With the advent of civilization coupled with urbanization and industrialization, present agricultural techniques such as irrigation, crop rotation, and application of fertilizers were developed. However, exponential increase in population demanded an increase in agricultural produce from the available land. The global necessity to increase agricultural production from a steadily decreasing and degrading land resource base has placed considerable strain on the agroecosystems (Tilak et al. 2005). Many synthetic fertilizers containing acids radicals, like sulfuric and hydrochloride radicals, tend to increase the soil acidity, adversely affect soil health and population of beneficial organisms, and interfere with plant growth. Generally, healthy soil contains enough nitrogen-fixing bacteria to fix atmospheric nitrogen, which is an essential nutrient for the growing plants, but also reduce the healthy atmosphere of soil by decreasing the population of beneficial strains and interfere with proper growth of plant. Given the negative environmental impacts of chemical fertilizers and their increasing costs, plant growth-promoting rhizobacteria (PGPR) can help reduce chemical fertilizers application and, economically and environmentally, benefit through lower production cost to achieve sustainable agriculture and restore fertility of the soil.

Although all parts of the plant are colonized by microorganisms, the rhizosphere represents the main source of microbiome with plant-beneficial activities. In this context the use of PGPR is being considered as an alternative or a supplement for reducing the use of agrochemicals in agriculture (De Weger et al. 1995). PGPR promotes plant growth in two ways: direct and indirect (Glick 1995). The positive effect of many soil bacteria on plants is mediated by a range of mechanisms including improvement of mineral nutrition, enhancement of plant tolerance to biotic and abiotic stress, modification of root development, as well as suppression of soilborne diseases (Kloepper et al. 1989). The bacterial traits involved in these activities include nitrogen fixation, phosphate solubilization, iron sequestration, synthesis of phytohormones, modulation of plant ethylene levels, and control of phytopathogenic microorganisms (Bhardwaj et al. 2014) (Fig. 5.1).

Plant growth-promoting rhizobacteria can be classified into extracellular plant growth-promoting rhizobacteria (ePGPRs) and intracellular plant growth-promoting rhizobacteria (iPGPRs) (Viveros et al. 2010). The ePGPRs may exist in the rhizosphere, on the rhizoplane, or in the spaces between the cells of root cortex, while iPGPRs are located generally inside the specialized structures of root cells like nodules. Bacterial genera such as *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, and *Serratia* belong to ePGPR (Ahemad and Kibret 2014). The iPGPR belongs to the family of *Rhizobiaceae* and includes species of *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium*, endophytes, and *Frankia* species all of which can

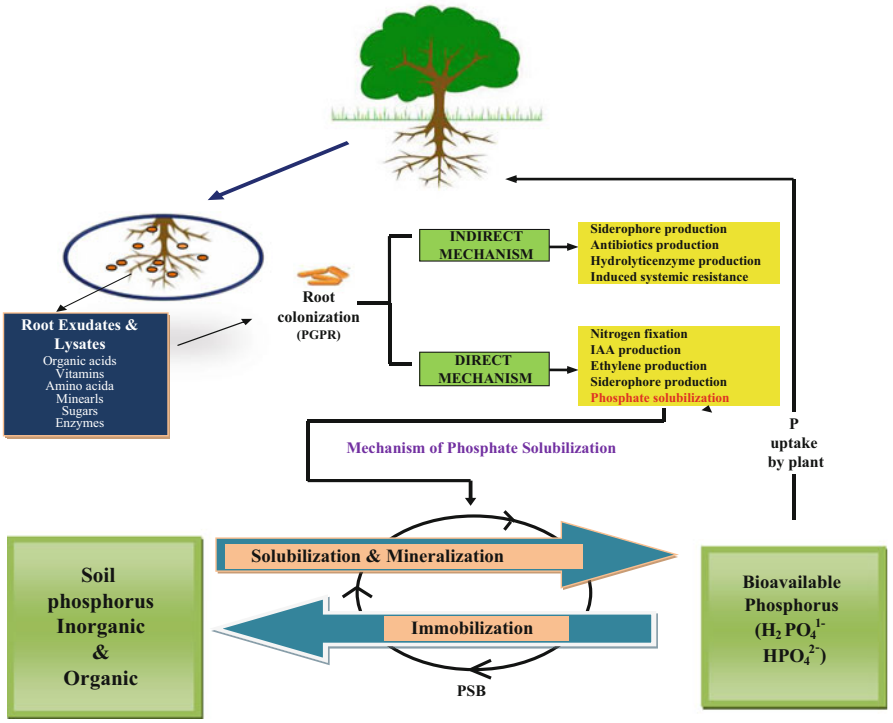

Fig. 5.1 Role of PGPR and mechanism of phosphate solubilization

symbiotically fix atmospheric nitrogen in association with the higher plants (Bhattacharyya and Jha 2012).

5.2 Demand and Supply of Phosphorus

It is mentioned that there is a shortage of phosphorus fertilizers, although phosphorus constitutes 0.1% of the lithosphere and the supplies are likely to outlast our species and possibly even the planet itself. The demand for phosphorus increased sharply in the mid-twentieth century with the success of the green revolution, when plant breeders successfully produced higher-yield versions of familiar field crops. Those higher yields required higher doses of fertilizers. The increase in the world's population, an expanding global affluence, and the resulting increased demand for more food, particularly meat, will only put more stress on the accessible supplies of phosphorus. Cordell et al. (2009) estimated that the amount of phosphorus that people consume in food is only one fifth that being mined, suggesting that huge amounts simply escape. For mined phosphorus, globally agriculture is by far the main user which accounts for between 80% and 90% of the total world demand (Childers et al. 2011).

5.2.1 Consumption of Phosphorus and Food Production

According to FAO data (2009), 60% of the global use of phosphate fertilizer is consumed by countries like China, India, and Europe. With 34% of world total, China is the largest consumer of phosphorus fertilizers in the world. India stands second with 19% of the global consumption (FAOSTAT 2012). Between 2002 and 2009, global use of P fertilizers increased by 12%. India mostly uses P fertilizers in agricultural fields and showed the largest increase in use to almost doubling in quantity between 2002 and 2009 (80% increase). Between 2002 and 2009, China also showed strong increases with 20% growth in P consumption. In contrast, Europe decreased use by 20% from 2002 to 2009 and reflecting increased environmental awareness (Tirado and Allsoapp 2012). According to FAO data, between 2012 and 2015, consumption of food in India was about 298.38 million tons, and this amount is expected to increase to 337.3 million tons by 2030. Hence, there is a need to increase production with a consequential increase in demand for the use of P fertilizers.

5.3 Forms of Phosphorus Present in Soil

P occurs in organic forms in most of the surface horizons of the soil. Among the soil P present in organic form, about 20 to 80% of P is available of which phytic acid (inositol hexaphosphate) is a major component (Richardson 1994). Phosphorus uptake by plants is limited to low availability of P in the bulk soil. The most soluble mineral such as potassium moves through the soil via bulk flow and diffusion, but P moves mainly by diffusion. According to Daniel et al. (1998), since the rate of diffusion of P is slow (10^{-12}–10^{-15} m^2 s^{-1}), high plant uptake rates create a zone around the root that is depleted of P. From the soil solution, plants obtain their P in the form of $H_2PO_4^-$ and HPO_4^{2-}, although HPO_4^{2-} uptake by plant appears to be slower than the uptake of $H_2PO_4^-$.

5.4 Availability of Phosphorus in Soil and Their Role in Plant Growth

The inorganic phosphate available for incorporation into biosynthetic purposes depends on not only the total amount of phosphorus in the environment but also its solubility, which in turn is accomplished by several chemical reactions and biological interactions in the soil. The diverse soil phosphorus (P) forms can be generally categorized as soil solution P, insoluble organic, and insoluble inorganic

P. In soil, the two reactions, fixation and immobilization, convert applied phosphorus into forms unavailable for the plant. More than 70–90% of the applied phosphatic fertilizers get fixed in the soil rendering them unavailable for plant uptake (Stevenson 1986). High percentages of soil P are also converted to organic forms of which inositol hexaphosphate is usually a major component and, thus, get immobilized and not available for plant uptake (Richardson 1994). The concept of conversion of inorganic unavailable phosphate into available forms, viz., $H_2PO_4^-$ and HPO_4^{2-} for plant uptake, is a phenomenon referred to as mineral phosphate solubilization (MPS). The form in which inorganic phosphate (Pi) exists also changes according to the soil pH. Below pH 6.0, most Pi will be present as monovalent $H_2PO_4^-$. The plant uptake is also high at the pH range of 5.0–6.0, which indicates that P is primarily taken up as monovalent form (Furihata et al. 1992). The average orthophosphate concentration in the soil solution of around 10^{-6} M is near the limit at which plants can absorb adequate phosphate.

Different intensive cultivation practices and irrigation have significantly disturbed soil nutrition balance. Phosphorus that may be available to plants is present in millimolar and micromolar amounts in soil (Anthony and Kloepper 2009). Phosphorus is present in soils at levels of 400–1200 mg/kg of soil (Begon et al. 1990). The quantity of P in the soil solution, even when at relatively high levels, is only in the range of 0.3–3.0 kg/ha (Ross 2013); a large fraction of this is in an insoluble form, and only $<10\%$ enters the plant animal cycle (Kucey et al. 1989). It has been estimated that at any point of time the dissolved/available form of P in many soils may only be from 0.01 to 0.06 ppm (0.02–1.33 kg P/hectare). The first systematic soil fertility map of India given in 1967 indicates that 4% samples are high in available P (Ramamurthy and Bajaj 1969). It is indicated that around 20% of soil samples are high in available P (Motsara 2002). The recently prepared GIS-based district-wise soil fertility maps of India (Muralidharudu et al. 2011) showed that soils of about 51% districts were low, 40% were medium, and 9% were high in available P. The high P status in some soils may be due to non-judicious use of P fertilizers by the farmers and its subsequent fixation and accumulation in the soil (Richardson 1994).

The phosphorus requirement of plants varies considerably. Tree crops have relatively low P requirements with the critical values ranging from 0.12% to 0.15%. Grasses have higher P requirements with critical values ranging from 0.20% to 0.25%. The P content of plants is initially high and declines with age, and since P is a fairly mobile element, deficiency generally occurs on older tissue. Phosphorus level in young plants can be very high, such as 0.50–1.00%. In some instances, high plant P levels may cause imbalances and deficiencies of other elements such as Zn, Cu, Fe, etc. Plant P content thus needs to be maintained within the sufficiency range by proper P fertilization and microbially mediated transformation in soil.

5.5 Biodiversity of P-Solubilizing Microorganisms

The use of P-solubilizing microorganisms as inoculants simultaneously increases P uptake by the plant and crop yield. Phosphate-solubilizing bacteria included in the genera *Arthrobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium*, and *Rhodococcus* are used as soil inoculants to improve plant growth and yield (Bhattacharyya and Jha 2012; Pradhan et al. 2014). However, the beneficial effects of the inoculation with P-solubilizing microorganisms used alone or in combination with other rhizospheric microbes have been also reported (Zaidi et al. 2009a, b). The microorganisms functioning similarly also include some fungi belonging to the genera *Penicillium*, *Aspergillus*, *Rhizopus*, *Fusarium*, and *Sclerotium* (Zhu et al. 2011).

5.6 Mechanism of P Solubilization

5.6.1 Inorganic P Solubilization

Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate [$(Ca_3PO_4)_2$], dicalcium phosphate ($CaHPO_4$), hydroxyapatite [$Ca_5(PO_4)_3(OH)$], and rock phosphate (Goldstein 1986). Soil microorganisms in this regard have generally been found to be more efficient in making P available to plants from both organic and inorganic sources by solubilizing (Toro 2007; Wani et al. 2007) and mineralizing complex P compounds (Bishop et al. 1994). Among the bacterial genera with this capacity are *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Enterobacter*, *Flavobacterium*, and *Erwinia*.

The main mechanism of phosphate solubilization is the action of organic acids produced by the soil bacteria (Halder et al. 1990), and these organic acids solubilize the insoluble phosphate by (1) lowering the pH, (2) enhancing chelation of the cations bound to P, (3) competing with P for adsorption sites on the soil, and/or (4) forming soluble complexes with metal ions associated with insoluble P (Ca, Al, Fe) and thereby releasing the P. Lowering pH of the medium suggests the release of organic acids by the P-solubilizing bacteria (Maliha et al. 2004) via the direct oxidation pathway that occurs on the outer face of the cytoplasmic membrane (Zaidi et al. 2009a, b). The quality of the acid is more important for phosphate solubilization than the total amount of acids produced by phosphate-solubilizing (PS) organisms (Scervino et al. 2010). These acids are the product of the microbial metabolism, mostly by oxidative respiration or by fermentation of organic sources (e.g., glucose) (Atlas and Bartha 1997), or such organic acids can either directly dissolve the mineral P as a result of anion exchange of

phosphate by acid anion or can chelate Fe, Al, and Ca ions associated with P (Omar 1998). Gluconic acid (GA) seems to be the most common agent for mineral P solubilization and is reported to be produced by P-solubilizing bacteria such as *Pseudomonas* sp. (Illmer and Schinner 1992), *Erwinia herbicola* (Liu et al. 1992), and *Pseudomonas cepacia* (Goldstein et al. 1993). Another organic acid identified in strains with phosphate-solubilizing ability is 2-ketogluconic acid, which is present in *Rhizobium leguminosarum* (Halder et al. 1990), *R. meliloti* (Halder and Chakrabartty 1993), and *Bacillus firmus* (Banik and Dey 1982). Strains of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* are found to produce mixtures of lactic, isovaleric, isobutyric, and acetic acids. Other organic acids, such as glycolic, oxalic, malonic, and succinic acid, have also been identified among P solubilizers (Banik and Dey 1982; Illmer and Schinner 1992) (Table 5.1). Chelating substances and inorganic acids such as sulfuric, nitric, and carbonic acid are considered as alternate mechanisms for P solubilization. However, the effectiveness and contribution to P release in soils seem to be less than organic acids.

Table 5.1 Organic acids produced by phosphate-solubilizing bacteria

Bacteria	Organic acids	References
Bacillus megaterium,	Lactic acid, malic acid	Taha et al. (1969)
Bacillus subtilis		
Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus atrophaeus, Paenibacillus macerans, Pseudomonas aeruginosa	Lactic acid, isovaleric acid, isobutyric acid, acetic acid	Vazquez et al. (2000)
Pseudomonas fluorescens	Citric acid, malic acid, tartaric acid, gluconic acid	Fankem et al. (2006)
Escherichia freundii	Lactic acid	Sperber (1958a, b)
Enterobacter sp. Fs-11	Malic acid, gluconic acid	Shahid et al. (2012)
Pseudomonas trivialis (BIHB 769)	Gluconic acid, 2-ketoglutaric acid, lactic acid, succinic acid, formic acid, malic acid	Vyas and Gulati (2009)
Arthrobacter sp. (CC-BC03)	Citric acid, lactic acid	Yi et al. (2008)
Pseudomonas striata	Malic acid, glyoxylic acid, succinic acid, fumaric acid, tartaric acid, α-ketobutyric acid	Gaur (1990)
Arthrobacter sp.	Oxalic acid, malonic acid	Banik and Dey (1982)
Bacillus firmus	2-Ketogluconic acid, succinic acid	Banik and Dey (1982)

5.6.2 Organic P Solubilization

A second major component of soil P is organic matter. Organic form of P may constitute 30–50% of the total P in most soils, although it may range from as low as 5% to as high as 95% (Paul and Clark 1988). Organic P compounds undergo mineralization, and the resulting P is taken up as nutrient by plants. Numerous soil microbes or rhizosphere microflora possess the ability to transform organic P into soluble forms of P (Rodriguez et al. 2006). Mineralization process is mediated by the enzymes especially phosphatases (Yadav and Tarafdar 2003) and phytases (Maougal et al. 2014), released by the soil microbes. The enzyme phosphatases (e.g., acid and alkaline phosphatases) released to the exterior of the cell (exo-enzymes) are nonspecific in nature and use organic P as a substrate to convert it into inorganic form (Beech et al. 2001). Of the two phosphatases, acid phosphatases are considered as the principal mechanism for mineralization of soil organic P (Hilda and Fraga 1999) where it catalyzes the release of inorganic P from organic P compounds such as inositol hexaphosphate (Nozawa et al. 1998). Another attractive application of P-dissolving enzymes is the mineralization of soil organic P through phytate degradation mediated by the enzyme phytase, which specifically releases P from phytic acid. Phytate is a major component of organic P in soil. Though the ability of plants to obtain P directly from phytate is very limited, the growth and phosphorus nutrition of *Arabidopsis* plants supplied with phytate was improved significantly when they were genetically transformed with the phytase gene (*phyA*) derived from *Aspergillus niger* (Richardson 2001). This led to an increase in P nutrition to such an extent that the growth and P content of the plant was equivalent to control plants supplied with inorganic phosphorus. Phosphonatases (Kumar et al. 2013) and C–P lyases (Salimpour et al. 2010) are the other enzymes that cleave the C–P of organophosphonates (Table 5.2). Once the inorganic or organic P compound is changed to soluble P, it can then easily be used up as P nutrient by plants, algae, cyanobacteria, and autotrophic bacteria and thereafter could be immobilized into organic cellular macromolecules, like DNA, RNA, and ATP. The microbial

Table 5.2 Enzymes produced by phosphate-solubilizing bacteria

Bacteria	Enzymes	References
Pseudomonas fluorescens	Acid phosphatase, phosphonoacetate hydrolase	Gügi et al. (1991)
		McGrath et al. (1995)
Enterobacter aerogenes	Acid phosphatase	Thaller et al. (1995)
Bacillus subtilis	Phytase	Richardson and Hadobas (1997)
Pseudomonas putida	Phytase	Richardson and Hadobas (1997)
Bacillus licheniformis	D-a-glycerophosphatase	Skrary and Cameron (1998)
Klebsiella aerogenes	C–P lyase	Ohtake et al. (1996)
Serratia marcescens	Acid phosphatase	Thaller et al. (1995)

mineralization of organic P is strongly influenced by environmental parameters, and moderate alkalinity favors the mineralization of organic phosphorus (Paul and Clark 1988). Considering the critical impact of such enzymes in dissolution of complex organic compounds into usable form of P, it is highly desirable to develop the bacterial/fungal inoculants with high phosphatase and phytase activity which in turn could possibly be of great practical value in sustainable crop production.

5.7 Genetics of Phosphate Solubilization

The mechanism of different P-solubilizing bacteria has shown that direct oxidation pathway provides the most effective P solubilization in Gram-negative bacteria via diffusion of the strong organic acids like gluconic acid produced in the periplasm, into the adjacent environment. Glucose is the precursor for synthesis of gluconic acid (Rodríguez and Fraga 1999). Goldstein (1996) proposed direct glucose oxidation to gluconic acid (GA) as a major mechanism for mineral phosphate solubilization. GA biosynthesis is carried out by the glucose dehydrogenase (GDH) enzyme and the cofactor, pyrroloquinoline quinone (PQQ) (Fig. 5.2). Among the best known examples are methanol dehydrogenase and glucose dehydrogenase. PQQ is an aromatic, tricyclic orthoquinone that serves as the redox cofactor for several bacterial dehydrogenases. This enzyme catalyzes the formation of gluconic acid from glucose by the direct oxidation pathway. PQQ is a prosthetic group required by several bacterial dehydrogenases, including methanol dehydrogenase (MDH) of Gram-negative methylotrophs and some glucose dehydrogenases. PQQ is derived from two amino acids, tyrosine and glutamic acid (Houck et al. 1991), but the pathway for its biosynthesis is unknown. Genes involved in PQQ synthesis have been cloned from *Acinetobacter calcoaceticus* (Goosen et al. 1989), *K. pneumoniae* (Meulenberg et al. 1992), and *M. extorquens* AM1 (Morris et al. 1994). In *A. calcoaceticus*, five *pqq* genes were identified and sequenced, designated IV, V, I, II, and III (Goosen et al. 1989). In *K. pneumoniae*, genes analogous to those were

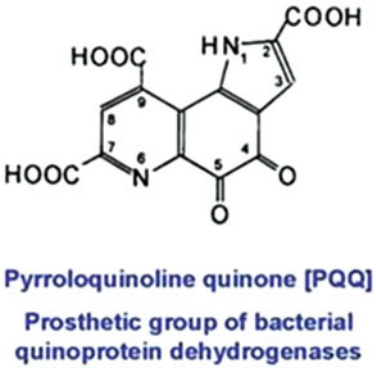

Fig. 5.2 PQQ (4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid) chemical structure (Source: http://www.ucc.ie/biomerit/simon%20image.gif)

identified and designated *pqqABCDE*, and in addition, a sixth gene was found immediately downstream of *pqqE*, designated *pqqF* (Meulenberg et al. 1990).

Following a similar strategy, a mineral phosphate solubilization gene from *Pseudomonas cepacia* was isolated (Babu-Khan et al. 1995). This gene (*gabY*), whose expression also allowed the induction of the mineral phosphate solubilization phenotype via gluconic acid production in *Escherichia coli* JM109, showed no apparent homology with the previously cloned PQQ synthetase gene (Liu et al. 1992), but it did with a permease system membrane protein. The *gabY* gene could play an alternative role in the expression and/or regulation of the direct oxidation pathway in *Pseudomonas cepacia*, thus acting as a functional mineral phosphate solubilization gene in vivo. Very little is known regarding the genetic regulation governing the mineral phosphate solubilization trait. In fact, the information about the genetic or biochemical mechanisms involved in the synthesis of the GDH–PQQ holoenzyme is scant, and variations between constitutive and inducible phenotypes are observed among several bacterial species (Goldstein 1996). Glucose, gluconate, mannitol, and glycerol are among the possible inducers of the holoenzyme activity (van Schie et al. 1987) (Fig. 5.3).

Different patterns of phosphatase activity are widespread in bacteria, particularly in those belonging to the family *Enterobacteriaceae*. The production of these enzymes is often controlled by complex regulatory mechanisms, so that the enzyme activity is detectable only under specific environmental conditions. The principal mechanism for the regulation of phosphatase production is the regulation by inorganic phosphate (Pi) concentration (i.e., phosphate-repressible phosphatases). This mechanism has been best studied in the alkaline phosphatase (gene *phoA*) of *E. coli* (Rosenberg 1987). The mechanism involves a Pi transport operon

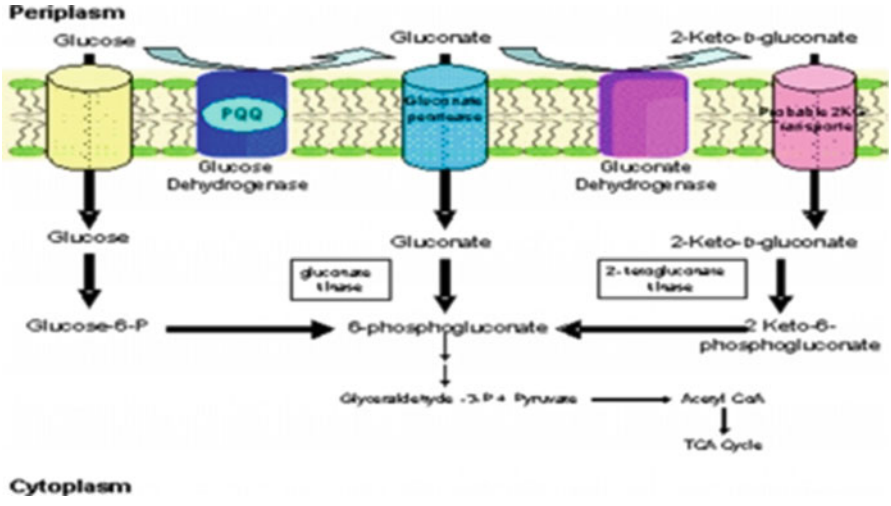

Fig. 5.3 Alternative extracellular oxidation pathway of glucose metabolism (Source: http://www.ucc.ie/biomerit/simon%20image.gif)

as a regulatory element, in addition to the sensor-activator operon. The genes controlled by Pi and activated by PhoB constitute the Pho regulon (Torriani-Gorini 1994; Santos-Beneit 2015). Hence, alkaline phosphatases found in the family *Enterobacteriaceae* are Pi-repressible, while many of the acid phosphatases are Pi-irrepressible. Other regulatory systems have been proposed for some bacterial phosphatases. In *Pseudomonas fluorescens* MF3, it was determined that the expression of the *apo* gene, which encodes an acidic phosphatase enzyme, was regulated by the growth temperature. Furthermore, the *apy* gene of *Shigella flexneri*, encoding an ATP diphosphohydrolase or apyrase, and other related alleles present in virulent *Shigella* spp. and enteroinvasive *E. coli* strains, is expressed in a thermoregulated manner (Bhargava et al. 1995).

5.8 P Solubilizers as Biofertilizers

Crop yields could be improved by enhancing the phosphate availability through the application of phosphatic fertilizers. However, the global energy crisis and dwindling resources have increased the cost of chemical fertilizers, and this trend is expected to continue. Increasing demand for food production without affecting the cost-benefit ratio is thus a challenging task ahead of scientists worldwide. Agronomists are therefore looking vigorously for an alternative source of phosphatic fertilizer to supplement, or to replace in some cases, the chemical fertilizers to ensure competitive yields of crops. So, an alternative to chemical phosphatic fertilizers is the exploitation of various microbial processes encompassed in the soil–root interface (rhizosphere). Microorganisms that colonize the rhizosphere are actively engaged in phosphorus transformation in soil and transport phosphate to the plants. Soil microorganisms play a vital role in accumulation of soil P to plants and their ability to solubilize and mineralize inorganic and organic soil P fractions (Adhya et al. 2015). The use of phosphate-solubilizing organisms in agronomic practices is advocated for several reasons. For example, they improve soil fertility through their sustained activities in the soil, increase plant growth and crop yield through increased nutrient availability, do not cause environmental pollution, improve soil heath and conditioning, protect plants against some soilborne pathogens, and involve low-cost technology for their production with a high cost-benefit ratio. For agronomic purposes, phosphorus is second only to nitrogen as the most limiting element for plant growth. Phosphorus promotes nitrogen fixation in legume crops and is essential for photosynthesis, energy, and sugar production (Saber et al. 2005). Microbial involvement in the solubilization of inorganic phosphate is well documented. Most of the studies on phosphate solubilization were, however, centered on the isolation of the microorganisms from the rhizospheric soil and then evaluation of their phosphate-solubilizing activity under in vitro conditions. The investigations into solubilization of phosphorus under field conditions and its uptake by plants were, however, started later. In this context, the beneficial effects of inoculation with

PSM for many crop plants have been described (Zaidi and Khan 2005). Nitrogen-fixing bacteria are, perhaps, the most promising group of PSM on account of their ability to fix nitrogen symbiotically (legumes) or asymbiotically (non-legumes), together with the ability of some strains to solubilize inorganic phosphatic compounds. Several reports have demonstrated that phosphate-solubilizing strains of *Rhizobium*, *Bradyrhizobium*, and *Azotobacter* increase growth and phosphorus content in both nonleguminous and leguminous plants. An alternative approach for the use of PSM as microbial inoculants is either the use of mixed cultures or co-inoculation with other microorganisms. Phosphate-solubilizing bacteria have already been applied as effective inoculants in agronomic practices to raise the crop productivity.

In saline–alkaline soil without causing any hazard to the environmental and population health, PSM technology improves the fertility of soil and helps in maintaining sustainable agriculture which ultimately lowers the continuous use of synthetic fertilizers (Elizabeth et al. 2017). Several scientists have demonstrated that in both non-leguminous and leguminous plants phosphate-solubilizing strains of *Rhizobium*, *Bradyrhizobium*, and *Azotobacter* increase growth and phosphorus content. In agricultural field, PSMs have applied effective inoculants in order to raise the crop productivity. For example, first commercial biofertilizer under the name "Phosphobacterin" was prepared using *Bacillus megaterium var. phosphaticum* in the former Soviet Union and later on was frequently applied in East European countries and India (Mohammad et al. 2007).

5.9 Use of Biotechnology to Develop More Potent P-Solubilizing Bacteria

Studies related to solubilization of insoluble P at molecular level brought out by PSM are inconclusive, and genetics knowledge of phosphate solubilization is still scanty (Rodriguez et al. 2006). However, some genes which are involved in mineral and organic phosphate solubilization have been isolated and characterized. Initially, manipulation of such genes achieved through genetic engineering and molecular biotechnology followed by expression in selected rhizobacterial strains opens a promising perspective for obtaining PSM strains with improved phosphate-solubilizing ability and, therefore, a more effective use of P-solubilizing bacteria as agricultural inoculants. Initially, cloning of gene involved in P solubilization from the Gram-negative bacteria *Erwinia herbicola* was achieved by Goldstein and Liu (1987). According to Fraga et al. (2001), the *napA* phosphatase gene of bacterium *Morganella morganii* isolated from soil was transferred to *Burkholderia cepacia IS-16*, using the broad-host range vector pRK293 and this strain was used as a biofertilizer. For healthy plants and greater performance, genetically modified PSMs have several advantages over transgenic plants: (1) It is far easier to modify a bacterium with current technologies, than complex higher organisms, (2) in a single

organism, several plant growth-promoting traits can be combined, and (3) when nonspecific genus like *Azospirillum* is used, a single, engineered inoculant can be used for several crops instead of engineering crops individually (Rodriguez et al. 2006). Molecular-based techniques offer new prospect to detect the abundance of specific microorganisms and for quantification of target gene expression with high levels of sensitivity in soil directly or in the rhizospheric region. For example, P-solubilizing microorganisms, including mycorrhizal fungi, *Penicillium* sp., and *Pseudomonas* sp. specific primers, have been described based on conserved regions (Oliveira et al. 2009). Microarrays provide further application for the estimation of diversity surrounding particular traits or functional groups of microorganisms (Richardson and Simpson 2011). Together, these tools deliver new opportunities in ecology of microbial community and assess the survival and perseverance of specific inoculants under diverse environmental conditions.

For more successful plant–microbe interaction, biotechnological and molecular approaches could possibly develop more knowledge about the mode of actions of PSM, and efforts should also be focused toward the use of PSM because biotechnology provides an excellent opportunity to reduce pesticide applications in agricultural field. In brief, development of PSM leads to environment-friendly phosphorus biofertilizer that can be used as supplements to chemical fertilizers.

5.10 Conclusion

The use of phosphatic fertilizer requires a greater input that cannot be afforded by the farmers of the developing nations. Microbiologists and soil scientists thus have a responsibility to society to find ways and means of making phosphorus available to crops, an economically efficient substitute for fertilization of crops. Though chemical fertilizers are not cost effective and most of the soils are deficient in plant-available phopsphorus, so there is an interest in using plant growth-promoting rhizobacteria (PGPRs) bestowed with phosphate-solubilizing ability as inoculants to mobilize phosphate from poorly available sources in soil. Although potential clearly exists for developing such inoculants, their widespread application remains limited by a poor understanding of microbial ecology and population dynamics in soil, and by inconsistent performance over a range of environments. Furthermore, promotion of growth of agronomically important plants, as a consequence of microbial inoculation, may not necessarily be associated with characteristics such as phosphate solubilization, which are manifest under laboratory conditions. Further, in order to ensure food security in developing countries, there is an urgent need for the sustainable intensification of agricultural production systems toward supporting productivity grains and income generation. In this context, novel, genetically modified soil and region-specific PSM and technologies for their ultimate transfer to the fields have to be developed, pilot-tested, and transferred to farmers in a relatively short time.

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Chapter 6
Arbuscular Mycorrhizal Fungi (AMF) for Sustainable Rice Production

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Abstract Arbuscular mycorrhizal fungi (AMF) exhibit multifunctional mutualistic symbiosis between plants and members of phylum *Glomeromycota*. This bipartite association improves uptake of water and nutrients such as phosphate, nitrogen, and micronutrients and also protects the plants from abiotic and biotic stresses. The trade-offs between plant colonization by AMF are controlled by physiological and/or genetic drivers in nature. The plant signal, different subsets of its genes, and a diffusible fungal signaling factor that triggers gene activation support the progress of AMF infection in successive root cell layers. The molecular understanding of AMF association in plants particularly in rice is very important to select an efficient and right species to reap the full beneficial effects from these fungi. The potential of AMF has been exploited vastly for most of the crop plants, but its role in sustaining rice cultivation is not much dissected, since there is a belief that these fungi may not work under lowland rice cultivation, which is one of the misconceptions. Rice is our staple food crop and demands huge amount of phosphorous (P), nitrogen (N), etc. In order to overcome the future P crisis and mitigate drought for sustainable rice cultivation, AMF can be appreciated as a "savior for rice" irrespective of different cultivations. In the above perspective, the present chapter discusses about the potentiality of AMF for rice cultivation and also confers the molecular insight and future perspective of this fungal association for sustainable rice production.

Keywords Rice · Arbuscular mycorrhizal fungi · Nutrient management · Molecular understanding

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6.1 Introduction

The word *mycorrhizae* is derived from two Greek words, *mycos* and *rhizos*, meaning fungus and root, respectively. Mycorrhizal association forms a very dynamic and beneficial relationship that exists between arbuscular mycorrhizal fungi (AMF) and roots of higher plants (vascular plants). More than 80% of terrestrial plants form association with mycorrhizal fungus except some plants belonging to non-mycorrhizal families like Amaranthaceae, Brassicaceae, Caryophyllaceae, Chenopodiaceae, Polygonaceae, and Urticaceae (Lambers and Teste 2013; Kumar et al. 2016). AMF belong to *Glomeromycota* phylum and require a host for completing its life cycle. Apart from its acknowledged role in P uptake by the host plant, uptake of other micronutrients like Cu, Zn, Al, Mn, Mg, and Fe has also been demonstrated (Kucey and Janzen 1987; Purakayastha and Chhonkar 2001; Hajiboland et al. 2009a; Panneerselvam et al. 2013). In addition, AMF play a vital role in wide range of functions from stress reduction to bioremediation in soils polluted with heavy metals (Hildebrandt et al. 2007). They are also involved in diverse functions such as soil health improvement, plant health improvement, and protection of plants against pathogen. Recent advances in "omics" sciences have allowed a deeper insight regarding AMF colonization and how it differs from symbiotic rhizobial association. AMF interaction with most of the terrestrial crops was found to be successful, and today it holds an integral part in farming system. AMF have potential to maintain profitability and sustainability of agricultural systems if managed properly. However, the potential of AMF interaction with monocots especially rice under different cultivation conditions/methods remains largely unexplored.

Application of AMF at nursery stage is found to increase rice yield by 14–21% (Solaiman and Hirata 1997). Secilia and Bagyaraj (1994b) reported that rice grain yield increased by 35–62% on inoculation with *Acaulospora* sp., *Glomus fasciculatum*, and *G. mosseae* in wetland rice. Recent evidences indicate that application of some herbicides like bispyribac sodium significantly enhances the mycorrhizal colonization of rice (Panneerselvam et al. 2016b). Knowledge on the interaction of rice plants with AMF will help develop AMF-based bio-inoculants for rice for efficient management of P and other nutrients under wide range of cultivation.

6.2 AMF in Plant Growth and Sustainability

6.2.1 AMF for Phosphorus Economy

Phosphorus is an essential nutrient for growth of living organisms for most of their cellular and physiological functions. Phosphorous is increasingly becoming a limiting nutrient, and by 2050, P crisis on a global scale is anticipated.

AMF-plant symbiotic relationship is very much beneficial for the growth and development of colonized host plants by facilitating a larger surface area for uptake of P via translocation through hyphae or mycelium to the host plant roots. It is well documented that AMF enhance P and other nutrient availability to plants (Panneerselvam et al. 2013; Sahoo et al. 2017), in particular inorganic (ortho) phosphate (Pi), by forming far-reaching extraradical mycelia that operate as functional extensions of the plant root system. In the absence of the symbiosis, Pi is taken up directly by plant roots in the form of orthophosphate which under diverse soil conditions remain bound to soil components, both inorganic and organic, thereby creating P-limiting conditions.

Soil phosphorous get solubilized by rhizosphere modification through the release of phosphatase enzyme, organic acids, and metabolites like siderophores (Shenoy and Kalagudi 2005). The greatest beneficial effect of AMF symbiosis has been related to P nutrition uptake and acquisition. Mycorrhizal plant can uptake P from poorly soluble P source, i.e., iron and aluminum phosphates and rock phosphate (Peterso and Massicotte 2004; Shenoy and Kalagudi 2005). The AMF association in host plants through indirect mechanism also cause changes in pH (Li and Christie 2001) and root exudation profile (Laheurte et al. 1990), thus altering the rhizosphere properties, to make it favorable for profuse development of microbial community and enhance P solubilization mechanism.

Sustainability of agricultural soil can be enhanced by introducing AMF in the field either through artificial inoculations or through AM-favoring crop and soil management practices (Panneerselvam and Saritha 2017; Sharma et al. 2012; Barea et al. 2005; Maiti et al. 2012). AMF association is a symbiotic one in which both partners are benefitted and facilitate plant uptake of water and nutrients from soil interphase and the fungus utilizes carbon provided by the plant for its growth, development, and other physiological functioning. AMF are not specific to its colonization but exhibit host preference in certain cases. Recent studies indicate that AMF diversity is being reduced by land use intensification (Oehl et al. 2003; Ciccolini et al. 2016). If this loss of fungal diversity pursues, it can impair plant productivity and ecosystem functioning. This shows that there exists a need to carry out research focused on increasing the benefits of AMF symbiosis in the field in order to manage P availability.

6.2.2 AMF in Disease Management

Management of diseases caused by root pathogen in crops has become one of the most concerned research areas in plant pathology. The concern about environment-related consequences of pesticide application has created interest in altering plant protection protocols. There exists a negative antagonistic interaction between AMF and various soilborne plant pathogens which is the reason why AMF are used as biocontrol agent. AMF play a vital role in reduction of plant pathogens (Saritha et al. 2015; Panneerselvam et al. 2006; Sukhada Mohandas and Panneerselvam

2016; Whipps 2004; St-Arnaud et al. 1994; Azcon-Aguilar and Barea 1997) such as *Rhizoctonia solani* (Yao et al. 2002), *Pythium ultimum*, and *Phytophthora* sp. (Trotta et al. 1996; Cordier et al. 1996). AMF have also been shown to reduce bacterial diseases in different crops (Dehne 1982). Generally, soilborne pathogens are controlled by using various agronomic and cultural practices including resistant cultivars, chemicals, soil fumigation, biocontrol agents, etc. However all these methods are associated with their own demerits such as residual effect of chemicals in soil that cause soil toxicity, lack of good sources of plant resistance (Azcon-Aguilar and Barea 1997), persistence of pathogen inoculum, etc. Hence there is a need to use alternate approaches that include manipulation or adding microorganism to enhance plant protection (Grosch et al. 2005). AMF compete with plant pathogen for nutrients and space by various means such as by producing secondary metabolites, by parasitizing pathogen, or by induced resistance in host (Berg et al. 2007). Various mechanisms have been demonstrated to explain biocontrol activity by AMF that includes biochemical changes, morphology of root system, stress alleviation, and anatomical changes (Hooker et al.1994). Several hypotheses have been proposed to elaborate the bio-protection mechanism by AMF to control various plant pathogens. Those are as follows: (i) higher competition for colonization sites and host photosynthates, (ii) anatomical changes in root system, (iii) modification of microbial community, (iv) plant nutrient uptake, (v) physiological and biochemical changes, and (vi) active plant defense mechanism.

6.2.3 AMF in Soil Health Improvement

Soil is an important component in any sustainable development. Interactions between plant and microbe that exist in the soil are primary determinants of plant health, soil health, and soil fertility. AMF act as the most important and beneficial microbial symbiosis for majority of plant species (Saritha et al. 2014). Along with other soil microorganisms, AMF present in soil regulate the biogeochemical cycling of organic and inorganic nutrients maintaining soil quality. AMF have the potential to influence economical benefits of agricultural systems via direct and indirect methods related to plant nutrition (Smith and Smith 2012), availability of moisture in water stress condition (Manoharan et al. 2010), building soil structure (Rillig and Mummey 2006), carbon sequestration in soil aggregates (Jastrow et al. 1998), and providing induced systemic resistance to crop plants (Azcon-Aguilar and Barea 1997).

In exchange of such useful support, plants provide the mycorrhizal fungus with photosynthetic carbon that is delivered to soil via fungal hyphae. In response to this, amount and activity of other soil biota are also stimulated. The main role of soil mycelium of AMF in the formation of water-stable aggregates is well documented (Andrade et al. 1998; Bethlenfalvay and Barea 1994; Miller and Jastrow 2000). The glomalin (i.e. glycoprotein) produced by AMF is deposited on the outer wall of hyphae of extraradical mycelium and adjacent soil particle, acts as a long-term soil-

binding agent (Wright and Upadhyaya 1998; Sathyarahini et al. 2015). As a result, extraradical hyphae together with fibrous root form a "sticky string bag" that causes soil particles' entanglement and enmeshment to form macroaggregates, a basic building block of soil structure and stabilization (Miller and Jastrow 2000).

6.2.4 *Production of Plant Growth-Regulating Substances by the AMF*

AMF are also involved in the production of growth hormones such as IAA, GA_3, cytokinin, and vitamins like vitamin B (Barea and Aguilar 1982). AMF take up hexose which is converted to trehalose and glycogen inside the mycelium. Trehalose and glycogen are carbon storage forms that may buffer the intracellular sugar concentration. Pentose is produced by oxidative pentose phosphate pathway which is used in the production of nucleic acid. Lipid biosynthesis also takes place in intraradical mycelium (Pfeffer et al. 1999). Allen et al. (1980) demonstrated that VA mycorrhizal infection increases the level of cytokinins in the host plant. The gibberellin- and cytokinin-like substances were also recorded in the culture filtrates of *G. mosseae* cultures (Barea and Azcón-Aguilar 1982).

6.2.5 *Role of AMF in Nutrient Management*

Mycorrhizal colonization of plant roots influences the phosphorus and nitrogen uptake of plant root along with certain micronutrients. Uptake of nutrients occurs through extraradical hyphae by absorbing nitrate, phosphate, and ammonium from soil. According to plant requirement for nutrient, AMF contribute much higher amount of plant phosphorus for uptake rather than plant nitrogen uptake. Arbuscules are the site where required material exchange occurs between fungus and the colonized plant. Vesicle serves as a storage organ. AM fungus has biochemical capabilities to increase supply of available phosphorus and other immobile nutrients. This capability may include excretion of chelating agents, root phosphatase activity, and rhizosphere acidification. But these mechanisms do not explain the effect of fungi on plant growth (Habte and Fox 1993).

6.2.6 *AMF in Water Management*

Moisture stress is a major limiting factor in crop production. Attaining maximum yield per drop of water (water productivity) is a major concern under limited water availability. In this regard, AMF play a key role in protecting plant against osmotic

stress by altering water movement in the host plants. AMF hyphae being very thin with a diameter of 2–5 nm can enter the soil pore which the root hairs cannot penetrate (10–20 nm diameter) and can absorb moisture inaccessible to plants (Allen 1982; Hardie 1985). The rate of water transport via extra radial hyphae to root can modify plant water relations (Allen 1991). Faber et al. (1991) estimated that the water transport rate ranges from 375 to 760 ml water per hour. Based on hyphal entry points/unit of root length, cross-sectional area of hyphae and water potential gradient of the rate of water uptake by extra radial hyphae were estimated (Filter 1985; George et al. 1992; Koide 1993). All these data in addition to other research studies show the positive effect of AMF on water uptake and plant growth. AMF perform water stress management by increasing dehydration avoidance and tolerance capabilities (Auge et al. 1987). In comparison to non-mycorrhizal plants, AMF-colonized plants avoid dehydration to a greater extent by increasing solute accumulation and alternately reducing bulk leaf symplastic and osmotic potential. AMF enhance root water uptake and provide adequate water to preserve plant physiological activity (Faber et al. 1991; Smith and Read 1997). In addition to this, it improves nutrient acquisition, conductance by stomata, photosynthesis, and proline accumulation. AMF colonization provides multifunctional effect on the host positively by improving growth, nutrient content, and root water uptake, thereby alleviating the stress condition (Abdul-Wasea and Elhindi 2010).

6.2.7 AMF for Salt Stress Management

Salinization of soil has a huge adverse impact on plant growth and yield and is increasing particularly in arid and semiarid areas (Giri et al. 2003; Al-Karaki 2006). Rainfall and rock weathering are the dominant sources of salt. Salinity affects growth, photosynthesis, protein synthesis, and plant metabolism that cause adverse effect on plant at different life stages. AMF alter the toxicity induced by salt stress. AMF are known to exist in saline environment due to improved mineral nutrition and different physiological processes like photosynthesis or water use efficiency, production of osmoregulators, higher K^+/Na^+ ratio, and bringing molecular changes through expression of gene (Ruiz-Lozano et al. 1996; Giri et al. 2003; Al-Karaki 2006: Evelin et al. 2009).

6.3 AMF for Sustainable Rice Production

Rice is the most important staple food crop of India. It feeds approximately more than 50% of the world's population. India has the largest area under rice in the world next to China. But the average productivity is very low. Rice alone contributes around 42% of the total food grain production and 45% of total cereal production in the country. In recent years, the importance of sustainable agriculture

has been realized to a great extent and became the most important issue in agriculture. The control of plant diseases and insects using pesticides and insecticides raises serious concern about food safety, pesticide resistance, environmental safety, and quality which has increased the need to find alternate methods of disease and pest management strategies. The effect of nutrients like N, P, K, Zn, B, Mn, etc. could affect the disease resistance or tolerance of plants to pathogens. Sustainable development in agriculture is a phenomenon that includes the management and utilization of agricultural system in order to maintain its productivity, regeneration capacity, biological diversity, vitality, and ability to function without harming other ecosystems.

With its multifaceted potential, AMF can be used as one of the tools for sustainable rice production. AMF play a vital role in nutrient management by providing rice with essential nutrient in its available form without extraneous application of fertilizers. Also AMF work both in flooded and non-flooded rice with improvement in production.

6.3.1 Role of AMF for Nutrient Management in Rice

Large amount of P fertilizers are used in rice fields as compared to other crop species. Generally rice is grown in different ecosystems, but 78% of the world's rice is grown under irrigated or rainfed lowland condition (Itao et al. 1999). Rice plants form mycorrhizal association rapidly under upland condition, but the infection is rare under submerged condition due to the prevalence of anoxic environment (Ilag et al. 1987). Mycorrhization of rice plant with *Glomus mosseae* causes significant mobilization of insoluble P from soil and their uptake by plants. AMF-inoculated rice plant through direct or indirect mechanism facilitates uptake of P from poorly soluble P (Panneerselvam et al. 2016a). These mechanisms involve change in pH (Li and Christie 2001) and root exudation pattern (Laheurte et al. 1990). In greenhouse experiment rice cultivar Pusa Basmati-1 grown in a Zn-deficient soil revealed that higher intensity of colonization in rice can be achieved when inoculated with *Glomus etunicatum* by raising seedlings in P- and Zn-deficient soil in nursery under aerobic conditions (Purakayastha and Chhonkar 2001). Good AM fungal root colonization was observed when same seedlings were transplanted into pots under waterlogged conditions.

Rice plants are generally found to be deficient in Zn in the arid and semiarid regions of India (Katyal and Vlek 1985). AMF can be used to enhance plant growth by improving the supply of plant nutrients which has low mobility in soil such as P, Zn, Cu, etc. (Abbott and Robson 1982; Harley and Smith 1983; Wellings et al. 1991; Ikram et al. 1992; Ho 1993; Tarafdar and Marschner 1994; Thompson 1996). Formation and activity of AMF can be affected by soil organic matter content because of saprophytic nature of AMF (Hepper and Warner 1983; Warner 1984) and alteration of various physical properties like water holding capacity and porosity of soil. This abovementioned AMF rice root association could increase

the availability of native and added Zn to rice. AMF absorb N, P, K, Ca, S, Fe, Cu, Mn, and Zn from soil and transfer these nutrients to the host plant (Table 6.1). The beneficial effect of mycorrhizal colonization in rice has been proven. When insoluble P was added to rice plants inoculated with either *G. mosseae* or *G. intraradius*, increase in P uptake was observed. The root colonization increased by 117% on AMF inoculation under flooded condition (Hajiboland et al. 2009b). Mycorrhizal colonization also had a major contribution in the uptake of P and K in plants (Hajiboland et al. 2009a). Even in the absence of P, shoot and root dry weight increased by 86–206%. Aerobic rice genotype inoculated with mycorrhizal inoculum showed 28–57% higher root colonization (Gao et al. 2007). In case of upland rice seedling, colonization with arbuscular mycorrhiza proved beneficial to achieve higher yields at optimum fertilizer dosage thereby decreasing the cost as well as environmental pollution. The effect of AMF on nutrient management is given in Table 6.1. The native flora of AMF of upland ecology has been found to be very efficient, and upland rice is generally responsive to AMF (Maiti et al. 2006). The effect of native AMF in rice cultivation is given in Table 6.2.

6.3.2 Role of AMF in Moisture Stress Management in Rice

About half of rice area in the world does not have sufficient amount of water to maintain flooded conditions, and hence yield is reduced to some extent by drought. Water stress at critical stages may result in considerable reduction in yield and crop failure. Hence there is a need for management of water for sustainable rice production. One possibility to maintain this sustainability is to increase plant water acquisition which can be achieved by inoculation of AMF symbiotically associated with plant roots and hence increasing crop productivity and plant growth under stressed conditions (Barea et al. 2005; Azcón and Barea 2010). Rice plants form mycorrhizal association readily under upland conditions, but very rarely infection occurs under submerged condition due to anoxic environment (Ilag et al. 1987). The rice cultivation under non-flooded condition and prevailing their aerobic environment in soil stimulates rice root colonization by AMF (Vallino et al. 2009; Ruíz-Sánchez et al. 2010).

Under well-watered condition, AMF rice exhibit high level of glutathione. But under drought stress condition, AMF rice has decreased level of glutathione. This decrease of glutathione content in AMF plant under drought condition enhances the lipid peroxidation (Ruíz-Sánchez et al. 2011). Also, inoculation with AMF itself increased ascorbic and proline as very effective protection compounds to cope with harmful effects of water limitation. Hence, AMF are effective for rice plants as an eco-friendly and acceptable technology to improve plant performance and development.

It has been reported that arbuscular mycorrhizal fungus *Glomus intraradices* enhanced growth response, photosynthetic efficiency, and antioxidative responses of rice plant toward drought stress (Ruíz-Sánchez et al. 2010). In addition, plant

Table 6.1 Effect of AMF on nutrient uptake in rice

Sl. no	Nature of experiment	Mycorrhizal species	Treatment condition	Effect of mycorrhizal inoculation on rice	General conclusion	References
1	Pot culture experiments	*Glomus mosseae* and *Glomus intraradices*	Flooded and non-flooded condition	Increased the uptake of phosphorus by 25–56% in rice plants	*Glomus mosseae* responded better in both flooded and non-flooded condition	Hajiboland et al. (2009b)
2	Field experiments	Indigenous AM fungal community	Flooded system and SRI	Phosphorus uptake was increased by 10–60% under SRI method of rice cultivation	P uptake was increased up to 120 days of crop growth	Watanarojanaporn et al. (2013)
3	Field experiments	Native VAM	Aerobic condition	Increased P uptake by 11–23%	P fertilizer recommendation can be reduced by 30% in AMF-inoculated crop rotation practices	Maiti et al. (2006, 2011, 2012) and Maiti (2015)
4	Pot culture and field experiments	Mixture of indigenous AMF (*Glomus* spp.)	Anaerobic condition	In shoots, N content increased by 12.25%; P, Zn, and Cu content decreased by 11%, 16%, and 14%, respectively, in VAM inoculation	N is highly mobilized in shoots compared to P, Zn, and Cu in VAM inoculation	Solaiman and Hirata (1997)
5	Pot culture experiments	*Glomus mosseae* and *Glomus intraradices*	Flooded condition	In P-deficient condition, P uptake of less efficient variety increased by 70% in mycorrhizal inoculation	Genotypic differences were present within rice varieties for P uptake in VAM inoculation	Hajiboland et al. (2009b)
6	Pot culture experiments	Indigenous AM fungi	Aerobic and anaerobic condition	VAM inoculation increased N content by 14%, no change in P content, and K content was decreased by 11.5% in shoots of rice	In general, N, P, and K uptake were higher in anaerobic condition than in aerobic condition under VAM inoculation	Solaiman and Hirata (1995)
				Similarly, in roots, N content was increased by 10%, K content was decreased by 9%, and no change in P change		

(continued)

Table 6.1 (continued)

Sl. no	Nature of experiment	Mycorrhizal species	Treatment condition	Effect of mycorrhizal inoculation on rice	General conclusion	References
7	Pot culture experiments	Inoculation of VAM *G. etunicatum*	Wetland condition	Increased the uptake of zinc from native or applied sources	Root biomass and yield were increased	Purakayastha and Chhonkar (2001)
8	Pot culture experiments	*Glomus mosseae* (BEG167) or *Glomus etunicatum* (BEG168)	Aerobic condition	Increased zinc uptake by 7–170% but genotype dependent and *G. etunicatum* showed better results	Less efficient Zn uptake genotypes responded more in mycorrhizal treatments	Gao et al. (2007)
9	Pot culture experiments	*Glomus mosseae* and *Glomus intraradices*	Flooded condition	In zinc-deficient condition, there were twofold increase in Zn uptake by efficient genotype and around 50% reduction in Zn uptake by inefficient genotype	Less efficient genotype showed reduced uptake in zinc-deficient condition	Hajiboland et al. (2009b)

Table 6.2 Effect of arbuscular mycorrhizal fungi (AMF) on rice cultivation

Sl. no	Nature of experiment	Mycorrhizal species	Treatment condition	Effect of mycorrhizal inoculation on rice	General conclusion	References
1	Pot culture experiments	18 different VAM fungi	Anaerobic condition	*Acaulospora* sp., *Glomus fasciculatum*, and *G. mosseae* were found to be efficient in wetland rice	Rice grain yield was increased from 35% to 62%	Secilia and Bagyaraj (1994b)
2	Pot culture and field experiments	Mixture of indigenous AMF (*Glomus* spp.)	Anaerobic condition	Nursery inoculation of VAM increased the yield of rice by 14–21%	Interestingly, grain yield and shoot biomass were not significantly influenced by AMF colonization under pot conditions	Solaiman and Hirata (1997)
3	Field experiments	Indigenous AM fungal community	Flooded system and SRI	*Glomus* and *Acaulospora* were dominating species in rice, and flooded rice had only *Glomus* sp.	Rice grown in SRI plots had more diverse AMF communities	Watanarojanaporn et al. (2013)
4	Field experiments	Indigenous AM fungi	High-input and intensively irrigated wetland	*Glomeraceae*, *Claroideoglomeraceae*, and *Paraglomeraceae* were dominating species	AMF significantly increased with growth stages of the rice crop in intensively irrigated rice systems	Wang et al. (2015)
5	Field experiments	Native VAM	Aerobic condition	Rice- and maize-based cropping system increased the infective propagules in rice	Cropping system of rice with maize increased the yield of the rice crop than sole rice crop	Maiti et al. (2006)
6	Field experiments	Native VAM	Aerobic condition	AMF inoculation in rice–maize-/horse gram-based crop rotation increased the phosphorus uptake by 11–22%	AMF inoculation had synergistic effect with crop rotation in rice and increased the rice yield by 25–34%	Maiti et al. (2011)

(continued)

Table 6.2 (continued)

Sl. no	Nature of experiment	Mycorrhizal species	Treatment condition	Effect of mycorrhizal inoculation on rice	General conclusion	References
7	Field experiments participatory mode with farmers	Indigenous AM fungal community	Aerobic condition	AM fungal colonization was maximum for maize–horse gram/rice rotation	Crop rotation increased the AM fungal colonization from 10% to 39% over control	Maiti et al. (2012)
8	Pot culture experiments	10 different VAM	Anaerobic condition	*Glomus intraradices* and *Acaulospora* sp. were considered efficient for inoculation into rice nurseries	AM fungi increased the yield of wetland rice	Secilia and Bagyaraj (1992)
9	Field experiments	*Glomus intraradices* and *G. fasciculatum*	Anaerobic condition	*Glomus intraradices* increased rice yield by 11% and *G. fasciculatum* increased yield by 8%	*G. intraradices* inoculation can reduce the amount of phosphate fertilizer applied to rice by 50% without yield reduction	Secilia and Bagyaraj (1994b)
10	Pot culture experiments	Indigenous AM fungi	Aerobic and anaerobic condition	Aerobic condition showed the highest mycorrhizal colonization, but 2–12% colonization level was also observed in flooded conditions at 60 days after transplanting	AMF accelerates the N and P transfer from soil and/or shoots to rice grains in aerobic and anaerobic rice cultivation system and increases the harvest index	Solaiman and Hirata (1995)
11	Pot culture experiments	Indigenous fungi (*Glomus mosseae*, *G. microcarpum*, *G. fasciculatum*, *G. caledonium*), and nonindigenous fungus (*G. etunicatum*)	Aerobic condition, high and low P content	Indigenous VAM fungi showed higher growth than nonindigenous VAM fungi in rice	VAM fungal colonization in rice is variety dependent, and indigenous VAM produces better results in both low and high P levels	Dhillion (1992)

growth can be efficiently increased by co-inoculation with PGPR and AMF due to synergistic effects on plant growth, particularly under growth-limiting conditions (Azcón and Barea 2010). Also, it has been reported that when rice plant is inoculated with AMF and *Azospirillum*, growth responses were significant. Hence, all these results demonstrate the importance of mycorrhization of rice plant both under well-watered and under drought stress conditions. The effect of AMF in abiotic stress management is given in Tables 6.3 and 6.4.

6.3.3 *Response of Rice Crop to Co-inoculation of Blue-Green Algae (BGA) and AMF*

Rice productivity can be improved by using chemical nitrogen fertilizer, but this has negative impact on the environment, as substantial amount of the fertilizer is lost through different mechanisms causing environmental pollution. This problem can be overcome by employing biological N_2 fixation (BNF) that can decrease dependence on application of fertilizer N, thereby reducing the environmental risks (Abbott et al. 1984; Bruce et al. 1994; Smith and Read 1997). Mycorrhizal fungi maintain the whole-plant nutrient balance (Abbott et al. 1984; Bruce et al. 1994; Smith and Read 1997). Use of an additional biofertilizer like BGA can replace use of chemical fertilizers, reducing the cost of crop production and restoring soil fertility status. Inoculation with BGA along with fertilizer N enhances plant N content (at booting stage), effective tillers, panicle weight, and plant height of rice crop (Yanni et al. 1984). The highest yield/fed (1 fed = 4200 m^2) was found in rice with application of 20 kg N + 10 kg *Nostoc commune* or 40 kg N + 5 kg *N. commune* (Ashoub et al. 1993). AMF can enhance plant growth even under low-fertility conditions, and the plant response was found sensitive to O_2 and CO_2 level in soil (Abbott et al. 1984; Bruce et al. 1994; Smith and Read 1997). Pot culture experiment revealed an increase in grain and straw yield of wetland rice and increase P and Zn content (Abbott et al. 1984; Bruce et al. 1994; Smith and Read 1997) in plants inoculated with AMF. Combined application of BGA+AMF+N fertilizer showed positive effects such as improvement in soil structure, increased grain yield ha^{-1}, straw yield ha^{-1}, enhanced nutrient availability, and alkali soil reclamation. The combined effect of biological N_2 fixation by BGA and production of plant growth regulators by AMF resulted in increased grain and straw yield ha^{-1} (Bagyaraj and Menge 1978; Kerni 1991; Hassouna and Aboul-Nasr 1992).

6.3.4 *Effect of AMF Inoculation in Dry and Wet Nursery*

The growth, establishment, and development of AMF are affected by several factors (Abbott et al. 1984; Bruce et al. 1994; Smith and Read 1997). It has been

Table 6.3 Effect of AMF on salinity and drought stress tolerance in rice

Sl. no	Nature of experiment	Mycorrhizal species	Treatment condition	Effect of mycorrhizal inoculation on rice	General conclusion	References
1	Pot culture experiments	*C. etunicatum* (EEZ 163)	Pots were watered regularly	Inoculated rice plants showed highest shoot biomass under salinity conditions Ratio of shoot Na^+ to root Na^+ was lower in VAM-inoculated plants	AMF symbiosis favors Na^+ extrusion from the cytoplasm and sequestration into the vacuoles, unloading of Na^+ from the xylem and its recirculation from photosynthetic organs to roots	Porcel et al. (2016)
2	Pot culture experiments	*C. etunicatum* (isolate EEZ 163)	Pots were watered regularly	Inoculated rice plants showed increased quantum yield of photosystem II under salinity	AMF rice plants had a higher photochemical efficiency for CO_2 fixation and solar energy utilization under salinity	Porcel et al. (2015)
3	Pot culture experiments	*Glomus intraradices*	Pots were watered regularly and withhold for drought stress	Shoot fresh weight increased up to 50% under drought stress in VAM inoculation compared to control	VAM inoculation reduced the oxidative stress to rice plants and increased the photosynthetic efficiency under drought stress	Ruíz-Sánchez et al. (2010)
4	Pot and field conditions	Native VAM fungi	Anaerobic condition	Increase in AMF spore density and infection decreased the sheath blight disease in rice	Organic amendments like green leaf manure increased the VAM colonization	Baby and Manibhushanrao (1996)

Table 6.4 Effect of AMF on heavy metal toxicity in rice

Sl. no	Nature of experiment	Mycorrhizal species	Treatment condition	Effect of mycorrhizal inoculation on rice	General conclusion	References
1	Pot culture experiments	*Glomus versiforme* (GV), *Glomus mosseae* (GM), and *Glomus diaphanum* (GD)	Aerobic condition (70% field capacity)	Inoculation resulted in protective effects against combined toxicity of Cd, Cu, Pb, and Zn	Mycorrhizal inoculation effectively partitioned the heavy metals only in roots; *Glomus mosseae* showed the highest tolerance among these isolates	Zhang et al. (2006)
2	Pot culture and hydroponics experiments	*G. mosseae*	Anaerobic condition	Inoculation resulted in accumulation of Cu in the cell wall of roots than in symplasm	Mycorrhizal inoculation altered the Cu transport between the apoplast and symplast and reduced the toxicity inside the cells	Zhang et al. (2009)
3	Pot culture experiments	*G. intraradices* (E31V)	Anaerobic and aerobic (80% field capacity)	Inoculation resulted in reduced ratio of As(V)/As(III) in shoots and increased ratio of As(V)/As (III) in roots	In low arsenic concentration, uptake were significantly reduced, and in high arsenic condition, uptake were reduced	Li et al. (2011)
4	Pot culture experiments	*Glomus geosporum* (Gg), *G. mosseae* (Gm), and *G. versiforme* (Gv)	Aerobic condition	Combination of Gg and Gm increased total P uptake and decreased the total As uptake in husk and rice grains	There was no significant correlations observed between AMF colonization rates and As uptake	Chan et al. (2013)
5	Pot culture experiments	*Glomus geosporum*, *G. versiforme*, and *G. mosseae*	Aerobic condition	In arsenic-rich soil, mycorrhizal inoculation increased the grain yield without increasing arsenic in the grain	Mycorrhizal inoculation significantly decreased As concentrations in husk, straw, and root in high arsenic soils (70 mg kg^{-1})	Wu et al. (2015)
6	Pot culture experiments	*G. mosseae*	Aerobic condition	In Cu-rich (0–200 mg kg^{-1}) and Pb-rich (0, 300, and 600 mg kg^{-1}) soils, mycorrhizal inoculation significantly increased the root biomass but increased the Pb content in shoots	There is an interaction between the Cu and Pb in mycorrhizal treatments, and it promotes the Pb partitioning in shoots and Cu in roots	Lin et al. (2014)

(continued)

Table 6.4 (continued)

Sl. no	Nature of experiment	Mycorrhizal species	Treatment condition	Effect of mycorrhizal inoculation on rice	General conclusion	References
7	Pot culture and hydroponics experiments	*Rhizophagus intraradices* (RI) and *Funneliformis mosseae* (FM)	Aerobic condition	FM reduced more Cd in rice shoots and roots compared to RI, and Cd were converted into less toxic form and stored in vacuoles of cells	AMF significantly decreased the Cd concentrations both in shoots and roots of rice	Li et al. (2016)
8	Pot culture experiments	*Glomus mosseae*	Aerobic condition	Chlorothalonil decreased the plant biomass, and the decreases were smaller in mycorrhizal inoculation	Chlorothalonil affected physiological processes in upland rice irrespective of inoculation	Zhang et al. (2006)

reported that there is an increasing trend in yield and nutrient acquisition in wetland rice by inoculation with AMF in both high- and low-fertility soils (Secilia and Bagyaraj 1992; Gupta and Ali 1993; Secilia and Bagyaraj 1994a; Solaiman and Hirata 1996). It has been observed that nursery inoculation technique influences AM fungal colonization and its colonization in rice root was significantly higher in dry nursery (DN) seedling than wet nursery (WN) seedling at all growth stages (Solaiman and Hirata 1996). The colonization level decreased when rice seedling reaches to maturity stage in DN. However, 28.4% colonization was observed for DN plots in comparison with WN (24.6%). DN seedlings recorded higher sporulation than WN seedling at all growth stages of rice crop under high soil fertility condition (Solaiman and Hirata 1998). Sporulation increased up to 70 days after transplanting, decreased gradually, and became low at harvest stage. Tillering significantly increased sporulation at all growth stages up to harvest, but root dry weight increased in early tillering, decreased gradually at panicle initiation stage, and remained completely unaffected at harvest stage following AMF inoculation. At harvest, grain N and P content in addition to shoot N content increased significantly when inoculated with AMF irrespective of nursery inoculated technique. Significant increase in root P uptake was observed in both DN and WN seedling. Under pot culture condition, the rice root colonization by *G. mosseae* reported to have increased P uptake in roots as compared to shoot at seedling stage (Gangopadhyay and Das 1984). It can be concluded that there is a significant increase in grain and root P nutrition at the harvest stage with decrease in shoot, contributing to a significant increase in total P uptake by plants. P translocation from root and shoot was accelerated to grain.

6.4 Molecular Understanding of AMF Colonization

The biochemistry behind AMF colonization is very well regulated and controlled at genetic level. The plant systems including both monocotyledons and dicotyledons form symbiotic associations with AMF. The morphology of colonization is similar except for a marked difference that exists in the organization of their root systems. Genomics, transcriptomics, and metabolomics reveal that the interaction between plants and fungal symbionts is characterized by a balanced nutritional exchange. A successful AMF colonization is strictly controlled by the plant. The receptor kinase of hypernodulation and aberrant root formation 1 (*HAR1*) gene, which controls the number of root nodules in legumes, also plays a role in the regulation of AMF symbiosis. This is evident by the report of Solaiman et al. (2000) that the *HAR1* mutants have higher fungal colonization rates than the wild-type plants. On the contrary, *ENOD40* overexpression leads to enhanced arbuscule formation (Staehelin et al. 2001). The mode of action of *HAR1* gene is still not well understood.

There exists a common pathway termed as the Sym pathway which is shared between AMF and rhizobial symbioses. The Sym pathway is constituted by seven

proteins, namely, a receptor-like kinase encoded by the gene SymRK/DMI2 (Endre et al. 2002; Stracke et al. 2002), nuclear porins encoded by *NUP85* and *NUP133* (Kanamori et al. 2006; Saito et al. 2007), putative cation channels encoded by two highly homologous genes *CASTOR* and *POLLUX*/*DMI1* (Ane et al. 2004; Imaizumi-Anraku et al. 2005), a calcium-/calmodulin-dependent kinase encoded by *CCAMK*/ *DMI3* (Levy et al. 2004; Mitra et al. 2004), and a protein encoded by the gene *CYCLOPS* (IPD3) (Chen et al. 2008; Parniske 2008). The genetic codes of *SymRK*, *CASTOR*, *POLLUX, NUP85*, and *NUP133* are mandate for Ca^{2+} spiking, an early response of root hairs to Nod factor application and to approaching AMF (Kosuta et al. 2008). The role of calcium-/calmodulin-dependent kinase *CCAMK*/ *DMI3* is to decipher the calcium signals (*DMI3*) as reported by Levy et al. (2004), Mitra et al. (2004), and Kosuta et al. (2008). *CYCLOPS* (*IPD3*) interacts with CCAMK and serves as a phosphorylation substrate for *CCAMK* (Messinese et al. 2007; Parniske 2008), which is primarily required for mycorrhizal colonization (Chen et al. 2008).

Though few workers clearly conclude about the existence of common Sym pathway, the temporal expression analysis of rice roots colonized by *Glomus intraradices* demarcated early- and late-induced genes. The expression of gene *AM11* is induced early and it relies on an intact Sym pathway. Interestingly the late-induced gene *AM14* depends on intact Ca^{2+} response and thus requires the involvement of *CASTOR*, *POLLUX*, and *CCAMK*, but it is purely independent of *CYCLOPS*. Similarly the expression of *PT11* gene is dependent on arbuscule formation. The dependence of genes *AM10* and *AM15* on Sym pathway signaling or on arbuscule formation is not yet understood. The early induced genes *AM1* and *AM2* do not require functional *CCAMK*, *POLLUX*, *CASTOR*, or *CYCLOPS* demonstrating the existence of an alternative AM signaling pathway (AP). AMF signaling pathway studies in rice opened new concepts. The rice mycorrhiza-specific phosphate transporter *PT11* (Paszkowski et al. 2002) served as a marker for arbuscule formation. In situ hybridization studies confirmed specific expression in arbusculated cells, with a specific role in P uptake at the periarbuscular interface within the root system (Harrison et al. 2002; Javot et al. 2007).

6.4.1 AMF-Specific Phosphate Transporters

In AMF symbiosis, the fungal hyphae penetrate through the intercellular spaces of the root and subsequently invade the inner cortical cells, forming structures called arbuscules within the cells (Parniske 2004; Harrison 2005). As many such arbuscule forms, the plant cell envelops it in a membrane, the periarbuscular membrane, and eventually results in an extensive plant–fungal interface specialized for nutrient exchange (Smith and Smith 1990). Phosphate (Pi) and carbon transfer occurs at the arbuscule/cortical cell interface. Pi is translocated through the AM fungal hyphae as polyphosphate (polyP), and after hydrolysis, in the arbuscule, Pi is exported from the AM fungus to the periarbuscular space (Kojima and Saito 2004; Javot et al.

2007). The import of Pi across the periarbuscular membrane, into the root cell, is then mediated by plant phosphate transporters.

So far, two classes of Pi transporters are implicated in Pi transport in AMF symbiosis: (a) mycorrhiza-induced Pi transporters of *Pht*1, subfamily III, and (b) - mycorrhiza-specific Pi transporters of *Pht*1, subfamily I. For example, members of subfamily III, such as *StPT*3 of potato, *Pht*1;6 of maize, and *LjPT3* of *Lotus japonicus*, are expressed in roots but show increased expression in symbiosis (Maeda et al. 2006). The other mycorrhiza-inducible genes encoding phosphate transporters in plants include *MtPHT1;4* in *Medicago* (Harrison et al. 2002); *StPHT1*;3, *StPHT*1;4, and *StPHT1*;5 in potato (Nagy et al. 2005); *OsPHT*1;11 and *OsPHT*1;13 in rice (Glassop et al. 2007); *SlPHT*1;3, *SlPHT*1;4, and *SlPHT*1;5 in tomato (XU et al. 2007); *GmPHT*1;7, *GmPHT*1;10, and *GmPHT*1;11 in soybean (Tamura et al. 2012); *BdPHT1*;3, *BdPHT1*;7, *BdPHT*1;12, and *BdPHT1*;13 in *Brachypodium distachyon* (Hong et al. 2012); *AsPHT*1;1 in *Astragalus sinicus* (Xie et al. 2013); and *ZmPHT*1;6 in *Zea mays* (Willmann et al. 2013).

In addition to *Pht* genes, the expression of two half-size ABC transporters, *STR1* and *STR2*, is triggered during AMF symbiosis, and these transporters are directly involved in arbuscule development (Gutjahr et al. 2012).

6.4.2 Relationship Between Strigolactone Biosynthesis and Mycorrhization

Certain biomolecules like strigolactones (SLs) and sugars also play a dominant role in AMF symbiosis which differentiates it from *Rhizobium* symbiosis. According to Akiyama et al. (2005), SLs are branching factors which led to a breakthrough in understanding the molecular mechanisms of the precontact stage of AMF symbiosis. SLs also act as plant hormones to regulate shoot branching (Domagalska and Leyser 2011) and are dispensable for arbuscule development (Gutjahr et al. 2012). Fungi release signaling molecules, in the form of "Myc" factors that trigger symbiotic root responses. Plant genes required for AMF development have been characterized. As per Parniske (2008), the genetic program for AMF has been recruited for other plant root symbioses during evolution. Moreover the functional adaptation of a plant receptor kinase that is essential for AMF symbiosis helps the nitrogen-fixing bacteria to form intracellular symbioses with plant cells.

6.4.3 A Versatile Monosaccharide Transporter in AMF Symbiosis

The AMF plants are profited from AMF association by the fact that mycorrhizal plants can rely exclusively on the symbiotic route for phosphate acquisition (Liu

et al. 1998; Smith et al. 2003). In turn the plant transfers carbohydrates to the fungus growing within the root cortex. The carbon transfer from the plant to the fungus was first demonstrated by Ho and Trappe (1973), who showed that ^{14}C accumulates in AMF spores derived from plants exposed to $^{14}CO_2$.

A typical MST2 transporter for the functioning of AMF symbiosis is highlighted. The inactivation of MST2 gene leads to fungal growth arrest. In MST2-silenced roots, arbuscule development is severely impaired in addition to an overall reduced degree of mycorrhization. This is due to the lower expression of periarbuscular membrane-located phosphate transporter PT4. Hence there exists a correlation between phosphate transporters and monosaccharide transporters. But phosphate is an important factor in the regulation of the transcriptional activation of MST2 in planta, and Xyl per se might be a signal that is able to induce MST2 expression.

The fungus within the root cortex itself induces a signal that increases the carbon sink. It promotes the availability of xylose and Xyl and then triggers MST2 expression: shortly after MST2 induction, PT4 activation occurs and subsequently arbuscule formation occurs in inner cortical cells. On the other hand, if the root is provided with sufficient phosphate, PT4 and MST2 expression were downregulated. It is well established that high phosphate negatively regulates not only PT4 expression (Breuillin et al. 2010) but also carbon partitioning to the root (Olsson et al. 2010). These conclude that high phosphate concentration in the soil might act by reducing the level of Xyl that induces MST2. Hence there exists a tight regulation of the exchange of phosphate for carbon that is mainly modulated at the arbuscular interface.

6.5 Future Thrust

- In general, most of the works in AMF are confined to specific genus, i.e., *Glomus*, *Gigaspora*, and *Acaulospora*, though ten different genera have been described so far. Hence, the other genus/species of AMF with beneficial role in rice need to be explored.
- The scientific understanding of AMF association in rice has different schools of thoughts. Some groups reveal that the performance of AMF is better in aerobic and uplands than submerged rice, but few findings report that some species of AMF are working excellently under submerged condition in rice; this needs further in-depth investigation.
- Some research findings revealed that the AMF performance is species specific meaning some species have host preference; if it's so there is a need to identify suitable AMF species specifically for rice under different ecological conditions.
- We should develop simple molecular markers to track the performance of AMF at different stages of rice plant growth.
- Clear understanding is needed on Pi transporters gene expression in relation to the association/colonization of different species of AMF in rice.

- The role of AMF and its mechanisms on other nutrients' mobilization, viz., N, K, Zn, and Fe, from soil to plants should be proven through molecular approaches.
- Additional attention should be paid toward biotic and abiotic stress management through intervention of AMF in rice.
- The mode of application and effective dose of AMF for rice and other field crops need to be re-looked and optimized.
- A simple AMF inoculum production technique with highly reproducible method of assessing quality AMF inocula and mode of delivery should be optimized for agricultural crops including rice.

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Chapter 7
Biological Nitrogen Fixation in Cereals Crops: A Bacterial Perspective

S. Garcha and P. K. Maan

Abstract Nitrogen (N_2) is one of the essential requirements of all living forms. The crop requirement is generally met by cheaply available chemical fertilizers. Endophytic bacteria of cereal crops as rice, wheat, etc. have the natural ability to fix atmospheric nitrogen. A few of them have established a mutually beneficial association with the plants. This chapter lists commonly encountered endophytic microbes of rice and wheat and the major enzymes catalyzing N_2 fixation, an energy intensive reaction. Nitrogenase enzyme is sensitive to the presence of oxygen. Microbial cells have devised a method to lower oxygen concentration for optimal performance of the enzyme. Further, genetic control of nitrogen fixation is explained mentioning the genes and their respective functions. Quality assurance and longer shelf life of such biological products can go a long way in consolidating market share of biofertilizers. Engineering plant microbe communication can facilitate manipulation for greater efficacy of nitrogen fixation.

Keywords Biological nitrogen fixation · Cereal crops · Associative nitrogen fixation · Nitrogen-fixing enzymes · Genetics of nitrogen fixation · Bio-inoculants

7.1 Introduction

Nitrogen (N) plays a crucial role in growth of all living forms. It oscillates between organic and inorganic forms. This change is brought about by phenomena of biological nitrogen fixation (BNF) carried out by microorganisms – free-living in the soil or present in associative or symbiotic relationship with the plants. These include *Rhizobium*, *Azorhizobium*, *Azotobacter*, *Azospirillum*, etc. Nonsymbiotic nitrogen-fixing bacteria that live in the rhizosphere (Dobereiner 1997) and/or endophytically (Hecht-Buchholz 1998) often increase yields of cereals and other

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crops. Free-living N-fixing bacteria play an important role in plant development on account of nitrogen fixation and supply of growth activators (Ahmed and Kibret 2014). BNF thus plays an important role in maintaining soil fertility (Vance and Graham 1995).

Wheat and rice along with maize are the cereal crops that are the mainstay of global food security. Unlike legumes, cereals do not have any close association with N_2-fixing bacteria. Hence these crops cannot be grown with reliance on BNF alone and are mostly dependent on chemical N fertilizer for their N needs. *Azotobacter*, a free-living nitrogen-fixing bacterium (Martinez-Toledo 1985) which is used as a biofertilizer in the cultivation of many cereal crops, fixes annually about 60–90 kg N/ha and may be used in crop production as a substitute for a part of mineral N fertilizer. Among the field crops, wheat and sugar beet are most responsive to N nutrition (Bogdanovic et al. 2005). Inoculation with *Azotobacter* replaced up to 50% of urea-N for wheat grown in a greenhouse trial under aseptic conditions, and the effect of inoculation with *Azotobacter* varies depending on the species and strain of N-fixing bacteria, physicochemical soil properties, N fertilizers applied, climatic conditions, and wheat cultivar (Milosevic and Jarak 2005). The increase is construed as a result of BNF, as well as the production of antibacterial and antifungal compounds, growth regulators, and siderophores (Pandey and Kumar 1989).

Azotobacter and *Azospirillum* were found to fix nitrogen in cereals and increase yield up to 27% in wheat (Khandan and Namvar 2013) and in the process they fixed 15–20 Kg N/ha. Cyanobacteria in rice field fixed N_2 in the range of 20–30 Kg N/ha (Singh 2014). Inoculation of rice with *Azospirillum lipoferum* increased yield up to 6.7 g/plant, nearly 1.8 t/ha (Balandreau 2002). *Azospirillum brasilense* and *A. lipoferum* contributed 7–12% of wheat plant N by BNF as was evidenced by N^{15} tracer studies (Malik et al. 2002).

7.2 Associative Nitrogen-Fixing Bacteria in Wheat and Rice: Diversity and Phylogeny

The N_2 requirement of cereals is well established and the N_2 requirement is met by application of chemical fertilizers. Unfortunately, intensive use of chemical fertilizers leads to eutrophication, leaching, pollution of groundwater, alteration of soil organic matter, etc. It also has adverse effect on soil microflora and fauna (Mano and Morisaki 2008). The need can be met at least partially by application of beneficial bacteria. Endophytic bacteria associated with cereals and grasses include *Achromobacter*, *Acetobacter*, *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Azomonas*, *Bacillus*, *Beijerinckia*, *Clostridium*, *Campylobacter*, *Corynebacterium*, *Derxia*, *Desulfovibrio*, *Enterobacter*, *Erwinia*, *Herbaspirillum*, *Klebsiella*, *Lignobacter*, *Mycobacterium*, *Methylosinus*, *Pseudomonas*, *Rhodospirillum*, *Rhodopseudomonas*, and *Xanthobacter* (Wani 1990). The various species isolated and identified from rice and wheat are summarized in Table 7.1.

Table 7.1 Endophytic species identified in rice and wheat

Crop	Endophyte	References
Rice	*Herbaspirillum seropedicae, H. frisingeuse, H. rubrisubalbicans*	Elbeltagy et al. (2000)
	Klebsiella pneumonia	
	Bacillus pumilus, Bacillus subtilis, Bacillus polymyxa	
	Azospirillum brasilense, Azospirillum lipoferum, A. amazonense, A. oryzae	Salamone et al. (2010), Elbeltagy et al. (2000), Vargas et al. (2012), Baldani et al. (1986), and Xie and Yokota (2005)
	Azorhizobium	
	Rhizobium leguminosarum	Yanni et al. (1997) and Mirza et al. (2001)
	Bradyrhizobium japonicum	
	Pseudomonas	Salamone et al. (2012)
	Burkholderia	Singh et al. (2006) and Vargas et al. (2012)
	Clostridium	Choudhury and Kennedy (2004)
	Azotobacter	Choudhury and Kennedy (2004)
	Serratia marcescens	
Wheat	*Azospirillum brasilense,*	Anastasia et al. (2011)
	Azospirillum zeae	Kennedy et al. (1997)
	Azotobacter vinelandii	Aly et al. (2012)
	Pseudomonas stutzeri	Anastasia et al. (2011)
	Klebsiella pneumoniae 342	Iniguez et al. (2004)
	Mycobacterium	Conn and Franco (2004)

Benefit of inoculation with diazotrophic bacteria *Burkholderia kururiensis* M130 and *Azospirillum brasilense* sp245 was observed to be influenced by plant and bacterial genotypes (Vargas et al. 2012; Malarvizhi and Ladha 1999; Shrestha and Ladha 1996). Early plant responses are believed to involve ethylene signaling (Vargas et al. 2012), and ethylene reponse (ER) pathway is demonstrated to participate in the early stages of the establishment of the association between Poaceae and beneficial diazotrophic bacteria. Different bacteria trigger different patterns of ethylene receptor function in various rice genotypes. Inoculation of rice cultivar IR 42 with *Azospirillum brasilense* sp245 resulted in an increase in the level of four ERs and also 143% increase in number of lateral roots. However, its response to inoculation with *B. kururiensis* M130 was different.

Members of Poaceae family, to which rice and wheat belong, have beneficial interaction with the genera of *Azospirillum*, *Burkholderia*, *Gluconacetobacter*, and *Herbaspirillum* (Suarez-Moreno et al. 2012). They colonize rhizospheric soils and surfaces of their hosts and are also endophytic in roots, intercellular spaces, and vascular tissue. However, they do not form any specialized symbiotic structures as nodules (James 2000). Rice is widely studied as model system in plant-microbe ecology (Hardoim et al. 2008). Wheat has been found to select specific subpopulation of *Bacillus polymyxa* from rhizosphere soil (Mavingui et al. 1992). Soil type and soil bacteria largely determine the nature of endophytic population in wheat

(Conn and Franco 2004). *Klebsiella* sp strain Kp342 has been found to fix nitrogen in wheat (Iniguez et al. 2004). It was shown to aggregate at lateral root junctions of wheat by Dong et al. (2003).

Use of bacterial bio-inoculant preparations in rice, containing *Azotobacter*, *Clostridium*, *Azospirillum*, *Herbaspirillum*, and *Burkholderia*, is well documented. They are believed to possess the ability to assimilate soil N and also exhibit PGPR traits (Biswas et al. 2000; Yanni et al. 1997). *Azotobacter* is an aerobic free-living heterotrophic nitrogen fixer. It has been reported to increase yields 7–20% and fix 11–15 Kg N_2/ha in cereals by Yanni and El-Fattah (1999). *Clostridium* sp., an anaerobic heterotroph capable of fixing nitrogen in the absence of oxygen in wetland rice (Elbadry et al. 1999), acts like that of *Azotobacter*. Population of *Clostridium* increases upon application of rice straw in fields (Kanungo et al. 1997), and application of rice straw is reported to stimulate N_2 fixation by *Clostridium*, up to 5–10 mg N/g of carbon consumed.

Azospirillum is a heterotrophic N_2-fixing bacterium (Roper and Ladha 1995). It can grow both in the rhizosphere soil of graminaceous crops and also intracellularly in the root (Baldani and Döbereiner 1980). *Azospirillum brasilense* sp245 and *Burkholderia kururiensis* M130 have demonstrated endophytic capability also (Suarez-Moreno et al. 2012; Baldani et al. 2000). *Azospirillum brasilense* and *A. lipoferum* have been isolated from the roots and stems of rice (Ladha et al. 1982). Another species, *A. amazonense*, has also been isolated from rice roots (Pereira et al. 1988). However, 85% of *Azospirillum* isolates from rice rhizosphere belong to *A. lipoferum* (Ladha et al. 1987) which results into 32–81% increase in yield under greenhouse (Malik et al. 2002) and around 22% increase under field conditions (Balandreau 2002). There is increase in the height and tiller number and also increased uptake of PO_4^{-3} and NH^{+4} by rice plants (Murty and Ladha 1988). *Azospirillum* is also reported to have biocontrol attributes as reduction in bacterial leaf blight was reported by Islam and Bora (1998). Amount of N fixed by *A. lipoferum* and *A. brasilense* was quantified by Mirza et al. (2000) using N^{15} isotope, and results demonstrate the ability of *Azospirillum* to meet 19–47% of N requirement of basmati and super basmati rice. Colonization of wheat by *Azospirillum brasilense* was demonstrated by Webster et al. (1998). Naringenin and other flavonoids stimulated colonization by *A. brasilense* (Jain and Gupta 2003).

Herbaspirillum is an endophytic diazotroph reported to colonize many plants including cereals like rice, maize, sugarcane, sorghum, etc. *H. seropedicae* was first isolated from Brazil colonizing rice roots (Baldani et al. 1986). In addition to fixing 31–54% of total rice plant Ndfa (N derived from the atmosphere), it also increased shoot and root length, grain yield and grain weight (Baldani et al. 2000), seed germination (Pereira et al. 1988), and root and shoot dry weight (James et al. 2002). Yield increase of 44–90% under greenhouse condition was reported in super basmati rice inoculated with *Herbaspirillum* spp. by Mirza et al. (2000). Basmati and super basmati recorded % Ndfa of 19.5–38.7 and 38.1–58.2, respectively. However, the amount of N_2 fixed by *Herbaspirillum* is reported to vary with rice variety (Gyaneshwar et al. 2002).

Few species of *Burkholderia* are capable of fixing nitrogen including *B. vietnamiensis*, *B. kururiensis*, *B. tubernum*, and *B. phynatum* (Vandamme et al. 2002; Estrada-de Los Santos et al. 2001). *Burkholderia kururiensis* M130 have demonstrated endophytic capability (Suarez-Moreno et al. 2012; Baldani et al. 2000). *B. vietnamiensis* when used in field trials of rice increased grain yield by 13–22% and reduced N fertilizer application by 25–30 Kg N/ha (Tran Van et al. 2000). Under gnotobiotic conditions, these spp. are reported to fix 19% of rice plant Ndfa. Other endophytic spp. of *Burkholderia* fixed 31% of rice plant Ndfa and increased rice plant biomass by 69% (Baldani et al. 2000). However, *B. cepacia* is reported to be of human health risk (Balandreau 2002), and *B. glumae* is known to cause grain and seed rot of rice (Nakata 2002).

Well-known legume symbiont *Rhizobium* influences growth physiology and root morphology of the rice plant. *R. leguminosarum* bv. *trifolii* has the ability to colonize rice (Yanni et al. 1997). It has been demonstrated to biologically fix nitrogen, increase shoot and root growth and grain yield, and decrease chemical N fertilizer usage (Biswas et al. 2000; Yanni et al. 1997). Inoculation of *Rhizobium* in cereals is accompanied by increased presence of phenolic acids like gallic, tannic, ferulic, and cinnamic acids in leaves (Mirza et al. 2001). *Azorhizobium caulinodans* and *Azorhizobium brasilense* enter rice through lateral root cracks and establish in intercellular space within the cortical cell layer of roots. Nodulation in nonlegumes like cereals has been reported by Al-Mallah et al. (1990). It involves facilitating the entry of rhizobia by enzymatically treating the roots.

Cultivated rice varities have exhibited higher N_2-fixing bacteria. Genotypes of the rice plants effect association between rice plants and N_2-fixing bacteria (Malarvizhi and Ladha 1999; Shrestha and Ladha 1996; Ladha et al. 1986, 1987). Magnitude of variation is attributed to soil N_2 status. A rice genotype IR42 is reported to have high BNF trait (Wu et al. 1995). Rice plants have demonstrated ability to utilize biologically fixed N_2 in soils having low fertility compared to soils having high fertility.

7.3 Types of Nitrogen-Fixing Enzymes: MO-, V-, Fe-Containing Types

7.3.1 Nitrogenase

Nitrogenase is a complex enzyme system that fixes N_2. It is very sensitive to oxygen. In legume nodules, the protection against oxygen is provided by leghemoglobin, whereas in many cyanobacteria it is provided by a special structure called the heterocyst. Free-living diazotrophs, e.g., *Cyanobacteria* and *Azotobacteraceae*, and symbiotic diazotrophs, e.g., *Rhizobia* and *Frankia*, are

among the organisms that synthesize nitrogenase (Herrero and Flores 2008). Nitrogenase catalyzes the conversion of N_2 to NH^{4+} through this reaction:

$$2N_2 + 8^{e-} + 10H^+ + nMgATP \rightarrow 2NH_4^+ + H_2 + nMgADP + nP_i \quad (n \geq 16)$$

Nitrogenase enzyme consists of two major protein components – dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein). Both of them contain iron (Fe), while dinitrogenase contains molybdenum (Mo) in addition. A cofactor called MoFe-cofactor or MoFe-co is bonded to iron (Fe) and molybdenum (Mo) present in dinitrogenase. MoFe protein is a 230 kDa $\alpha_2ß_2$ tetramer. MoFe protein tetramer contains two pairs of metalloclusters, i.e., two molydenum-iron-sulfur-homocitrate clusters (FeMo-co) and two [Fe_8S_7] clusters (P-cluster) each. The P clusters consist of two iron-sulfur partial cubanes. The P-clusters are located at the interface of α and ß subunits. FeMo cofactor (FeMo-co) contains two components ([Fe_4S_3] and [$MoFe_3S_3$]) bridged by three sulfide bonds. Homocitrate is bonded to the Mo atom. FeMo-co is the actual site of nitrogen reduction. It is enclosed by the three domains of the α subunit. P clusters act as intermediates in the electron transport pathway (Peters et al. 1997; Howard and Rees 1996).

Dinitrogenase (MoFe protein; molecular mass 220,000) is joined with one or two molecules of dinitrogenase reductase (Fe protein; molecular weight 64,000). Fe protein (dinitrogenase reductase) has four iron atoms and is rapidly and irreversibly inactivated by O_2. Fe protein is 64 kDa. A single, regular [Fe_4S_4] cubane is symmetrically coordinated between the subunits by Cys97 and Cys132 from each subunit. This [Fe_4S_4] cluster is the redox-active center involved with electron transfer to MoFe protein. The [Fe_4S_4] cluster of Fe protein oscillates between the reduced and the oxidized state during electron transfer (Merrick 1992). Each Fe protein dimer can bind to two nucleotide molecules (Burgess and Lowe 1996). Binding of Mg-ATP at these sites results in a conformational change in Fe protein. The two subunits rotate toward each other, extruding the [Fe_4S_4] cluster toward the protein surface by 4°A which is an important step in the catalytic cycle of nitrogenase (Schindelin et al. 1997).

The redox potential of the $[Fe_4S_4]^{2+/1+}$ couple is changed from −300 mV to nearly −450 mV by binding of Mg-ATP to reduced Fe protein. An associated Mg-ATP-induced conformational change promotes interaction of Fe protein with MoFe protein. A second conformational change in the Fe protein changes the redox potential of the Fe_4S_4 cluster by another −200 mV. Transfer of single electron from the Fe protein to MoFe protein is now energetically possible. This electron transfer is coupled with the hydrolysis of Mg-ATP (bound to Fe protein) to Mg-ADP and Pi. The nitrogenase complex dissociates in the rate-limiting step of the cycle, after electron transfer and Mg-ATP hydrolysis. Fe protein is then reduced by a low potential electron donor like ferredoxin or flavodoxin in vivo. Mg-ADP is exchanged for Mg-ATP. The catalytic cycle continues till required numbers of electrons have been transferred to completely reduce the FeMo-cobound substrate. Fe protein is the obligate electron donor for MoFe protein in all characterized nitrogenase systems (Burgess and Lowe 1996).

Nitrogenase enzyme functions under anaerobic conditions, i.e., in the absence of oxygen. This character is attributed to the low redox potential required and high reactivity of this enzyme to oxygen. It has a complex oxygen protection system in place. It is evident from the X-ray crystallographic structures for the Fe protein (Schlessman et al. 1998) and MoFe protein (Peters et al. 1997) as well as for two complexes between the two proteins (Rees et al. 1998; Schindelin et al. 1997).

Nitrogenase activity is determined by many factors like temperature, pH, available soil moisture, and presence of oxygen. Nitrogenase activity in wet soils is higher than that observed in soils having moderate to low level of moisture. Also, high levels of available nitrogen are inhibitory to nitrogen fixation. The available nitrogen content of wet, marshy soils is low due to denitrification and leaching of nitrates. As expected, greater rate of acetylene reduction is observed in marshy soils in which crops like rice are grown, compared to soils where other cereal crops are grown. The decreased combined nitrogen in the soil enhances nitrogen fixation (Postgate 1998).

7.3.2 Alternative Nitrogenase

Under certain growth conditions, some nitrogen-fixing bacteria synthesize nitrogenases that contain vanadium (and iron) or only iron in the place of molybdenum. These non-molybdenum nitrogenases are called alternative nitrogenase. Alternative nitrogenases are not synthesized if molybdenum is present in sufficient quantity as the molybdenum nitrogenase is normally the main nitrogenase in the bacterial cell. Alternative nitrogenases serve as a backup mechanism to ensure that fixation of nitrogen can still take place even if molybdenum is not available. Vanadium nitrogenase (VNase) was first described by Bishop et al. (1980) in *Azotobacter vinelandii* and later it was isolated from *A. chroococcum*.

VNase consists of an iron protein, a homodimer with a total molecular mass of $M_r = 64\ 000$, and an iron-heterometal (FeV) protein of $M_r = 240\ 000$ (Fallik and Robson 1990). A [4Fe–4S] ferredoxin join the two subunits of the iron protein. It has two binding sites for Mg^{2+} ATP. VFe protein has an $\alpha_2ß_2\delta_2$ subunit structure. It has two P clusters located at the interface of the α and ß subunits and two M clusters which are located in the α subunits. The M cluster FeVco is the site for substrate activation. The metal clusters of he VFe protein are analogous to those in the Mo nitrogenase (Chen et al. 1993).

Protein residues required in binding the M and P clusters are conserved as discovered by amino acid sequencing of α and ß subunits of the Mo and V nitrogenases (Fallik and Robson 1990). Close homology of structure as well as function of the clusters has been elucidated by spectroscopic and extrusion studies (Eady 1996; Chen et al. 1993). The P clusters are double cubanes of the composition $[\{Fe_4S_3(Cys)_2\}_2(\mu\text{-}S)_2(\mu\text{-}Cys)_2]$ in the reduced form. V is the component of a complex Fe-S system. It is combined to three iron centers by binding three times to sulfide. It is then bonded to histidine and adjacent alkoxide and carboxylate of homocitrate.

Another type of VNase has been discovered in *A. vinelandii* where α subunit is not present. This nitrogenase has only one FeVco. Also, half of one of the P clusters is missing. The remaining fragment is bonded to a (4Fe–4S) ferredoxin. EPR and redox properties of the α ß$_2$ variant of the VNase are also different (Tittsworth and Hales 1996). Few cyanobacteria and *Anabaena variabilis* have these two alternative nitrogenases (Thiel et al. 1998). One of them is V-dependent nitrogenase. It is encoded by *vnfH*, *vnfDG*, *vnfK*, *vnfE*, and *vnfN* and functions only in the absence of Mo (Lyons and Thiel 1995). The *vnf* genes are expressed only in resistant heterocysts form. Their expression and also that of Mo-dependent *nif1* genes are controlled similarly. The *vnf* genes do not require vanadium for transcription. They are repressed by Mo. The *vnf* genes are constitutively expressed in cells grown in absence of fixed N_2 in Mo lacking mutants. They fix N_2 using V nitrogenase.

7.3.3 Hydrogenase

Many diazotrophs evolve dihydrogen (H_2) during N_2 fixation, which in turn inhibits the N_2 fixation reaction. This inhibitory action of H_2 is overcome by the hydrogenase enzyme which recycles H_2 produced by nitrogenase enzyme in nitrogen-fixing system.

Physiological H_2 uptake has been demonstrated to be similar in *A.vinelandii* and *A. chroococcum*. H_2 is produced when N_2 is reduced to NH^{+4}. It is reoxidized to H^+ by a Ni-dependent hydrogenase. This enzyme is encoded by the 16-gene *hup* cluster in *A. chroococcum* and *hox*/*hyp* cluster in *A. vinelandii* (Enon et al. 1992). Hydrogenase activity is beneficial in *A. chroococcum*. Hup^+ strain MCD-1 has greater ability to survive in controlled, carbon-limited conditions than Hup^- mutant (Yates and Campbell 1989). Nearly equal amounts of protein were produced in chemostat cultures of Hox^+ and Hox^- strains of *A. vinelandii*. Both had similar respiratory activites. Some amount of total respiratory activity was due to H_2 dependent O_2 consumption in the wild type. While fixing N_2 in carbon-limited growth conditions, the hydrogenase enzyme does not prove useful in *A. vinelandii*.

Two types of NiFe hydrogenases are present in cyanobacteria. Genes of these two hydrogenases have been characterized in *Anabaena* sp. PCC 7120, *A. variabilis* and *Nostoc* sp. PCC 73102 (Happe et al. 2000). One type of hydrogenase is an uptake hydrogenase. It has a large subunit encoded by *hupL* and a small subunit encoded by *hupS*. The uptake hydrogenase acts after the start of N_2 fixation (Happe et al. 2000). It is found in the thylakoid membranes of heterocysts. It utilizes the H_2 produced by nitrogenase for energy generation. Uptake hydrogenase lacking mutants, *hupSL* in *A. variabilis* and *hupL* in *Anabaena* sp. PCC 7120, generate greater amount of H_2 after N_2 fixation has begun than the wild-type strains.

The second type of hydrogenase is a bidirectional $NAD(P)^+$-reducing hydrogenase. It can lead to the formation as well as uptake of H_2. Dihydrolipoamide:NAD oxidoreductase component of this enzyme, also known as diaphorase, is encoded by *hox(E)FU*. The hydrogenase component is encoded by *hoxHY*. Nitrogen-fixing

cyanobacteria, e.g., *Anabaena* sp. PCC 7120 and *A. variabilis* possess this enzyme (Axelsson and Lindblad 2002). It is not present in all unicellular cyanobacteria. Whenever present in filamentous cyanobacteria, it can be found in both vegetative cells as well as heterocysts.

7.4 Genetic Basis of Nitrogen Fixation and Its Regulation

Genetical, biochemical, and physiological studies done on *Klebsiella pneumoniae* N_2 fixation system have revealed a number of basic concepts that are common to many diazotrophs (Burris and Roberts 1993; Dean and Jacobson 1992; Merrick 1992). Nitrogen fixation has also been studied extensively in various other diazotrophs such as *Azotobacter* spp. photosynthetic bacteria (Roberts and Ludden 1992), cyanobacteria (Haselkorn and Buikema 1992), *Azospirillum* spp. (Elmerich et al. 1992), Rhizobia (de Philip et al. 1992), and methanogenic bacteria (Lobo and Zinder 1992). Genes involved in nitrogen fixation are summarized in Table 7.2.

nod genes are those genes which direct specific nodulation events in a legume by a strain of *Rhizobium* that along with specificity genes (genes restricting a *Rhizobium* strain to a particular host plant) are borne on large plasmids called "Sym" plasmids. The *nod* gene products are required for the early steps in nodule formation. Ten *nod* genes have been identified in this species, namely, *nod M*, *nod L*, *nod E*, *nod F*, *nod D*, *nod A*, *nod B*, *nod C*, *nod I*, and *nod J*. The *nod ABC* genes are responsible for the synthesis of oligosaccharides, called nod factors, which induce root hair curling and trigger root cell division eventually resulting in the nodule formation. Nod factors contain a backbone of N-acetylglucosamine to which different substituents are linked. Host specificity is determined by the precise structure of the nod factor of a given *Rhizobium* spp. They often show variations in the structural components of their nod factors. However, *nod ABC* genes direct the synthesis of nod factor backbone, whereas variation components are synthesized under the direction of other nod genes. Roots of leguminous plants, unlike those of other plants, secrete large amounts of flavonoids, which act as inducer molecules and presumably trigger *nod* gene expression in nearby rhizobial cells in the soil. Some flavonoids, which are structurally very closely related to *nod* gene inducers (luteolin and eriodictyol), are considered to inhibit *nod* gene expression in certain *Rhizobium* spp.

The *nif* and *fix* genes of *A. caulinodans*, *B. japonicum*, and *R. meliloti* are organized in distinct clusters whose structure and genomic location are specific to the species. Linkage between nitrogen fixation genes in rhizobia is not as firm as in *K. pneumoniae*. In this organism 20 adjacent *nif* genes are organized in eight operons within *ca.* 24 kb of DNA (Arnold et al. 1988).

In *R. leguminosarum* and certain other species of *Rhizobium*, *nif* genes are plasmid-borne. *R. meliloti* carries two extremely large plasmids (megaplasmids) of about 1400 kb (pSym-a or megaplasmid 1) and 1700 kb (pSym-b or megaplasmid 2) (Honeycutt et al. 1993). Both cluster I (consists of 12 genes

Table 7.2 Functions of *NOD*, *NIF*, and *FIX* genes

Gene	Function	References
***nod* gene**		
nod M	Nod factor synthesis	Merrick (1992)
nod L	Determine host range	Gottfert (1993)
nod E	Determine host range	Gottfert (1993)
nod F	Encodes a specific acyl carrier protein used to acylate the nod factor specified by *nod A*	Merrick (1992)
nod D	Encodes a regulatory protein called Nod D that controls transcription of other *nod* genes	Merrick (1992)
nod A	Direct synthesis of nod factor backbone	Gottfert (1993)
nod B	Direct synthesis of nod factor backbone	Gottfert (1993)
nod C	Direct synthesis of nod factor backbone	Gottfert (1993)
nod I	Membrane proteins that help in exporting nod factors	Kondorosi et al. (1991)
nod J	Membrane proteins that help in exporting nod factors	Kondorosi et al. (1991)
***nif* gene**		
nifD	***α*** subunit of dinitrogenase. Forms an $\boldsymbol{\alpha}_2$ $ß_2$ tetramer with ß subunit interface. FeMo-co, the site substrate reduction, is present buried within the ***α*** subunit of dinitrogenase	Dean and Jacobson (1992)
nifK	ß subunits of dinitrogenase. ß clusters are present at ß subunit interface	Dean and Jacobson (1992)
nifH	Fe protein subunit of dinitrogenase reductase. Obligate electron donor to dinitrogenase during dinitrogenase turnover. Also is required for FeMo-co biosynthesis	Dean and Jacobson (1992)
nifN	Required for FeMo cofactor biosynthesis	Allen et al. (1994) and Dean and Jacobson (1992)
nifV	Encodes a homocitrate synthase. Homocitrate is an organic component of FeMo cofactor	Hawkes et al. (1984) and Hoover et al. (1987)
nifB	Required for FeMo cofactor biosynthesis. Metabolic product. NifB-co is the specific Fe and S donor to FeMo-co	Allen et al. (1994) and Shah et al. (1999)
nifQ	Incorporation of Mo into FeMo cofactor. Proposed to function in early MoO_4^{2} processing	Imperial et al. (1984)
nifE	Forms $\alpha_2ß_2$ tetramer with *nifN*. Required for FeMo cofactor biosynthesis	Allen et al. (1994) and Dean and Jacobson (1992)
nifX	Not essential for nitrogen fixation; required for FeMo cofactor biosynthesis	Shah et al. (1999)
nifU	Involved in mobilization of Fe-S cluster synthesis and repair	Yuvaniyama et al. 2000
nifS	Involved in mobilization of Fe-S cluster synthesis and repair	Zheng et al. (1993)
nifY	Associates with MoFe protein and dissociates upon FeMo cofactor insertion	Homer et al. (1993)
nifM	Required for the maturation of *nifH* and Fe protein maturation. Putative peptidyl-prolyl cis/trans isomerase	Dean and Jacobson (1992)

(continued)

Table 7.2 (continued)

Gene	Function	References
nifW	Involved in stability of dinitrogenase. Proposed to protect dinitrogenase from O inactivation	Kim and Burgess (1996)
nifF	Flavodoxin required for electron transfer to the Fe protein, Physiologic electron donor to *nifH*	Thorneley et al. (1992)
nifJ	Pyruvate flavodoxin (ferredoxin) oxidoreductase involved in electron transport to nitrogenase; couples pyruvate oxidation to reduction of the *nifF* product	Shah et al. (1988)
nifA	Positive regulator of *nif* transcription	Dixon (1998)
nifL	Negative regulatory protein	Dixon (1998)
***fix* gene**		
fix LJ	Oxygen-responsive two-component regulatory system involved in positive control of *fixK* and *nifA*	David et al. (1988)
fix K	Positive regulator of *fixNOQP*, *nifA*; negative regulator of *nifA* and *fixK*	Batut et al. (1989)
fix NOQP	Microaerobically induced, membrane-bound cytochrome oxidase	Boistard et al. (1991)
fix GHIS	Redox process-coupled cation pump	Kahn et al. (1989)
fix ABCX	Unknown function; required for nitrogenase activity; FixX shows similarity to ferredoxins	Earl et al. 1987
fix R	Unknown function; not essential for nitrogen fixation	Thony et al. (1987)

nifHDKE, *nifN*, *fixABCX nifA nifB frdX*) and cluster II (consists of 10 genes *fixLJ*, *fixK*, *fixNOQP*, *fixGHIS*) are located on megaplasmid 1 (David et al. 1987). The cluster II genes map at about 220 kb downstream of the *nifHDKE* operon and are transcribed in opposite orientation to it. A functional duplication of the region spanning *fixK* and *fixNOQP* is present at ca. 40 kb upstream of *nifHDKE* (Renalier et al. 1987). A cluster of *nod* genes including the common nod genes (*nodABC*) is located in the 30-kb region between *nifE* and *nifN* (Long 1989). Additional genes required for an effective symbiosis are located on megaplasmid 2 and on the chromosome (Honeycutt et al. 1993).

The *nifD* and *nifK* genes specify α and ß subunits, respectively, of the $\alpha_2ß_2$ FeMo protein (dinitrogenase or component I; Mr 220,000). The homodimeric Fe protein component or dinitrogenase reductase (Mr 60,000) is encoded by *nifH*. In *R. meliloti nifHDK* genes are organized in an operon along with *nifE*. For the synthesis of the FeMo cofactor of component I, the products of the *nifE*, *nifN*, and *nifB* genes are required. The exact biochemical functions of the respective proteins are not known (Dean et al. 1993). The amino acid sequences of the NifE and NifN proteins show a significant similarity to those of NifD and NifK, respectively. It was suggested that the *nifEN* genes originated from duplication of the *nifDK* genes and that the *NifEN* complex may provide a scaffold for FeMo cofactor biosynthesis (Brigle et al. 1987). Downstream of the *nifB* genes of both *R. meliloti* and *B. japonicum* are the genes *fdxN* and *frxA*, respectively, encoding ferredoxin-

like electron transfer proteins. In contrast to *frxA* of *B. japonicum*, the *R. meliloti fdxN* gene is absolutely essential for nitrogen fixation (Ebeling et al. 1988). Another ferredoxin-like protein is encoded by *fixX*; this gene is located downstream of *fixC* in *Rhizobium* spp.

FeMo cofactor is synthesized by the expression of several genes in *Klebsiella* including *nifN*, *nifV*, *nifB*, *nifQ*, *nifE*, *nifX*, *nifU*, *nifS*, and *nifY*. Genes *nifS* and *nifU* play a part in the assemblage of Fe-S clusters (Hu and Fay 2007). Maturation of Fe protein is brought about by the products of *nifH*, *nifM*, *nifU*, and *nifS*. *nifE* and *nifN* products function as scaffold for FeMo-co biosynthesis. Various genes have their own specific functions – *nifB* gene product acts as iron and sulfur-containing precursor of FeMo-co, gene *nifQ* is a molybdenum sulfur-containing precursor of FeMo-co, and gene *nifV* encodes homocitrate synthase. It is required for the synthesis of FeMo-co. The gene *nifW* protects the dinitrogenase protein from oxygen inactivation thus stabilizing it (Cheng 2008). *Klebsiella* also contains the genes that mediate electron transport to nitrogenase. The *nifF* gene encodes flavodoxin which transfers electrons to nitrogenase, *nifJ* encodes pyruvate oxidoreductase that transfers electrons to flavodoxin from the pyruvate, *nif A* encodes positive regulatory protein that serves to activate transcription of other genes, and *nifL* acts as repressor of nitrogenase (Beringer and Hirsch 1984).

The *fixABCX* genes were first identified in *R. meliloti* (Earl et al. 1987) in *B. japonicum* (Gubler and Hennecke 1986), *A. caulinodans* (Kaminski et al. 1988), *R. leguminosarum* bv. *viciae* (Gronger et al. 1987), *R. leguminosarum* bv. *trifolii* (Iismaa et al. 1989), and *R leguminosarum* bv. *phaseoli* (Michiels and Vanderleyden 1993). They are organized in a single operon. In *B. japonicum fixA* and *fixBCX* form distinct transcriptional units present in clusters II and I, respectively. The *B. japonicum fixBCX* operon includes a proximal open reading frame (ORF35), which is not essential for nitrogen fixation activity but whose translation significantly stabilizes *fixBCX* mRNA (Gubler et al. 1989). Mutations in any one of the *fixABCX* genes of *R. meliloti*, *B. japonicum*, and *A. caulinodans* totally eliminate nitrogen fixation. It has been proposed that the *fixABCX* gene products may have a role in electron transport to nitrogenase (Earl et al. 1987, Gubler and Hennecke 1986).

The *fixNOQP* genes were first described in *R. meliloti* as a duplicated *fix* region that is linked to the regulatory genes *fixLJ* and *fixK* and whose expression is induced under symbiotic conditions (Renalier et al. 1987). Homologous genes were then identified in *B. japonicum* (Preisig et al. 1993), *R leguminosarum* bv. *viciae* (Hynes et al. 1992), and *A. caulinodans* (Mandon et al. 1993). They are probably organized in an operon in all three species. *R. meliloti* mutant strains deleted for both *fixNOQP* regions and *B. japonicum fixNOQP* mutants have flawed symbiotic nitrogen fixation. A corresponding mutant of *A. caulinodans* retained 50% of wild-type nitrogenase activity under both symbiotic and free-living conditions (Mandon et al. 1993; Renalier et al. 1987). In addition, *B. japonicum* mutants are affected in bacteroid development and exhibit a decreased whole-cell oxidase activity when grown microaerobically or anaerobically. The amino acid sequences of the FixNOQP proteins imply that they constitute a membrane-bound, cytochrome

c-containing heme/copper cytochrome oxidase. It is postulated that this oxidase complex is required to support bacteroid respiration under conditions of low oxygen present in root nodules (Preisig et al. 1993).

Four tightly linked genes, named *fixGHIS*, have been identified by mutational analysis and subsequent DNA sequence determination downstream of the fixNOQP operon in cluster II of *R. meliloti* (Kahn et al. 1989). On the basis of hybridization experiments, homologous genes are found to exist in various members of the genus *Bradyrhizobium*, in *A. caulinodans*, and in *R. leguminosarum* bv. *viciae* and bv. *phaseoli*. FixG is likely to be involved in a redox process, because it contains two cysteine clusters typical of iron-sulfur centers present in bacterial ferredoxins. FixI is homologous to the catalytic subunit of prokaryotic (bacterial) and also eukaryotic ATPases which are involved in cation pumping. It is speculated that FixI is a symbiosis-specific cation pump whose function is coupled to a redox reaction catalyzed by the FixG subunit (Kahn et al. 1989). Further biochemical analysis is required to define the function of the *fixGHIS* gene products in rhizobial nitrogen fixation.

In *B. japonicum* a gene termed *fixR* is located upstream of the regulatory *nifA* gene, and the two genes form an operon (Thony et al. 1987). No *fixR*-like gene has been described so far in other rhizobia, but interspecies hybridization experiments indicate the existence of homologous DNA regions in other slow-growing rhizobia and in the nonsymbiotic bacterium *Rhodopseudomonas palustris* (Thony 1989).

Nitrogen fixation is regulated at the transcriptional level. It is in response to environmental oxygen and ammonium levels. The nitrogenase components are oxygen labile. Bacteria suppress transcription when oxygen levels are high. It is also advantageous to repress the expression of the metabolically expensive nitrogenase system when the cellular level of fixed nitrogen is adequate. The degree to which each stimulus affects transcription is characteristic of the particular diazotroph. Nitrogenase expression in symbiotic diazotrophs is insensitive to ammonium. It is due to the fact that export of ammonium to their symbiont represses ammonium levels.

The expression of *nif* genes in free-living diazotrophs is more sensitive to cellular ammonium levels (Merrick 1992). The paradigm of transcriptional regulation is based on studies on *K. pneumoniae*. In this model, regulation of *nif* gene expression is considered to be based on two elements, an external system involving *ntr* genes and an internal system mediated by *nif* A and *nif* L genes. The *ntr* gene system is responsible for transcription of *nif* genes, while *nif* A and *nif* L genes act as regulatory system through a "switch on" and "switch off" mechanism. Gene *nif* A produces Nif A protein, which activates the *nif I* genes transcription; whereas *nif* L genes produces Nif L protein, which inhibits the *nif* gene transcription.

The interrelationship between external (*ntr* genes) and internal (*nif* A and *nif* L genes) systems in *Klebsiella pneumonia* is represented in a simplified way. The protein Ntr A (the product of *ntr* A gene) is a factor of RNA polymerase and allows the latter to bind at *nif* promoters to begin *nif* gene transcription (i.e., N_2 fixation). When ammonia is in an excess in the environment, it inhibits nitrogen fixation through Ntr C protein (the product of *ntr* C gene) and Nif L protein (the product of

nif L gene). In NH_3 excess condition, the Ntr C protein represses the functioning of Ntr L protein, thus "switching off" N_2 fixation. The activity of Ntr C protein is regulated by Ntr B (product of *ntr* B gene). Ntr B is an enzyme that functions both as a protein kinase and as a phosphate; the kinase or phosphatase activity of Ntr B is regulated by the nitrogen status of the cell. When ammonia is limiting, Ntr C protein is activated and promotes the transcription of *nif* A gene to produce *Nif* A protein that "switches on" the N_2 fixation.

Nitrogenase activity is also inhibited, as mentioned earlier, by oxygen. It is to note that Nif L protein contains a molecule of FAD (ferredoxin adenine nucleotide) that is critical for oxygen sensing by the protein. When oxygen exceeds the required level, Nif L protein shuts down the transcription of *nif* genes, and as a result, the synthesis of oxygen-sensitive nitrogenase stops.

Ammonia also regulates nitrogenase activity in certain nitrogen fixers. This phenomenon is called ammonia "switch off" effect. In this case, excess ammonia modifies the structure of dinitrogenase reductase (Fe protein) leading to a loss of nitrogenase activity. When ammonia returns to limiting level, the modified dinitrogenase reductase (Fe protein) converts back to its active form, and the activity of nitrogenase resumes.

The control of *nif* gene expression is focused on NifA (the *nif*A gene product), a σ^{54} (*rpoN* gene product)-dependent transcriptional activator. It is responsible for control of all major *nif* gene cluster transcription. Transcription of *nifA* is under the control of the *ntrBC* gene products. They comprise a global two-component transcriptional activator system which is responsible for cellular nitrogen regulation (Merrick 1992). In the model organism *K. pneumoniae*, the *nifA* gene is co-transcribed with *nifL*. This gene encodes a redox- and nitrogen-responsive regulatory flavoprotein (NifL) which acts as a negative regulator of NifA. This adds another level of regulation in response to oxygen and fixed nitrogen. Oxidized NifL is also sensitive to the nucleotides present in vitro. It exhibits increased inhibition especially in response to ADP (Hill et al. 1996). The means by which NifL inhibits NifA remain unclear.

Deviations from the *K. pneumoniae* model have been found in approximately all nitrogen fixation organisms of interest. In *A. vinelandii* and *Rhodospirillum rubrum*, expression of *nifA* is not regulated by *ntrBC* gene products. It is still not known whether *nifA* expression is under nitrogen control. In *Rhizobium meliloti*, redox-dependent control of *nifA* expression exists in response to *fixL* and *fixJ*, which encode for a two-component regulatory system responsive to oxygen (Merrick 1992). This system is replaced by the *ntrBC* control found in *K. pneumoniae*. *R. meliloti* also lacks NifL. NifA is inhibited by oxygen stimulus (Krey et al. 1992). Interestingly, there is no evidence for NifL in *Rhodobacter capsulatus*. This organism contains *nif*-related genes analogous to *ntrBC*, but the expression of an *rpoN*-like gene is found to be sensitive to oxygen and amount of fixed nitrogen available (Merrick 1992). *R. capsulatus* contains two copies of *nifA*, which respond differently to ammonium (Klipp and Paschen 1998). Nitrogenase transcriptional control mechanisms are separate for different diazotrophs.

An added level of nitrogenase regulation is present in a few free-living diazotrophs due to the metabolically demanding nature of nitrogen fixation. To prevent unproductive nitrogen fixation during energy-limiting or nitrogen-sufficient conditions, the nitrogenase complex is rapidly and reversibly inactivated by ADP-ribosylation of Fe protein. The ADP-ribosylation system has been identified in *R. rubrum* and *R. capsulatus* (purple and non-sulfur photosynthetic bacteria), in *Azospirillum brasilense* and *Azospirillum lipoferum* (microaerophilic, associative bacteria), and also in *Chromatium vinosum* (a purple sulfur bacterium) (Ludden and Roberts 1989). *R. rubrum* remains the model organism under study. The posttranslational nitrogenase regulation was first identified in this organism. The ADP-ribosylation in *R.rubrum* exhibits the roles of the NAD^+-dependent enzyme, dinitrogenase reductase ADP-ribosyltransferase (DRAT), and its partner, dinitrogenase reductase-activating glycohydrolase (DRAG).

ADP-ribosylation of Fe protein occurs at a specific arginine residue (Arg101 in *R. rubrum*). It occurs by the formation of a α-N-glycosidic bond between the guanidino nitrogen atom of arginine and the terminal ribose of ADP-ribose (Ludden and Roberts 1989). Structurally, this ADP-ribose is similar to the modifying groups attached by bacterial ADP-ribosylating toxins. These toxins are the causative agents of cholera and diphtheria. The presence of the ADP-ribose group in nitrogenase inhibits association of Fe protein with MoFe protein instead of blocking electron transfer between complexed Fe protein and MoFe protein (Ludden and Roberts 1989). ADP-ribosylated Fe protein differs from unmodified Fe protein with respect to a few characteristics. The two subunits of the inactive Fe protein dimer are not equivalent because ADP-ribosylation occurs on only one subunit. Modified Fe protein retains the native $[Fe_4S_4]$ cluster. It can be chemically oxidized and reduced and it also retains the oxygen accountability of the active Fe protein. ADP-ribosylated Fe protein cannot hydrolyze Mg-ATP, but it still has the ability to bind Mg-ATP and also to undergo the conformational change that gives access of the $[Fe_4S_4]$ cluster to chelators (Ludden and Roberts 1989). It also plays a role in synthesis and insertion of FeMo-co into MoFe protein (Shah et al. 1988).

The genes encoding DRAT (draT) and DRAG (draG) are co-transcribed from a non-nif operon. This operon includes a third gene (*draB*) of unknown function. The configuration of the draTGB operon is conserved in both *A. brasilense* and *A. lipoferum* (Inoue et al. 1996). *R. capsulatus*, however, lacks draB (Masepohl et al. 1993). DRAT is a 30-kDa monomer with high specificity toward oxidized Mg-ADP-bound Fe protein. It has been found to possess no measurable activity with other arginine residues or even water as the ADP-ribose acceptor (Ludden and Roberts 1989; Halbleib et al. 2000). The amino acid sequence of DRAT is not really comparable to those of the bacterial toxins. Some key amino acid residues are conserved. The Fe proteins from *K. pneumoniae* and *A. vinelandii* are better substrates for *R.rubrum* DRAT than the *R. rubrum* Fe protein itself. These organisms lack the dra operon. There are no measurable reverse or glycohydrolytic reactions catalyzed by DRAT. The removal of the ADP-ribose group is catalyzed by dinitrogenase reductase activating glycohydrolase (DRAG). It restores fully active Fe protein with an intact Arg101 side chain. DRAG is a 32-kDa monomeric

binuclear manganese enzyme that is capable of cleaving the α-N-glycosidic bond of a number of analogs of ADP-ribosylarginine (Ludden and Roberts 1989). Only the reduced Mg-ATP-bound form of ADP-ribosylated Fe protein is a substrate for DRAG (Ludden and Roberts 1989; Halbleib et al. 2000). The exact modes of interaction of DRAT and DRAG with Fe protein are not defined, but it is supposed that each binds the same surface of Fe protein like MoFe protein. Overexpressed DRAT inhibits cellular nitrogenase activity (Grunwald et al. 1995).

The method of regulation of DRAT and DRAG is not well understood. However, it is known that the activity of each enzyme is regulated in vivo (Liang et al. 1991). It is widely known that the regulatory signals involve either negative effectors or known assay components. DRAT and DRAG have opposite specificities for Mg-ADP and Mg-ATP-bound Fe protein. Nitrogenase activity regulation is subject to cellular concentration in ATP and ADP levels during cycles of inactivation/activation (Ludden and Roberts 1989). Sensitivity of DRAT and DRAG toward the redox state of Fe protein suggests that DRAT and DRAG may be regulated by sensing the cellular energy and redox status directly from the state of Fe protein (Halbleib et al. 2000). The cellular NAD^+ concentration may also be a positive effector for DRAT (Norén et al. 1997). It appears that the unregulated variants of DRAG have altered divalent cation affinities.

The nitrogenase-inactivating conditions of nitrogen sufficiency (NH^{4+}) and energy limitation give rise to convergent signal transduction pathways. Inhibition of glutamine synthetase unsettles both responses. The cellular concentration of glutamine, however, is relatively unaffected by the modification and demodification of Fe protein (Kanemoto and Ludden 1987). Genetic changes of nitrogen control genes (*glnB, ntrBC*) yield results that do not support the model of narrowly confined signal transduction pathways. It is so because the effects on ammonia response seem to be independent of the darkness response (Zhang et al. 1995).

The response of DRAT and DRAG activities to exogenous inactivation effectors is not species specific. It was evidenced by plasmid-borne *draTG* genes from *A. brasilense* that restored the wild-type phenotype to dra mutants in *R. rubrum* (Zhang et al. 1992). The Fe protein was inactivated in response to darkness and not absence of oxygen. Mutants of *K. pneumonia* having a plasmid with *draTGB* from *R. rubrum* reversibly ADP-ribosylate Fe protein in the presence of exogenous ammonium. Future work in nitrogen fixation will be centered around regulatory strategies in all beneficial agricultural bio-inoculants.

7.5 Impact of Using N_2-Fixing Bio-inoculants: Prospects and Challenges

Bio-inoculants are microbial preparations containing live or latent cells of efficient strains of nitrogen-fixing, phosphate-solubilizing, or cellulolytic microorganisms. They can be applied to seed, soil, or composting areas with the objective of

increasing the population of such microorganisms and accelerating those microbial processes which augment the availability of nutrients to plants. Biofertilizers play a crucial role in long-term sustainability and aid in mitigating the environmental pollution associated with indiscriminate use of agrochemicals. They are seen as one of the best modern tools for sustainable agriculture. An indispensable requirement of sustainable agriculture is the continuous renewal of soil structure and fertility with renewable resources, lessening the need for chemical fertilizers, thus reducing their environmental burden. Intensive agriculture that involves heavy and continuous use of fertilizers has ensured high crop productivity from shrinking agricultural land. The use of fertilizers, including chemical fertilizers and manures, to enhance crop productivity has often negatively affected the complex system of the biogeochemical cycles (Steinshamn et al. 2004). Fertilizer use has caused leaching and runoff of nutrients, especially nitrogen (N) and phosphorus (P), leading to adverse environmental impact (Gyaneshwar et al. 2002).

Microbes are essential in all phases of agricultural practices as in maintenance of soil structure and fertility (Nakas and Hagedorn 1990), in preservation and processing of crops, and in recycling crop residues (Hanson 1996). They contribute a wide range of essential services to the sustainability of all ecosystems, by acting as the primary driving agents of nutrient cycling, regulating the dynamics of soil organic matter, modifying soil physical structure, enhancing the efficiency of nutrient acquisition by the vegetation, and enhancing plant health. These services are not only essential to the functioning of natural ecosystems but constitute an important resource for the sustainable management of agricultural and environmental ecosystems. Microbial intervention in soil fertility, biocontrol, and plant growth promotion involves the introduction of natural or transgenic microbial inoculants. Microbial inoculants or biofertilizers are promising tools in integrated solutions to agro-environmental issues. They possess the capacity to promote plant growth, enhance nutrient availability and uptake, and support the health of plants (Adesemoye et al. 2008). Thus, the incorporation of biofertilizers plays an important role in improving soil fertility, yield attributing characters of crops, and thereby crop yield. In addition, their application in soil improves soil biota and decreases the use of chemical fertilizers.

7.6 Future Outlook

Elucidation of the regulation of the nitrogen-fixation process in bacteria has advanced considerably due to extensive work done on wide range of model systems as well as expansion in the knowledge of molecular microbiology. This understanding has helped to derive physiological benefits from beneficial rhizospheric microbes. At the molecular level, the understanding of the regulatory processes is increasing especially in those organisms that have a NifA-dependent mode of control. The harvest of pure active NifA proteins (particularly of the O_2 sensitive group) is still a major road block. Newer information on broad outline of the major

signal transduction pathways may be in the public domain very shortly. A lot is yet to be unraveled about Gram-positive diazotrophs. To summarize, the challenge now is to amalgamate the existing knowledge into a whole-cell perspective on the genetic, biochemical, and physiological processes that contribute to successful diazotrophy. While plant biotechnology is making efforts to transfer N-fixing genetic potential in cereals to aid in their N-economy, it may take another 50 years to fructify on a field scale. Till that time, use of N_2-fixing microbes either in close association or as endophyte are good options for a sustainable agriculture.

Many varieties of various plants provide conducive environment to support the growth of endophytes and derive benefits as nitrogen fixation from them (Iniguez et al. 2004; Gutierrez-Zamora and Martinez-Romero 2001; Engerhard et al. 2000; Shrestha and Ladha 1996; Urquiaga et al. 1989). There are still a number of uncertain aspects that need to be focused on as soil microbiomes, soil metagenomics, transcriptomics, and key genes of active microorganisms associated with nodulation, growth regulators, disease suppressing, and nutrient cycling in soil. Development of biofertilizers using microbial consortia containing effective, competitive, and stress-tolerant microbial strains is required. The potential of biofertilizers to supply micronutrients and for biofortification of food crop is yet to be explored. For the development of biofertilizers, public sector should take steps in the future research like monitoring the quality of biofertilizers and their effects on plants and humans. Extension, training program, or short-term diploma programs may be initiated for motivating the farmers to exploit full potential of the biofertilizer technology.

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Chapter 8
Biological Control as a Tool for Eco-friendly Management of Plant Pathogens

Mamta Sharma, Avijit Tarafdar, Raju Ghosh, and S. Gopalakrishanan

Abstract Crop protection is pivotal to maintain abundant production of high quality. Over the past 100 years, use of chemical fertilizers and pathocides and good agronomical practices enabled growers to maintain improved crop productivity. However, extensive use of chemicals during the last few decades in controlling pests and diseases resulted in negative impacts on the environment, producing inferior quality and harming consumer health. In recent times, diverse approaches are being used to manage and/or mitigate a variety of pathogens for control of plant diseases. Biological control is the alternative approach for disease management that is eco-friendly and reduces the amount of human contact with harmful chemicals and their residues. A variety of biocontrol agents including fungi and bacteria have been identified but require effective adoption and further development of such agents. This requires a better understanding of the intricate interactions among the pathogen, plants and environment towards sustainable agriculture. Beyond the field assessment, the analysis of microbial communities with culture-independent molecular techniques including sequencing technologies and genomics information has begun a new era of plant disease management.

Keywords Biocontrol agent · Plant-pathogen interaction · Eco-friendly plant disease management · Sustainable agriculture · Socio-economic impact

8.1 Introduction

During the last 40 years, the world population has increased by 90%, while food production has increased only by 25% per head. It is estimated that 39% more production is needed worldwide to feed an additional 1.5 billion mouths by 2020

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and the production needed to be doubled by 2050. However, attack by pest and diseases causes a loss to the tune of 40% of the gross crop production. Further, with the rapid change in climatic factors, plant pathogens are becoming more aggressive, breaking the plant resistance, and inhibit the crops to reach its optimum yield. Current practices for integrated disease management are largely based on genetic host resistance and synthetic chemicals. Continuous use of those chemicals in controlling plant diseases has negative effects on the environment, causes pollution in the biosphere and harms the human beings. Further, those chemicals themselves are acting as selective agents, making the pathogens more resistant, and help these pathogens to persist as they are slowly becoming resistant to these agents. Thus, there was a necessity to execute new methods which would supplement conventional strategies for plant disease control and are competent to minimize adverse effects of chemical pathocides on human health and the environs. Control of plant diseases using biological agents like live microbial cells, or byproducts produced by them, is a powerful alternative way, called biological control. Biological control is eco-friendly, and the diversified microbial world provides endless resources for biologically active molecules which can stably inhabit the environment as nondominant species but maintain their effectiveness in suppression of plant pathogens. For instance, in the 1880s, the cottony cushion scale in citrus was the major threat to citrus industry in California. Vedalia beetle (*Rodolia cardinalis* Mulsant), a predatory insect, was introduced in California to cease the effect of the pest (*Icerya purchasi* Maskell). That was the first success story of the biological control. Since this success, scientists have developed diverse techniques to manage a variety of pests and pathogens using diverse biological agents. In recent years, they diverted their attention towards the potential of beneficial microbes. Therefore, dynamic research efforts for developing and exploring innovative tools for the control of diseases have become imperative.

8.2 Why Eco-friendly Management Is Important to Control Plant Pathogen?

Control of the diseases is very important for securing human food sources and agriculture-based industries. There are two main ways to manage diseases and pests, using chemicals (chemical control) and by predators or parasites (natural control/biological control). Controlling of diseases in economically important crops with chemicals has long been practiced in agricultural settings, and use of this method is more acceptable by the farming community, as it is typically less expensive and immediate than natural control methods. But extensive use of those chemicals for an extended period has long lasting negative effects on the environment, including human life and other living organisms existing in the ecological niches. Being detrimental to both beneficial and harmful organisms, they can damage the ecological balance and also contaminate the food chain

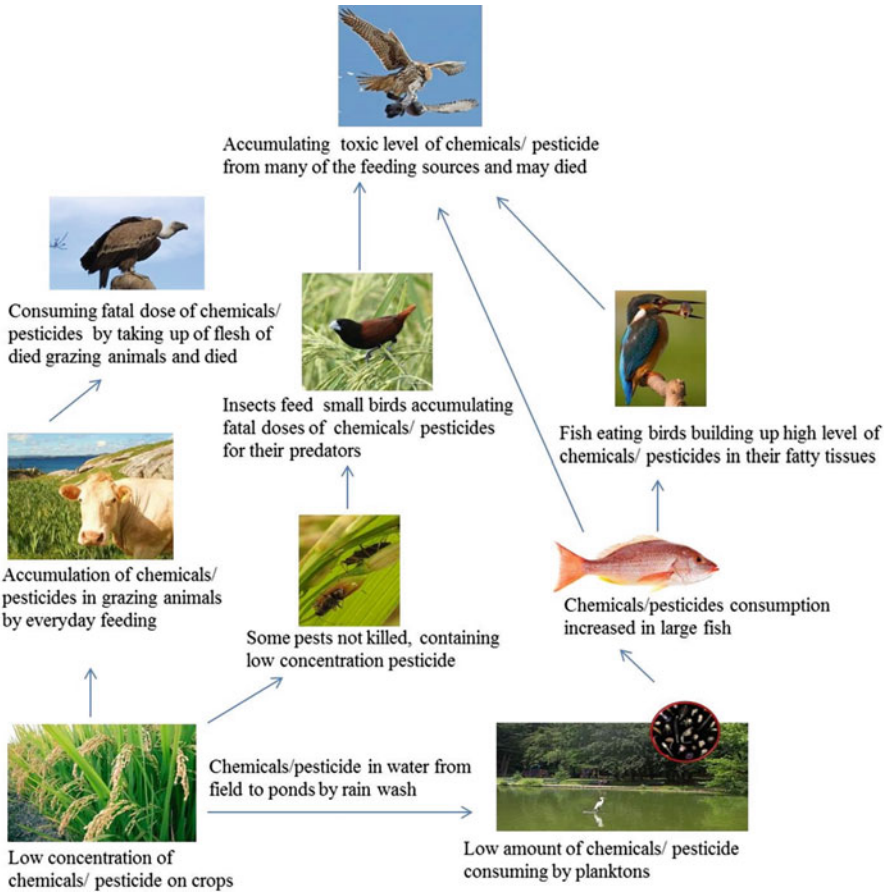

Fig. 8.1 Harmful chemicals enter the food chain and deposition increased tissues of the organisms belonging to the higher tropic level

through bioaccumulation of toxic residues. In this way the chemicals become worse for the organisms belonging to the higher tropic levels (Fig. 8.1).

The term 'biocontrol agent' (biopesticide), as a generic definition, has been applied with a narrow focus on preparations containing living microorganisms, through to a wider definition that includes botanical compounds and semiochemicals (e.g. pheromones) (Kiewnick 2007). The biocontrol agents and the process of biological control have several other benefits.

1. Biocontrol agents are safer both for the environment and the persons who are applying them and avoid environmental pollution (soil, air and water) by leaving no toxic residues.
2. It is comparatively easier to manufacture biocontrol agents, sometimes less expensive than chemical agents.

3. The biggest advantage of using biocontrol agents is that it can eliminate the specific pathogens effectively from the site of infection and can be used in combination with biofertilizers.
4. Biocontrol agents are very effective for a large number of soil-borne pathogen where using of chemical fungicide is not possible.
5. Biocontrol agents do not cause any toxicity to the plants; rather these increase crop yields by enhancing the root and plant growth through the encouragement of beneficial microflora in rhizosphere. It also helps in the mobilization of plant nutrients and makes it available to the plant.
6. Biocontrol agents avoid problems of resistance and also induce systemic resistance among the crop species.
7. Biological control is self-regulating, does not require any intricate management and helps to preserve the ecosystem.

However, despite the fascinating advantages of biocontrol of plant diseases, there might be few adverse effects on humans and the environment. Increasing the population of a certain biological agents artificially could be the reason of paying unexpected concerns. An organism that has been introduced from another area to destroy a pathogen in a new habitat may itself become a pathogen or predator for some beneficial organisms present in natural habitat or crops. Other than that it has the following limitations.

1. Biocontrol agents work slowly and less effectively in comparison to the chemical pesticides, as their efficacy almost completely depends on environmental conditions.
2. Biocontrol agents are mainly used against specific diseases as a preventive measure, not as a curative measure.
3. The antagonists and shelf life of biocontrol agents are short. For example, the shelf life of *Pseudomonas fluorescens* is 3 months and of *Trichoderma viride* is 4 months only. To maintain the effective level of biocontrol agents in cropping area, periodical checking is needed and this requires skilled persons.
4. Skilled persons are also required for multiplying and supplying the biocontrol agents without contamination.
5. At present, biocontrol agents are available only in a few places and in less quantities.

8.3 Groups of Biological Control Agents

After the development of the first commercial biological agent, a range of microorganisms, including virus, bacteria, actinomycetes, fungi, oomycetes, protozoa, etc., were identified for the purpose of plant disease management. Many organisms are found to be very effective against a variety of plant diseases. A few of those organisms are now being used for successful disease management in plants at fields and greenhouse conditions (Table 8.1).

Table 8.1 Groups of successful biocontrol agents that effectively control diseases in economically important crop species

Groups	Biocontrol agent	Target pathogens	Crops	Disease/pest	Experimental condition	References
Virus	*Tomato mosaic virus* (mild strain)	Severe strains of same	Tomato	Mosaic	Field	Fletcher (1978)
	Zucchini yellow mosaic virus (mild strain)	Severe strains of same	Cucarbits	Mosaic	Field	Lecoq and Lemaire (1991)
	Papaya ringspot virus (mild strain)	Severe strains of same	Papaya	Ringspot disease	Field	Tennant et al. (1994)
	Potato virus X (mild strain)	Severe strains of same	Potato	Viral	Field	Webb et al. (1952)
	Mild strain of *Citrus tristeza virus* (mild strain)	Severe strains of same	Citrus	Tristeza	Orchard	Folimonova (2013)
Actinobacteria	***Streptomyces***					
	S. violaceusniger	*Pythium ultimum*	Sugar beet	Damping-off	Field	Trejo-Estrada et al. (1998)
	S. janthimts, *S. cinerochromogenes*	*Pythium coloration*	Carrot	Cavity spot	Field	El-Tarabily et al. (1997)
	Streptomyces species	*Phytophthora medicaginis* and *Phytophthora sojae*	Alfalfa and soybean	Root rot	Controlled condition	Xiao et al. (2002)
	S. lydicus	*P. ultimum*	Cotton and pea	Root and seed rot	Growth chamber	Yuan and Crawford (1995)
	Non-*Streptomyces*					
	Actinoplanes spp.	*P. ultimum*	Beet	Damping-off	Field	Khan et al. (1997)
	A. missouriensis	*Phytophthora megasperma* f. sp. *glycinea*	Soybean	Root rot	Greenhouse	Sutherland and Lockwood (1984)
		Plectosporium tabacinum	Lupin	Root rot	–	El-Tarabily (2003)
	A. philippinensis	*P. coloratum*	Carrots	Cavity spot	–	El-Tarabily et al. (1996a)
		Pythium aphanidermatum	Cucumber	Damping-off	Field	El-Tarabily (2006)

(continued)

Table 8.1 (continued)

Groups	Biocontrol agent	Target pathogens	Crops	Disease/pest	Experimental condition	References
	A. utahensis, *Amorphosporangium auranticolor*, *Micromonospor*a sp.	*P. megasperma* f. sp. *glycinea*	Soybean	Root rot	Greenhouse	Filonow and Lockwood (1985)
	Actinomadura sp.	*Phytophthora cinnamomi*	Snapdragon	Root rot	–	You et al. (1996)
	A. rubra	*P. coloratum*	Carrots	Cavity spot	Field	El-Tarabily et al. (1997)
	Micromonospora sp.	*Gaeumannomyces graminis* var. *tritici*	Wheat	Take all	Field	Coombs et al. (2004)
		Fusarium oxysporum f. sp. *lycopersici*	Tomato	Wilt	–	Smith (1957)
	M. carbonacea	*P. cinnamomi*	Banksia sp.	Root rot	–	El-Tarabily et al. (1996b)
		P. coloratum	Carrots	Cavity spot	Field	El-Tarabily et al. (1997)
		Sclerotinia minor	Lettuce	Basal drop	Greenhouse	El-Tarabily et al. (2000)
	M. chalcea	*P. aphanidermatum*	Cucumber	Damping-off	Field	El-Tarabily (2006)
	M. globosa	*Fusarium udum*	Pigeon pea	Wilt	In vivo	Upadhyay and Rai (1987)
	Microbispora rosea	*P. aphanidermatum*	Cucumber	Damping-off	Field	El-Tarabily (2006)
	Nocardia globerula	*Helminthosporium solani*	Potato	Silver scurf	In vivo	Elson et al. (1997)
	Nocardioides sp.	*Phytophthora fragariae* var. *rubi*	Raspberry	Root rot	In vivo	Valois et al. (1996)
		G. graminis var. *tritici*	Wheat	Take all	Field	Coombs et al. (2004)
	Streptosporangium albidum, *Streptoverticillium netropsis*	*P. coloratum*	Carrots	Cavity spot	Field	El-Tarabily et al. (1997)

	Others					
	Arthrobacter sp.	*Fusarium* sp.	Carnation	Wilt	Greenhouse	Koths and Gunner (1967)
		F. moniliforme var. *subglutinans*	Pine	Pitch canker	Tissue-specific site	Barrows-Broaddus and Kerr (1981)
Bacteria	***Pseudomonads***					
	Pseudomonas chlororaphis	*F. oxysporum* f. sp. *radicis-lycopersici*	Tomato	Foot and root rot	In vitro and in vivo	Bolwerk et al. (2003)
	P. fluorescens	*Phytophthora infestans*	Potato	Late blight	In vitro	Hunziker et al. (2015)
		Sarocladium oryzae	Rice	Sheath rot	Field	Saravanakumar et al. (2009)
		Banana bunchy top virus	Banana	Bunchy top disease	Greenhouse and field	Kavino et al. (2008)
		Dematophora necatrix	Avocado	Root rot	Growth chamber	Cazorla et al. (2006)
		F. culmorum	Rye	Vascular wilt	Greenhouse	Kurek and Jaroszuk-Sciseł (2003)
		Rhizoctonia solani	Rice	Sheath blight	Field	Radjacommare et al. (2004)
		P. ultimum/Sphaerotheca fuliginea	Cucumber	Damping-off/powdery mildew	In vivo	Vogt and Buchenauer (1997)
		P. ultimum	Maize	Damping-off	Field	Callan et al. (1991)
		P. ultimum	Cotton	Damping-off	In vivo	Howie and Suslow (1991)

(continued)

Table 8.1 (continued)

Groups	Biocontrol agent	Target pathogens	Crops	Disease/pest	Experimental condition	References
		Phoma betae, *P. ultimum*	Cotton	Damping-off	Growth chamber	Walther and Gindrat (1988)
		P. ultimum	Sugar beet	Damping-off	–	Howell and Stipanovic (1980)
	P. putida	*Fusarium* sp.	Radish	Wilt	Greenhouse	de Boer et al. (2003)
		Different pathogens in soil micro-flora	Cucumber	Non-specific disease	Greenhouse	Loper and Henkels (1999)
	Bacilli					
	Bacillus pumilus, *B. amyloliquefaciens*, *B. subtilis*	*Cucumber mosaic virus*	Tomato	Mosaic disease	Greenhouse	Zehnder et al. (2000)
	B. amyloliquefaciens, *B. subtilis*, *B. pumilus*	*Tomato mottle virus*	Tomato	Tomato mottle	Field	Murphy et al. (2000)
	B. pumilus	*Erwinia tracheiphila*	Cucumber	Bacterial wilt	Field study	Zehnder et al. (2001)
		Peronospora tabacina	Tobacco	Blue mould	Control condition	Zhang et al. (2002)
	B. subtilis, *B. pumilus*	*Sclerospora graminicola*	Pearl millet	Downy mildew	Greenhouse and field	Niranjan Raj et al. (2003)
	B. cereus	*Corynespora cassiicola*	Tomato	Foliar diseases	Greenhouse	Silva et al. (2004)
		R. solani	Cotton	Root rot	In vivo	Pleban et al. (1997)
	Bacillus sp.	*Phytophthora capsici*	Bell pepper	Blight	Field	Jiang et al. (2006)
		Magnaporthe grisea	Rice	Blast	Greenhouse	Naureen et al. (2009)
		P. capsici	Squash	Blight	Greenhouse	Zhang et al. (2010)

	B. amyloliquefaciens	*F. oxysporum*	Maize	Wilt	In vivo	Koumoutsi et al. (2004)
		Botrytis cinerea	Pepper	Blight	Greenhouse	Park et al. (1999)
	Others					
	Aeromonas caviae	*R. solani* and *F. oxysporum* f. sp. *vasinfectum*	Cotton	Root rot/wilt	Greenhouse	Inbar and Chet (1991)
		Sclerotium rolfsii	Beans	Southern blight		
	Brevibacillus brevis	*B. cinerea*	Cucumber	Grey mould	Field	Konstantinidou-Doltsinis et al. (2002)
	Enterobacter agglomerans	*R. solani*	Cotton	Root rot	Greenhouse	Chernin et al. (1995, 1997)
	Enterobacter cloacae	*Pythium* sp.	Sugar beet	Damping-off	In vitro	Howell et al. (1988)
	Burkholderia sp.	*Fusarium verticillioides*	Maize	Maize rot	Greenhouse	Hernández-Rodríguez et al. (2008)
	Paenibacillus sp.	*F. oxysporum*	Sorghum	Wilt	In vitro	Budi et al. (2000)
	Lysobacter enzymogenes	*F. graminearum*	Wheat	Wilt	–	Li et al. (2008)
	Serratia plymuthica	*B. cinerea*	Many host	–	In vitro	Frankowski et al. (2001)
	Stenotrophomonas maltophilia	*P. ultimum*	Sugar beet	Damping-off	Field	Dunne et al. (2000)
	Agrobacterium radiobacter	*Agrobacterium tumefaciens*	Many hosts	–	Greenhouse	Vicedo et al. (1993)
	Collimonas fungivorans	*F. oxysporum*	Tomato	Wilt	Greenhouse	Kamilova et al. (2007)
	L. enzymogenes	*Pythium aphanidermatum*	Cucumber	Root and crown rot	Greenhouse	Folman et al. (2003)

(continued)

Table 8.1 (continued)

Groups	Biocontrol agent	Target pathogens	Crops	Disease/pest	Experimental condition	References
	Burkholderia cepacia	*Pythium* sp. and *Aphanomyces* sp.	Damping-off, root rot	Peas	Field	Heungens and Parke (2000)
Fungi	***Trichoderma***					
	T. harzianum, *T. harzianum*	*Crinipellis perniciosa*	Witches' broom	Cocoa	–	Marco et al. (2003)
	T. lignorum	*R. solani*	Damping-off	Bean	Greenhouse	Aziz et al. (1997)
	T. viride, *T. harzianum*	*Aspergillus flavus* and *Fusarium moniliforme*	Seed associated	Many hosts	In vitro	Calistru et al. (1997)
	T. harzianum	*Sclerotinia sclerotiorum*	Root rot	Soybean	Greenhouse	Ghisalberti and Sivasithamparam (1991)
	Trichoderma spp.	*Sclerotium rolfsii*	Rot	Common Vegetables	In vitro	Mukherjee and Raghu (1997)
	T. harzianum	*Pyrenophora tritici-repentis*	Tan spot and leaf blotch	Wheat	In vitro	Perello et al. (2003)
	T. virens	*R. solani*, *Pythium ultimum* and *Meloidogyne incognita*	Damping-off	Cucumber	Greenhouse	Yedidia et al. (1999)
	T. viride	*Colletotrichum truncatum*	Brown blotch	Cowpea	Field	Bankole (1996)
	T. viride, *T. pseudokoningii*, *T. koningii*	*Sclerotium cepivorum*	White rot	Onion	In vivo	Clarkson et al. (2002)
	T. harzianum	*R. solani*	Wet root rot	Chickpea	Field	Prasad and Rangeshwaran (2000)
	T. harzianum	*F. udum*	Wilt	Pigeon pea	Field	Prasad et al. (2002)

	T. harzianum	*Penicillium expansum*	Blue and grey mould	Apple	In situ	Batta (2004)
	T. virens, *T. harzianum*	*R. solani*	Stem canker or black scurf	Potato	Field	Brewer and Larkin (2005)
	T. koningii, *T. aureoviride*, *T. longibrachiatum*	*Sclerotinia sclerotiorum*	Head rot	Sunflower	Field	Escande et al. (2002)
	T. asperellum	*F. oxysporum*	Wilt	Tomato	Greenhouse	Cotxarrera et al. (2002)
	T. harzianum	*P. capsici*, *P. erythroseptica*	Crown rot/leaf blight	Chilli	Greenhouse	Khan et al. (2004)
	T. koningii	*Gaeumannomyces graminis* var. *tritici*	Take-all disease	Wheat	Greenhouse	Simon (1989)
	T. virens	*Verticillium dahliae*	Wilt	Cotton	Greenhouse	Hanson (2000)
	Yeast groups					
	Cryptococcus albidus var. *aerius*, *Pichia guilliermondii*, *Debaryomyces hansenii*	*Botryodiplodia theobromae*	Mango	Post-harvest rot	In vitro	Sugiprihatini and Wiyono (2011)
	P. guilliermondii	*Aspergillus flavus*	Soybeans	Post-harvest infection	In vitro	Wisniewski et al. (1991)
	P. membranefaciens, *C. albidus*	*Monilinia fructicola*, *Penicillium expansum*, *Rhizopus stolonifer*	Apple	Post-harvest infection	In vitro	Chan and Tian (2005)
	Rhodotorula glutinis, *C. laurentii*	*P. expansum*, *B. cinerea*	–	–	In vitro	Castoria et al. (1997)
	D. hansenii	*M. fructicola*	Peach	Brown rot	In situ	Stevens et al. (1997)
		Penicillium digitatum	Tangerine	Tomato and sweet potato		
		Rhizopus stolonifer		Soft rot		

(continued)

Table 8.1 (continued)

Groups	Biocontrol agent	Target pathogens	Crops	Disease/pest	Experimental condition	References
	P. guilliermondii	*P. digitatum*	Citrus	Decay of citrus fruit	Large scale in storage house	Droby et al. (1993)
	C. laurentii	*M. fructicola*, *P. expansum*	Peach		In vitro	Yao and Tian (2005)
	Chaetomium globosum	*Phoma betae*, *P. ultimum*, *R. solani*	Sugar beet	Damping-off	Growth chamber	Walther and Gindrat (1988)
	Oomycetes					
	Pythium oligandrum	*Sclerotinia sclerotiorum*	Wheat	White mould/stem rot	Field	Madsen and de Neergaard (1999)
		Pythium spp.	Sugar beet, cress	Damping-off	Greenhouse	McQuilken et al. (1998)
		Verticillium dahliae	Pepper	Wilt	Greenhouse	Rekanovic et al. (2007)
		Pythium dissotocum	Tomato	Root rot	Greenhouse	Vallance et al. (2009)
		F. oxysporum f. sp. *radicis-lycopersici*	Tomato	Wilt	Greenhouse	Benhamou et al. (1997)
		P. parasitica	Tomato	Buckeye rot	Greenhouse	Picard et al. (2000)
		B. cinerea	Tomato	Grey mould	Greenhouse	Lou et al. (2011)
		R. solani	Potato	Black scurf	Field	Ikeda et al. (2012)
		P. ultimum	Cress	Damping-off	Field	Al-hamdani et al. (1983)
		Aphanomyces cochlioides	Sugar beet	Root rot	Greenhouse, field	Takenaka and Ishikawa (2013)

		Cercospora beticola	Sugar beet	Leaf spot	Field	Takenaka and Tamagake (2009)
		Phytoplasma	Tobacco	–	Greenhouse	Lherminier et al. (2003)
	Phytophthora cryptogea	*P. infestans*	Potato	Late blight	In vivo	Stromberg and Brishammar (1991)
	Mycorrhiza					
	Glomus mosseae	*Thielaviopsis basicola*	Tobacco	Root infection	In vivo	Baltruschat and Schoenbeck (1975)
	Vesicular arbuscular mycorrhiza	*R. solani*	Rape	Damping-off	–	Iqbal et al. (1977)
	Glomus fasciculatus	*S. rolfsii*	Peanut	Southern blight	–	Krishna and Bagyaraj (1983)

8.4 Plant Extract

Plants are capable of synthesizing an overwhelming variety of small organic molecules, the secondary metabolites, which help the plants overcome from pathogen infection. Identification of novel effective secondary metabolites as fungicide or insecticide is essential to inhibit increasing resistance rates of the pathogens. The botanical extracts are more effective as insecticidal compounds (Table 8.2). But nowadays plant extracts are being used as effective biocontrol agents for inhibiting fungal diseases of plants. The plant extracts from *Cymbopogon proximus*, *Allium sativum*, *Carum carvi*, *Eugenia caryophyllus* and *Azadirachta indica* were found to have inhibitory effects on some phytopathogens including *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *lycopersici* and *Rhizoctonia solani* (Alkhail 2005). The methanolic plant extracts from *Salvadora persica*, *Lantana camara*, *Thymus vulgaris*, *Ziziphus spina-christi* and *Zingiber officinale* have antifungal properties

Table 8.2 Botanical pesticides used to control different pests and pathogens

Botanical compounds	Insect pests	Mode of actions	Plant source
Nicotine	Aphids, thrips, caterpillars	Cholinergic acetylcholine nicotinic receptor Agonist/antagonist	*Nicotiana* spp., *Haloxylon salicornicum*, *Stemona japonicum*
Rotenone	Bugs, aphids, potato beetles, spider mites, carpenter ants	Inhibitor of cellular respiration (mitochondrial complex I electron transport inhibitor, METI)	*Lonchocarpus* spp.
Ryania	Codling moths, potato aphids, onion thrips, corn earworms, silkworms	Affect calcium channels	*Ryania* spp.
Sabadilla	Grasshoppers, codling moths, armyworms, aphids, cabbage loopers, squash bugs	Affect nerve cell membrane action	*Schoenocaulon officinale*
Pyrethrum	Caterpillars, aphids, leafhoppers, spider mites, bugs, cabbage worms, beetles	Sodium and potassium ion exchange disruption	*Chrysanthemum cinerariaefolium*
Essential oils	Caterpillars, cabbage worms, aphids, whiteflies, land snails	Inhibition of acetylcholinesterase (AChE)	*Azadirachta indica*, *Mentha* spp., *Lavendula* spp., *Cedrus* spp., *Pinus* spp., *Citronella* spp., *Eucalyptus* spp.
Neem products/ azadirachtin	Armyworms, cutworms, stem borers, bollworms, leaf miners, caterpillars, aphids, whiteflies, leafhoppers, psyllids, scales, mites and thrips	Hormonal balance disruption	*Azadirachta indica*

against *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium aphanidermatum* (Hussin et al. 2009). Ethyl acetate extracts of *Lantana camara* showed inhibitory effects against *Colletotrichum gloeosporioides* which causes anthracnose in papaya (*Carica papaya* L.).The mother tincture extract of *Myroxylon balsamum* showed antifungal activity against the filamentous fungi *Fusarium guttiforme* and *Chalara paradoxa*, causing pineapple fusariosis.

8.5 Different Mechanisms of Biological Control

8.5.1 Direct Antagonism

8.5.1.1 Parasitism

Parasitism is an interactive mechanism in which two phylogenetically unrelated organisms live together over a prolonged period of time. In this type of relationship, one organism, usually benefitted, called the 'parasite' and the other called the 'host', is harmed. For instance, *Trichoderma* is a parasite of a range of fungi and oomycetes in the soil, which produce toxic metabolites and cell wall-degrading enzymes and inhibit the growth of others.

8.5.1.2 Hyperparasitism

Hyperparasites are the agents that are parasites of harmful plant pathogens. A classic example is the *Hypovirus*, a hyperparasitic virus on *Cryphonectria parasitica*, a fungus causing chestnut blight. The hypovirulence of *Hypovirus* reduces the disease-producing capacity of *C. parasitica* (Tjamos et al. 2010). Some strains of fungi have hyperparasitic activity against other fungi. The fungus *Ampelomyces quisqualis* grows on mildew pathogen; similarly *Nectria inventa* and *Gonatobotrys simplex* are parasites of *Alternaria* (Kiss et al. 2004). The fungus *Phlebiopsis gigantea* is used to control *Heterobasidion annosum*, a fungal pathogen that causes rots in freshly cut stumps of pine trees that can spread subsequently to intact trees by root-to-root contact (Pratt et al. 1999). The fungal species, *Acremonium alternatum*, *Acrodontium crateriforme*, *Cladosporium oxysporum* and *Gliocladium virens*, have the capacity to parasitize powdery mildew pathogens and be used as biocontrol agent (Heydari and Pessarakli 2010).

8.5.1.3 Commensalism

Commensalism is a unidirectional association between two unrelated species by living together, in which one population (commensals) benefits from these relationships, while the other (the host) is not harmed. Microbes present in the rhizosphere

control soil-borne pathogens through competition for nutrients and production of antibiotics and help the plants survive pathogen infection (Kumar et al. 2016a, b). On the other hand, the microbes have an important role on the growth of the plant by increasing solubilization of minerals or by synthesizing amino acids, vitamins and growth regulators that stimulate the plant growth.

8.5.2 Mixed-Path Antagonism by Synthesis of Allochemicals

8.5.2.1 Siderophores

Siderophores are ligands with low molecular weight having high affinity to sequester iron from the micro-environment. It has the ability to sequester ferric ion and competitively acquire iron from iron-limiting microenvirons, thereby preventing growth of other microorganisms. Two major classes of siderophores, classified on the basis of their functional group, are catechols and hydroxamate. A mix of carboxylate-hydroxamate group of siderophores is also reported (Hider and Kong 2010) (Table 8.3). Numerous strains of *Streptomyces* spp. have been reported as siderophore producers, namely, *S. pilosus* (Muller et al. 1984; Muller and Raymond 1984), *S. lydicus* (Tokala et al. 2002) and *S. violaceusniger* (Buyer et al. 1989). Biological control of *Erwinia carotovora* by several siderophore-producing and plant growth-promoting *Pseudomonas fluorescens* strains A1, BK1, TL3B1 and B10 was reported for the first time as an important mechanism of biological control (Kloepper et al. 1980). On the other hand, increased efficiency of iron uptake by the commensal microorganisms is thought to dislocate pathogenic microorganisms from the possible infection sites by aggressive colonization in plant rhizosphere.

Table 8.3 Examples of siderophores produced by various bacteria and fungi

Types of siderophores	Siderophore	Organism
Hydroxamate	Ferrichrome	*Ustilago sphaerogena*
	Fusarinine C	*Fusarium roseum*
	Desferrioxamine B	*Streptomyces pilosus*, *Streptomyces coelicolor*
	Desferrioxamine E	*Streptomyces coelicolor*
	2,3-Dihydroxybenzoylglycine	*Bacillus subtilis*
	Ornibactin	*Burkholderia cepacia*
	Rhodotorulic acid	*Rhodotorula pilimanae*
Catecholate	Enterobactin	*Escherichia coli*, *enteric bacteria*
	Bacillibactin	*Bacillus subtilis*, *Bacillus anthracis*
Mixed ligands	Azotobactin	*Azotobacter vinelandii*
	Pyoverdine	*Pseudomonas aeruginosa*
	Yersiniabactin	*Yersinia pestis*

Sneh et al. (1984) and Elad and Baker (1985) showed a direct correlation between in vitro inhibition capacity of chlamydospore germination of *F. oxysporum* and siderophore synthesis in fluorescent pseudomonads.

8.5.2.2 Antibiosis

The term 'antibiosis' came from the term antibiotics, which refers to organic substances produced by microorganisms that affect the metabolic activity of other microbes and inhibit the growth (Roshan et al. 2013). The result of antibiosis is often death of microbial cells by endolysis and breakdown of the cell cytoplasm. *Agrobacterium radiobacter* K-84, produced commercially as Agricon 84, was first recognized as a valuable control agent of crown gall since 1973. It is very effective against *A. tumefaciens* attacking stone fruit (e.g. plums and peaches), but not effective against *A. tumefaciens* strains that attack grapes, pome fruit (e.g. apples) and some ornamentals. A variety of antibiotics have been identified, including compounds such as 2,4-diacetylphloroglucinol (DAPG), amphisin, oomycin A, hydrogen cyanide, pyoluteorin, phenazine, tensin, pyrrolnitrin, cyclic lipopeptides and tropolone produced by pseudomonads and kanosamine, oligomycin A, xanthobaccin and zwittermicin A produced by *Streptomyces*, *Bacillus* and *Stenotrophomonas* spp. (Kumar et al. 2014) (Table 8.4). For instance, antibiotic 2,4-diacetyl phloroglucinol is reported to be involved in the suppression of *Pythium* spp., iturin suppresses the pathogens *Botrytis cinerea* and *Rhizoctonia solani*, and phenazine carboxylic acid antagonist the pathogen *Rhizoctonia solani* in rice (Padaria et al. 2016) and phenazines control *Gaeumannomyces graminis* var. *tritici* in wheat.

8.5.2.3 Volatile Substances

Apart from the production of antibiotics, some biocontrol agents are also known to produce volatile compounds as tools for pathogen inhibition. Common volatile compounds are hydrocyanic acid (HCN), certain acids, alcohols, ketones, aldehydes and sulphides (Bouizgarne 2013). HCN production is reported to play a role in disease suppression (Wei et al. 1991), for instance, Haas et al. (1991) reported HCN production by strains of *P. fluorescens* that helped in the suppression of black root rot of tobacco. Reports on the production of HCN by beneficial microbes in order to minimize the deleterious effect of pathogenic fungi and bacteria are available (Ahmad et al. 2008; Gopalakrishnan et al. 2011a, b, 2014).

8.5.2.4 Lytic Enzyme Production

Many microorganisms secrete and excrete lytic enzymes that can hydrolyse a wide range of polymeric compounds, including hemicellulose, cellulose, chitin, DNA

Table 8.4 Selected examples of antibiotics produced by biocontrol bacteria

Antibiotic	Source	Target pathogen
2,4-Diacetyl phloroglucinol	*Pseudomonas fluorescens* F113	*Pythium* spp.
2,4-DAPG	*Pseudomonas* sp.	*Xanthomonas oryzae* pv. *oryzae*
2-Hexyl, 5-propyl resorcinol	*P. fluorescens*	*Rosellinia necatrix*
Agrocin 84	*Agrobacterium radiobacter*	*A. tumefaciens*
Amphisin	*P. fluorescens*	*Pythium ultimum* and *Rhizoctonia solani*
Bacillomycin D	*Bacillus subtilis* AU195	*Aspergillus flavus*
Bacillomycin, fengycin	*B. amyloliquefaciens* FZB42	*Fusarium oxysporum*
Cyclic lipopeptides	*Pseudomonas* sp.	*Phytophthora infestans*
Geldanomycin	*Streptomyces hygroscopicus* var. *geldonus*	*R. solani*
Gliotoxin	*Trichoderma virens*	*R. solani*
Herbicolin	*Pantoea agglomerans* C9-1	*Erwinia amylovora*
Iturin A	*B. subtilis* QST713	*Botrytis cinerea*, *P. ultimum*, *R. solani*, *F. oxysporum*, *Sclerotinia sclerotiorum* and *Macrophomina phaseoli*
Iturin A and surfactin	*B. subtilis*	*R. solani*
Kanosamine	*B. cereus*	*Phytophthora medicaginis*
Kasugamycin	*S. kasugaensis*	*Pyricularia oryzae*
Mycosubtilin	*B. subtilis* BBG100	*Pythium aphanidermatum*
Oligomycin A	*S. libani*	*B. cinerea*
Phenazines	*P. fluorescens* 2–79 and 30–84	*Gaeumannomyces graminis* var. *tritici*
	P. chlororaphis	*F. oxysporum*
	P. aureofaciens	*Sclerotinia homeocarpa*
Phenazine-1-carboxamide	*P. chlororaphis*	*F. oxysporum* f. sp. *radicis-lycopersici*
Polyoxin D	*S. cacaoi*	*R. solani*
Pyoluteorin	*P. fluorescens*	*P. ultimum*
Pyoluteorin, pyrrolnitrin	*P. fluorescens* Pf-5	*P. ultimum* and *R. solani*
Pyrrolnitrin, pseudane	*Burkholderia cepacia*	*R. solani* and *Pyricularia oryzae*
	P. fluorescens	*Gaeumannomyces graminis* var. *tritici*
		R. solani
	Enterobacter agglomerans	*A. tumefaciens*, *Clavibacterium michiganense*, *Xanthomonas campestris*, *Pseudomonas syringae* pv. *syringae*

(continued)

Table 8.4 (continued)

Antibiotic	Source	Target pathogen
Polyenes	*S. violaceusniger*	*P. ultimum*
Citrinin	*Penicillium citrinum*	*B. cinerea*
Viscosinamide	*P. fluorescens*	*R. solani*, *P. ultimum*
Xanthobaccin A	*Lysobacter* sp. strain SB-K88	*Aphanomyces cochlioides*
Zwittermicin A	*B. cereus* UW85	*P. medicaginis* and *P. aphanidermatum*
	B. cereus and *B. thuringiensis*	*Phytophthora* spp.
	Bacillus spp.	*S. sclerotiorum*
	B. cereus	*Phytophthora parasitica* var. *nicotianae*

and proteins (Table 8.5). These extracellular hydrolytic enzymes play an important role in the suppression of plant pathogens. Chitinase secreted by *Streptomyces* sp., *Paenibacillus* sp. and *Serratia marcescens* was found to be inhibitory against *Sclerotium rolfsii*, *Botrytis cinerea* and *Fusarium oxysporum* f. sp. *cucumerinum*. Similarly, modifying plant growth substratum with chitosan inhibits the root rot in tomato caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. β-1,3-Glucanase produced by *Actinoplanes philippinensis* and *Micromonospora chalcea* was found to hydrolyse *Pythium aphanidermatum* in cucumber (El-Tarabily 2006).

8.5.2.5 Unregulated Waste Products

Few soil microbes release a range of unregulated waste products or harmful gases, e.g. ethylene, methane, nitrite, ammonia, hydrogen sulphide, other volatile sulphur compounds, carbon dioxide, etc., and suppress the growth of other pant pathogenic bacteria. This interaction between two species is called ammensalism. *Bacillus megaterium* produces ammonia and has an inhibitory effect on the growth of *Fusarium oxysporum* (Shobha and Kumudini 2012).

8.5.2.6 Detoxification and Degradation of Virulence Factor

Biological control by detoxification involves production of a protein that binds with the pathogen toxin and detoxifies pathogen virulence factors, either reversibly or irreversibly, ultimately decreasing the virulence potential of pathogen toxin. For example, the biocontrol agents *Alcaligenes denitrificans* and *Pantoea dispersa* are able to detoxify albicidin toxin produced by *Xanthomonas albilineans*. Similarly, strains like *B. cepacia* and *Ralstonia solanacearum* can hydrolyse fusaric acid, a phytotoxin produced by various *Fusarium* spp. The protein has the ability to bind reversibly with the toxins of both *Klebsiella oxytoca* and *Alcaligenes denitrificans*, as well as irreversibly with the toxin albicidin in *Pantoea dispersa*.

Table 8.5 Examples of lytic enzymes produced by biocontrol bacteria

Enzyme	Producing bacteria	Target phytopathogen	Host plant
Chitinases	*Aeromonas caviae*	*Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *vasinfectum*	Cotton
		Sclerotium rolfsii	Beans
	Arthrobacter sp.	*Fusarium* sp.	Carnation
		F. moniliforme var. *subglutinans*	Southern pines
	Streptomyces sp.	*Macrophomina phaseolina*	Sorghum
	Enterobacter agglomerans, *Bacillus cereus*	*R. solani*	Cotton
	B. circulans and *Serratia marcescens*	*Phaeoisariopsis personata*	Peanut
	E. agglomerans, *B. cereus*	*R. solani*	Cotton
	Paenibacillus illinoisensis	*R. solani*	Cucumber
	Pseudomonas sp.	*F. oxysp*orum f. sp. *cucumerinum*	Cucumber
	Serratia plymuthica	*Botrytis cinerea* and *Sclerotinia sclerotiorum*	Cucumber
	Serratia marcescens	*S. rolfsii*	Beans
		R. solani	Cotton
	Streptomyces lydicus	*Pythium* and *Aphanomyces*	-
	Serratia plymuthica	*Botrytis cinerea*	Many host
Glucanases	*Streptomyces* sp.	*Phytophthora fragariae*	Raspberry
	Pseudomonas cepacia	*R. solani*, *S. rolfsii*, *Pythium ultimum*	
	Actinoplanes philippinensis and *Micromonospora chalcea*	*Pythium aphanidermatum*	Cucumber
	Lysobacter enzymogenes	*Pythium*	Sugar beet
	Paenibacillus, *B. cepacia*	*F. oxysporum*, *R. solani*, *S. rolfsii* and *Pythium ultimum*	-
Chitinases and glucanases	*Serratia marcescens*, *Streptomyces viridodiasticus*, *Micromonospora carbonacea*	*Sclerotinia minor*	Lettuce
	L. enzymogenes	*F. graminearum*	Wheat
	Streptomyces sp. and *Paenibacillus* sp.	*F. oxysporum* f. sp. *cucumerinum*	Cucumber
Chitinases, proteases and cellulases	*B. subtilis*, *Erwinia herbicola*, *Serratia plymuthica* and *Actinomycete*	*Eutypa lata*	Grapevine
Proteases	*Stenotrophomonas maltophilia*	*P. ultimum*	Sugar beet
Laminarinase	*Pseudomonas stutzeri*	*F. solani*	-

8.5.3 Indirect Antagonism

8.5.3.1 Competitive Root Colonization

From the microbial perspective, living plant surfaces and soils are often nutrient-restricted environments. Nutrient limitation is an important mode of action of some biological control agents. Carbon plays an important role for competition of root colonization for nutrients such as *Trichoderm*a spp. (Sivan and Chet 1989). Carbon competition between pathogenic and non-pathogenic strains of *F. oxysporum* is one of the main mechanisms in the suppression of *Fusarium* wilt (Alabouvette et al. 2009). The disease suppression of bacterium *Erwinia amylovora* causes fireblight by the closely related saprophytic species *E. herbicola* due to competition of the nutrient on the leaf surface. Competition between rhizosphere bacteria and *Pythium ultimum*, a common cause of seedling damping-off for the same carbon source, has resulted in an effective biological control of the latter organism in several crops. Germination of the conidia of *Botrytis cinerea* is inhibited by *Pseudomonas* species due to competition for amino acids. This mechanism may not be useful in suppressing biotrophs such as powdery mildews and rusts, because they do not require exogenous nutrients for host infection.

8.5.3.2 Plant Growth Promotion Through SAR and ISR

Chemical stimuli are produced by some biocontrol agents, i.e. non-pathogenic plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF), or by soil- and plant-associated microbes. Such stimuli can either induce a sustained change in the plants which increase the capacity of tolerance to infection by pathogens or induce the local and/or systemic host defences of the whole plant against broad-spectrum pathogens. This phenomenon is known as induced resistance. Two types of induced resistance are distinguished in plants, systemic acquired resistance (SAR) and induced systemic resistance (ISR). The first of the two pathways is mediated by salicylic acid (SA) which is frequently produced after pathogen infection and induces the expression of pathogenesis-related (PR) proteins that include a variety of enzymes. The second method is mainly jasmonic acid (JA) and/or ethylene mediated following the applications of some nonpathogenic rhizobacteria (Fig. 8.2). The SAR-induced resistant was observed when *Trichoderma harzianum* was inoculated in roots and leaves of grapes, and it provides control of diseases caused by *Botrytis cinerea* from the site of application of *T. harzianum* (Deshmukh et al. 2006). It was found that the biocontrol agent *P. fluorescens* strain CHAO induces accumulation of salicylic acid and by inducing SAR-associated proteins confers systemic resistance to a vira1 pathogen in tobacco. Colonization of *Glomus intraradices* on the roots of *Oryza sativa* conferred resistance through induction of defence-related genes (Campos-Soriano et al. 2012). *Penicillium simplicissimum* enhanced the resistance of barley to *Colletotrichum orbiculare* by inducing salicylic acid accumulation, formation of active oxygen species, lignin deposition

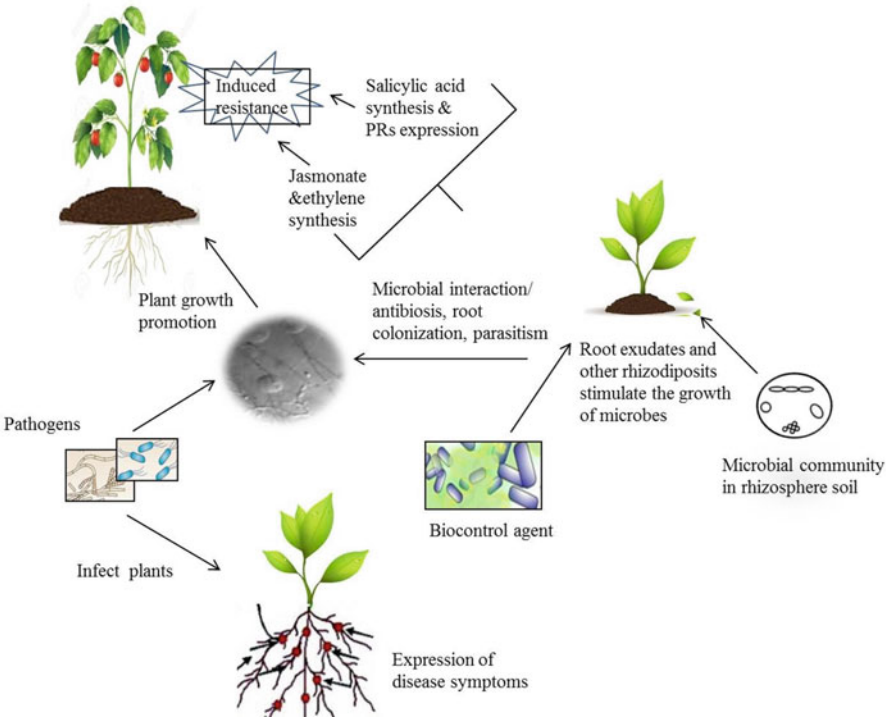

Fig. 8.2 Interaction within the plants, pathogenic microorganisms and biocontrol

and activation of defence genes. In addition, *Fusarium equiseti* and *Phoma* sp. elicited *Arabidopsis thaliana* systemic resistance *against Pseudomonas syringae* pv. tomato and *Pythium oligandrum* against *Ralstonia solanacearum*. However, different ISR elicitors like secondary metabolites and proteins involved in mycoparasitism and antibiosis have also been identified. Secondary metabolites like trichokinin, alamethicin, harzianopyridone, harzianolide and 6-pentyl–αpyrone have antagonist effects at high doses but in low doses act as ISR inducers. Expression of endochitinase *Ech42* of *Trichoderma atroviride* was found to act as an ISR inducer in barley, resulting in an increased resistance to *Fusarium* sp. infection. Similarly, chitinase *Chit42* of *T. harzianum* expression increased resistance in potato and tobacco against the foliar pathogens, *B. cinerea*, *Alternaria solani* and *A. alternata*, and soil-borne pathogen, *Rhizoctonia solani*.

8.6 Genomic Approaches of Biocontrol Agent

Recent advances in molecular technologies have brought a revolution in microbial worlds and unzipped the immense diversity in microbial population helping scientific community to find out novel biocontrol agents (Kumar et al. 2014; Sharma

et al. 2016). Utilizing bioinformatics tools and inexpensive sequencing techniques has led to the assembly of genomic data for microbial biocontrol agents and exploring the untapped and novel microbial isolates for important secondary metabolites and enzymes.

Seventy-eight percent of the genes functionally associated with antagonism were found to be distributed in *Trichoderma* species, described as the best fungal biocontrol agent till date. This is followed by *Coniothyrium*, *Pythium* and *Clonostachys* with 6, 5 and 4%, respectively. The way of antagonism is different in different microbes and sometimes depends on the pathogens. The genes associated with antagonism are diverse and involved in antibiosis, signalling, parasitism or transport. Of the identified genes, 44% are related to mycoparasitism, and 26% were for the antibiosis, whereas ISR-, signalling- and competition-related genes represent only 12, 11 and 5%, respectively. The role of different glucanases and chitinases during mycoparasitism is demonstrated with the functional characterization by gene-by-gene study in *Trichoderma* spp. (Daguerre et al. 2014). However, molecular mechanisms involved in the antagonism are not well known for all the cases. Now metatranscriptomic analyses appear as a more powerful tool as they provide generous information on different aspects of the antagonism allowing for comparison from the early stages to the later ones. The use of metatranscriptomic analyses prior to functional characterization seems to be the most sensible strategy. However, functional characterization is needed for verifying and ensuring the molecular mechanisms of antagonism.

The use of advanced molecular technique and genomic approaches in the identification of novel biocontrol agent is in its initial stages, but in the near future, latent biochemical products may arise as the key of antagonism of major phytopathogens as well as PGP in crops. For example, a total number of six genera of actinobacteria, viz. *Corynebacterium*, *Mycobacterium*, *Arthrobacter*, *Frankia*, *Rhodococcus* and *Streptomyces*, have been sequenced and analysed for potential secondary metabolite and gene diversity (James and William 2013).

8.7 Commercially Available Eco-friendly Biological Agents

Formulation of biopesticide based on a variety of microorganisms, e.g. nematodes, protozoa, fungi, bacteria, viruses, etc., is known as microbial pesticides or biocontrol agents. Predominantly five microbes, *P. fluorescens*, *B. subtilis*, *Gliocladium* spp., *Verticillium lecanii* and *Trichoderma* spp., are used for the purpose of commercial microbial pesticides. Several biopesticides are commercially available (Table 8.6) globally. However, in India only 35 microbes have been included in the Insecticides Act (1968) till now for commercial production of biocontrol agent, since the first biopesticide was notified in the *Gazette of India* dated 26 March 1999. In India, Singh (2006) identified novel *Trichoderma* strain with enhanced nematicidal, fungicidal and growth promotion property and used for developing biocontrol agent. The technology was transferred to Department of Agriculture, Government of UP, for its commercial production. Later on, this technology has also been

Table 8.6 List of biocontrol products commercially available for the control of plant pathogens

Product	Biocontrol agent	Target disease/organism	Crop	Manufacturer
Actinovate	*Streptomyces lydicus*	Soil-borne disease	Greenhouse and nursery crops, turf	Natural Industries, Inc., USA
Avogreen	*Bacillus subtilis*	*Pseudocercospora purpurea*	Avocado	Stimuplant, South Africa
Alfa guard	*Aspergillus flavus* AF36	*Aspergillus flavus*	Cotton	Circle One Global, USA
AQ10 Biofungicide	*Ampelomyces quisqualis* isolate M10	Powdery mildew	Apples, cucurbits, grapes, ornamentals, strawberries and tomato	Ecogen, Inc., USA
Aspire	*Candida oleophila* I182	*Botrytis* spp., *Penicillium* spp.	Citrus, pome fruit	Ecogen, Inc.
Ballad Plus	*Bacillus pumilus*	Rust, powdery mildew, cercospora, brown spot	Soybean	AgraQuest, USA
Biobest	*Bacillus subtilis*	Sheath blight, blast, brown spot	Rice	Appliedchem, Thailand
BioJect SpotLess	*Pseudomonas aureofaciens* strain TX-1	*Pythium*, *Rhizoctonia solani*	Vegetables and ornamentals in greenhouses	Eco Soil Systems, Inc.
Biosave 10LP, 110	*Pseudomonas syringae*	*Botrytis cinerea*, *Penicillium* spp., *Mucor piriformis*, *Geotrichum candidum*	Pome fruit, citrus, cherries and potato	Village Farms LLC
BlightBan A506	*Pseudomonas fluorescens* A506	*Erwinia amylovora* and russet-inducing bacteria	Almond, apple, apricot, blueberry, cherry, peach, pear, potato, strawberry, tomato	NuFarm Inc.
Cedomon	*Pseudomonas chlororaphis*	Leaf stripe, net blotch, *Fusarium* sp., spot blotch, leaf spot and others	Barley and oats; potential for other cereals	BioAgri AB
Companion	*Bacillus subtilis* GB03, other *B. subtilis*, *B. licheniformis*, *B. megaterium*	*Rhizoctonia*, *Pythium*, *Fusarium*, *Sclerotinia* and *Phytophthora*	Greenhouse, nursery and ornamental crops	Growth Products, USA
Contans WG, Intercept WG	*Coniothyrium minitans*	*Sclerotinia sclerotiorum* and *S. minor*	All types of crops	Prophyta Biologischer Pflanzenschutz GmbH, Germany

Cyd-Xe	Codling moth granulosis virus	Codling moth	Apple, pear, walnut and plum	Thermo Trilogy Corp., Columbia
Deny	*Burkholderia cepacia type*	*Rhizoctonia*, *Pythium*, *Fusarium* and disease caused by lesion, spiral, lance and sting nematodes	Alfalfa, barley, beans, clover, cotton, peas, grain sorghum, vegetable crops and wheat	Stine Microbial Products
EcoGuard	*B. licheniformis*	Dollar spot, anthracnose	Turf	Novozymes, Denmark
Frostban	*P. fluorescence* strain A506	Fire blight, bunch rot	Fruit crop, tomato, potato	Plant Health Technologies
Galltrol	*Agrobacterium radiobacter* strain 84	*Agrobacterium tumefaciens*	Fruit, nut and ornamental nursery stock	AgBioChem Inc., USA
GB34	*Bacillus subtilis* strain GB34	*Rhizoctonia*, *Fusarium*	Soybean	Gustafon, USA
HiStick N/T	*Bacillus subtilis* MBI600	*Fusarium*, *Rhizoctonia*, *Aspergillus*	Soybean, alfalfa, dry/snap beans, peanuts	Becker Underwood Inc., UK
Intercept	*Burkholderia cepacia*	*Rhizoctonia solani*, *Fusarium* spp., *Pythium* sp.	Maize, vegetables, cotton	Soil Technologies Corp.
Kodiak	*Bacillus subtilis* GB03	*Rhizoctonia solani*, *Pythium*, *Fusarium* spp., *Alternaria* spp. and *Aspergillus* spp. that attack roots	Wheat, barley, peas, cotton, legumes, soybean and vegetable crops	Bayer CropScience, USA
Larminar	*B. subtilis*	*Alternaria* spp., *Botryodiplodia* sp., *Colletotrichum* sp., *Corticium* sp., *Fusarium* spp., *Phytophthora* spp.	Vegetables, fruit trees, ornamentals, rice and field crops	Appliedchem, Thailand
Messenger	*Erwinia amylovora* HrpN harpin protein	Many	Field, ornamental, and vegetable crops	EDEN Bioscience Corporation,
Mycostop	*Streptomyces griseoviridis* strain K61	*Fusarium* spp., *Alternaria brassicola*, *Phomopsis* spp., *Botrytis* spp., *Pythium* spp. and *Phytophthora* spp. that cause seed, root and stem rot, and wilt disease	Field, ornamental, vegetable crops and tree seedlings	Kemira Agro Oy, Finland
Nogall	*Agrobacterium radiobacter* K1026	*Agrobacterium tumefaciens*	Fruit, nut, and ornamental nursery stock	Biocare Technology, Australia

(continued)

Table 8.6 (continued)

Product	Biocontrol agent	Target disease/organism	Crop	Manufacturer
Primastopsoil guard	*Gliocladium catenulatum*	Soil-borne pathogens that cause seed, root and stem rot and wilt disease	Ornamental, vegetable, spices and tree crops	Kemira Agro Oy, Finland
Rhapsody	*B. subtilis*	*Rhizoctonia*, *Fusarium*, *Pythium*, *Phytophthora*	Turf, ornamental, vegetables and fruits in greenhouse	AgraQuest, USA
RootShield, PlantShield	*Trichoderma harzianum* strain KRLAG2 T22	*Pythium* spp., *Rhizoctonia solani*, *Fusarium* spp.	Trees, shrubs, transplants, all ornamentals, cabbage, tomato, cucumber	Bioworks Inc., USA
Serenade	*Bacillus subtilis* QST716	Powdery mildew, downy mildew, *Cercospora* leaf spot, early blight, late blight, brown rot, fire blight, *Botrytis* and *Sclerotinia* disease and others	Cucurbits, grapes, hops, vegetables, peanuts, pome fruits, stone fruits and others	AgraQuest Inc., USA
SoilGard	*Gliocladium virens*	Damping-off and root rot pathogens especially *Rhizoctonia solani* and *Pythium* spp.	Ornamental and food crop plants grown in greenhouses, nurseries	Certis Inc., USA
Sonata	*B. pumilus*	Rusts, powdery and downy mildews	Vegetable and fruit crops	AgraQuest, USA
Subtilex	*B. subtilis*	*Rhizoctonia*, *Fusarium*, *Aspergillus*	Field, ornamental and vegetable crops	Becker Underwood, USA
Taegro	*B. amyloliquefaciens*	*Rhizoctonia*, *Fusarium*	Tree seedlings, ornamentals and shrubs	Novozymes, Denmark
Trichodex	*Trichoderma harzianum* T-39	*Botrytis cinerea*	Most of the food crops	Bio works, USA
YiedShield	*Bacillus pumilus* GB34	Soil-borne fungal pathogens causing root diseases	Soybean	Gustafson Inc., USA

transferred to Gujarat State Fertilizer and Chemicals Limited (GSFC), Gujarat Green Revolution Company Limited (GGRC) and Balaji Crop Care Pvt. Ltd., Hyderabad, for commercial production. The products 'Sardar Eco Green Biofungicide' and 'TRICHA' based on a potential strain of *Trichoderma harzianum* NBRI-1055 are in market for controlling phytopathogenic fungi. A talc-based formulation of *Trichoderma viride* strain 2953 has recently been transferred to Balaji Crop Care Pvt. Ltd., Hyderabad, for large-scale production.

8.8 Socio-economic Impact, Ethical Issues Winding with the Biocontrol

Global assessment of biocontrol agents' commercial availability in markets shows that the percentage of users and land have steadily increased since the late 1990s and the projected growth is continuing at a rate of 15.6% per year (Glare et al. 2012). Lehr (2010) reported that the global sales of biocontrol agents were estimated at US$ 396.48 million in 2003 and have continued to increase with projections to reach up to US$ 1.068 billion by 2010. With the successful implementation of biological agents in field for integrated plant disease management, demand for commercial biocontrol agent is increasing within the growers. There are approximately 225 microbial biocontrol agents which were manufactured in 30 member countries and registered by the Organization for Economic Development and Cooperation (Kabaluk and Gazdik 2007) for commercialization. The rest of the global market share is distributed among the countries within the Oceania at 20%, Latin and South American countries at 10% and less than 5% each accredited to Asia and India (Thakore 2006). The chances of future market expansion within the latter countries are likely to be variable. Organic and conventional producers are anticipating the use of alternative biocontrol products that pose a lower-risk exposure to human health than synthetic chemicals. Worldwide evolutionary exploration with the microbial products and the illustrating actions of the government personnel within the country, the growers and the industry have led to changes in strategy, management and research initiatives. On the other hand, legislation concurrently is supporting to make new policy that encouraged the registration of lower-risk pest control products.

Quality of the inoculants available in the market, however, needs to be carefully monitored as the formulation available in the market should contain sufficient population of the biocontrol microbes to produce an economic gain. Many countries such as the Netherlands, Thailand, Russia, France, Australia, Canada and the UK have regulations for inoculant quality which lead to improvements in the quality of commercial inoculants (Bashan et al. 2014). Canada and France have set norms that formulated products should have 10^6 viable cells per seed with no detectable contaminants (Catroux et al. 2001). However, that is not the case in developing countries as most of the inoculants produced are of poor or sub-optimal quality.

Brockwell and Bottomley (1995) observed that most of the inoculants produced in the world is of relatively poor quality and 90% of all inoculants has no practical effect on the productivity of crops for which it is used. Further, the presence and nature of contaminants encountered in inoculants may represent a risk for humans, plants and for the environment, which remains to be assessed. Hence, quality of inoculants available in the market needs to be closely monitored, and make sure that farmers use the quality inoculants so that they will have trust on biocontrol.

8.9 Future Prospects for Biocontrol

In the past five decades, an increasing number of chemical fertilizer and biocidal molecules were the main cause for a substantial increase in crop production and quality. Because of environmental issues and health concerns, continuous and extensive use of those molecules has raised serious debate, and often various biological control methods based on natural pest and pathogen-suppressing organisms are being recommended as a substitute. Globally the registrations of microbial biocontrol agents are increasing significantly. The changes in legislation in the country level, development of new policies and management structures to address the reduction of chemical uses are the expanding scope of biocontrol agents. On the other hand, the researchers worldwide have been supported to discover new biocontrol agents to reinforce for entering in the industry. Being practical, at present biocontrol agents are not comparable to chemical pesticides in meeting efficacy which is needed for market expectations, but they still have a promising future if knowledge and methods of various fields of biotechnology are utilized. The availability of recent molecular technologies has significantly facilitated for surveying and identification of candidate agents, and helped to interpret the modes of action after field applications. These new technologies like proteomics and functional genomics will give new possibilities for insights in ecological constraints and will help to see hitherto unseen possibilities to determine the physiological status and expression of crucial genes present within the biocontrol agents during mass production, formulation, storage and application.

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Chapter 9
Biological Control of Insect Pests for Sustainable Agriculture

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Abstract Maintenance of agricultural productivity is currently based mainly on extraneous application of fertilizers and pesticides. However, indiscriminate use of agrochemicals for controlling the pests and diseases led to pollution of soil, water, and food sources, poisoning of nontarget beneficial insects, and development of insect population resistant to insecticides. To obviate the pollution problem and obtain higher yields in a sustainable manner, biological control of insect pests using specific antagonistic microorganisms is an effective alternate approach with minimum deleterious effects. Microorganisms have been obtained from the rhizosphere of different crop plants that inhibited insect pests by producing toxins, bacteriocins, siderophores, hydrolytic enzymes, and other secondary metabolites. Moreover, plant hormones salicylic acid, jasmonic acid, and ethylene orchestrate a complex transcriptional programming that eventually leads to pest-induced SAR (systemic acquired resistance) and ISR (induced systemic resistance) in many plant species. Microbial genes involved in the biosynthesis of secondary metabolites and enzymes have been cloned and transferred to other microorganisms and plants to enhance the suppression and killing of insects. The efficiency of these biocontrol products can be further increased through genetic improvement, manipulation of the soil and plant environment, using mixtures of biocontrol agents, and optimization of formulations and by integration of biocontrol agents with other alternative methods that provide additive and synergistic effects. Thus, the application of effective biocontrol agents may reduce the use of chemical insecticides and support sustainable agriculture in an eco-friendly manner in tandem with improved crop productivity.

Keywords Biological control · Insect pests · Rhizosphere microorganisms · Sustainable agriculture

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9.1 Introduction

Global crop yields are reduced by 20–40% annually due to pests and diseases (FAO 2012). For the control of insect pests in agriculture, farmers have mostly relied on the application of synthetic pesticides, and the global pesticide market is presently growing at a rate of 3.6% per year (Lehr 2010). However, indiscriminate use of chemical pesticides to control the pathogens/insects has generated several problems including resistance to insecticides/fungicides, outbreak of secondary pests, as well as safety risks for humans and domestic animals. Moreover, the long persistence of applied pesticides in soil leads to contamination of groundwater and soil, and the residual toxic chemicals enter into the food chain. Excessive pesticide application also decreases the biodiversity due to destruction of nontarget entomofauna. These problems have increased the interest of scientists for development of eco-friendly microbe-based insecticides or biocontrol agents, which act differently from known chemicals (Ruiu et al. 2013). Sustainable agriculture in the twenty-first century will rely increasingly on alternative interventions for pest management that are environment-friendly and will reduce the human contact with chemical pesticides. Therefore, microorganisms are currently being explored for their possible use as biocontrol agents in the integrated pest management programs.

Some of the microorganisms obtained from the rhizosphere of crop plants provide the frontline defense to plant roots against the attack by various plant pathogens and insects (Compant et al. 2005). Several microorganisms including bacteria, actinomycetes, fungi, viruses, protozoa, and nematodes have been identified to control various root, foliage, and postharvest diseases of agricultural crops (Glick and Bashan 1997), and many microorganisms have been found to act as potential entomopathogens (Vaga and Kaya 2012; Lacey et al. 2015; Mascarin and Jaronski 2016). Among the various microbial control agents (MCAs), *Bacillus thuringiensis* (*Bt*), *Pseudomonas fluorescens*, *Serratia marcescens*, *Streptomyces* sp., *Lecanicillium lecanii*, *Trichoderma virens*, *Metarhizium* sp., *Beauveria bassiana*, and nuclear polyhedrosis virus are popularly used in plant protection (Mascarin and Jaronski 2016; Sindhu et al. 2016). So far, about 175 biopesticide active ingredients and 700 products have been registered worldwide.

9.2 Characterization of Microorganisms Involved in Biological Control of Insect Pests

Several microorganisms inhabiting either the soil or plant rhizosphere have been identified to act as entomopathogens, and some of the microbes have also been found to suppress the diseases of agricultural crops (Borneman and Becker 2007; Lacey et al. 2015). The microorganisms isolated from the rhizosphere soil could be screened for their biocontrol activities for subsequent use as biocontrol agents (Fig. 9.1). Li et al. (2015) demonstrated distinct variations in the microbial

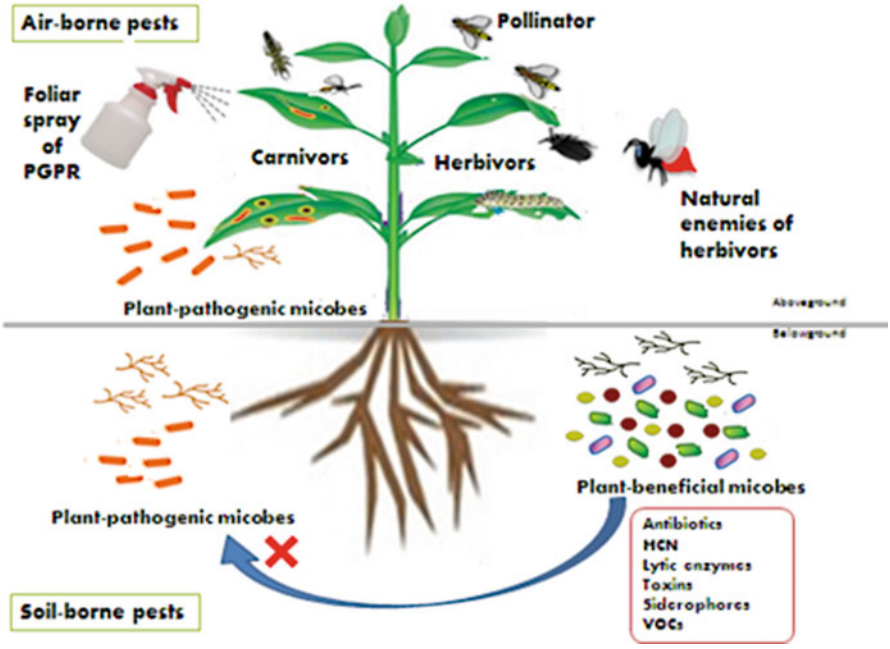

Fig. 9.1 Interactions of pathogens, insects, and biocontrol agents affecting the plant growth

community of cotton rhizosphere between monocropped (4 and 15 years) rhizosphere soils and fallow (control) agricultural soil. The monocropped soils significantly influenced the composition of root exudates and also reduced soil suppressiveness to Fusarium wilt. A significant correlation existed between the presence of certain amino acids (e.g., glutamic acid and alanine) and predominant bacterial taxa in the rhizosphere, indicating that some constituents in root exudates influenced the microbial compositions of the cotton rhizosphere to manage the disease status of plants in monocropped soils.

9.2.1 Antagonistic Bacteria

Rhizobacteria have been found to inhibit the growth of various pathogenic bacteria, fungi, and insects resulting in suppression of the diseases caused by different pathogens and insect pests (Sindhu et al. 2014). *Bacillus thuringiensis* (*Bt*) is the most studied entomopathogenic species for biological control of insect pests, and some of the toxin-producing strains have shown high mortality against specific insects compared to conventional insecticides used in the microbial pest management (Vega and Kaya 2012). The insecticidal proteins produced by *B. thuringiensis* are highly specific insect gut toxins and do not affect the nontarget organisms

(Lacey and Goettel 1995). *B. thuringiensis* subspecies *kurstaki* strain HD-1 (De Barjac and Lemille 1970) is most widely used for the management of lepidopteran pests in agriculture and forestry, whereas *B. thuringiensis* subspecies *israelensis* and *Lysinibacillus sphaericus* are the pathogens used for medically important pests including dipteran vectors. Strains of *B. thuringiensis* subsp. *aizawai* (*Bta*) (i.e., ABTS-1857) are used against armyworms and diamondback moth larvae. Similarly, *Bacillus* strains belonging to the subsp. *israelensis* (*Bti*) and *tenebrionis* (*Btt*) have been employed for the management of mosquitoes and simulids and against coleoptera, respectively (Glare and O'Callaghan 2000).

Other *Bacillus* species have also shown potential for insect pest management. Spherical endospore-producing bacteria *B. sphaericus* (Alexander and Priest 1990) produce parasporal crystals located within the exosporium, and these strains showed toxicity against mosquitoes. The equimolar ratio of the two homologous binary protein toxins (Bin), BinA and BinB, found in parasporal bodies acts in a similar fashion as Cry proteins (Charles et al. 2000). Other entomopathogenic bacteria that possess potential against diverse insect pests include *B. popilliae* with *B. lentimorbus*, the causal agents of milky disease in phytophagous scarab larvae (Zhang et al. 1997). *Serratia entomophila* contains a specific plasmid (pADAP) encoding genes implicated in pathogenicity against the grass-grub, *Costelytra zealandica* (White) (Jackson et al. 1992). *Clostridium bifermentans* serovar *malaysia* has been found effective against mosquitoes and blackflies (Nicolas et al. 1990). The mosquitocidal activity in this bacterium was associated to the production of a protein with homology to *Bt* δ-endotoxins (Cbm71). The encoding gene of this protein was cloned and induced for expression by transformed *Bt*, which exhibited toxicity against mosquitoes (Barloy et al. 1996).

Another group of entomopathogenic bacteria includes the endosymbionts of insecticidal nematodes, especially the members of the genera *Xenorhabdus* and *Photorhabdus* (Burnell and Stock 2000). These entomopathogenic bacteria and the nematodes produce a variety of metabolites that enable them to colonize and reproduce in the insect host. The metabolites produced include enzymes such as proteases, lipases, and phospholipases to maintain a food supply during reproduction (Bowen et al. 2000). Antifungal and antibacterial agents prevent the degradation or colonization of the insect carcass, while the bacteria and nematodes reproduce. Bowen (1995) reported that a soluble protein fraction purified from *P. luminescens* culture medium possessed sufficient insecticidal activity to kill *Manduca sexta* upon injection. The novel protein toxin secreted by bacterium *Xenorhabdus nematophila* was found effective against *Galleria mellonella* and *H. armigera*, cabbage white caterpillar *Pieris brassicae*, mosquito larva *Aedes aegypti*, and mustard beetle *Phaedon cochleariae* (Sergeant et al. 2006). These bacteria were found effective on most of the economically important lepidopteran, dipteran, and coleopteran insect orders, suggesting wide scope of these organisms for application in insect pest management.

Common soil organism *B. cereus* has also been found pathogenic to insects and has been isolated from several insect species (Kuzina et al. 2001; Sezen et al. 2005). Various bacterial isolates, i.e., *B. cereus* (Ags1), *Bacillus* sp. (Ags2), *B. megaterium*

(Ags3), *Enterobacter aerogenes* (Ags4), *Acinetobacter calcoaceticus* (Ags5), *Enterobacter* sp. (Ags6), *Pseudomonas putida* (Ags7), *Enterococcus gallinarum* (Ags8), and *Stenotrophomonas maltophilia* (Ags9), were identified from the flora of *Agrotis segetum* (Sevim et al. 2010), and these isolates caused 60% insect mortality after 8 days of application. *B. cereus*, *B. sphaericus*, *Morganella morganii*, *Serratia marcescens*, and *Klebsiella* species isolated from the predatory larvae of the antlion species *Myrmeleon bore* (Neuroptera, Myrmeleontidae) were found to kill 80% or more cutworms *S. litura* (Nishiwaki et al. 2007). The bacterial flora *Leclercia adecarboxylata* of Colorado potato beetle showed highest insecticidal effect (100% mortality) within 5 days (Muratoglu et al. 2009) and thus showed a potential for the control of several coleopteran pests.

Pseudomonas entomophila showed insecticidal properties against insects in different orders and triggered a systemic immune response in *Drosophila melanogaster* Meigen after ingestion (Vodovar et al. 2006). Similarly, biopesticidal potential of *Brevibacillus laterosporus* Laubach has been reported against insects in different orders, such as coleoptera (Boets et al. 2004), lepidoptera (Oliveira et al. 2004), mosquitoes and blackflies (Rivers et al. 1991), and houseflies (Ruiu et al. 2006), nematodes (Singer 1996), and against phytopathogenic fungi (Saikia et al. 2011). *Chromobacterium subtsugae* showed its insecticidal potential after ingestion against diverse insect species in different orders (i.e., coleoptera, lepidoptera, hemiptera) (Hoshino 2011). These insects included Colorado potato beetle (*Leptinotarsa decemlineata* Say), Western corn rootworm (*Diabrotica virgifera* Le Conte), Southern corn rootworm (*Diabrotica undecimpunctata* Mannerheim), small hive beetle (*Aethina tumida* Murray), diamondback moth (*Plutella xylostella* L.), sweet potato whitefly (*Bemisia tabaci* Gennadius), and Southern green stink bug (*Nezara viridula* L.) (Martin et al. 2007).

Subterranean termites have been found to cause extensive damage to major agricultural crops and forest plantation trees. Khan et al. (1985) reported that a commercial preparation of *B. thuringiensis* (Thuricide-HP concentrate) exhibited 100% mortality within 6 days of exposure against *H. indicola*, *M. championi*, and *Bifiditermes beesoni* (Gardner) (Kalotermitidae). Similarly, the colonies of *M. championi*, *H. indicola*, and *B. beesoni* exposed to suspensions of the spore-forming bacterium *Serratia marcescens* Bizio succumbed completely 7–13 days following infection (Khan et al. 1977). Khan et al. (1992) showed that mortality of *M. championi*, *H. indicola*, and *Coptotermes heimi* (Wasmann) (Rhinotermitidae) termites ranged from 25–52% after 7 days postinoculation to 84–100% 25 days after postinoculation due to the pathogenicity of *Pseudomonas aeruginosa* (Schroeter) in the laboratory. Osbrink et al. (2001) isolated biological control agents from dead termites and revealed the presence of 15 bacteria and 1 fungus in dead termites. Bacteria isolated from termite substrata included *Corynebacterium urealyticum* Pitcher, *Acinetobacter calcoacet/baumannii/Gen2* (Beijerinck), *S. marcescens*, and *Enterobacter gergoviae* Brenner. Devi et al. (2007) observed killing of *Odontotermes obesus* subterranean termites under in vitro conditions by three HCN-producing rhizobacterial species, i.e., *Rhizobium radiobacter*, *Alcaligenes latus*, and *Aeromonas caviae*. Rakshiya et al. (2016) reported that

Table 9.1. Entomopathogenic fungi having deleterious effects on different insects

Fungus	Insect	References
Beauveria bassiana	Red flour beetle (*Tribolium castaneum*)	Akbar et al. (2005)
B. brongniartii, *B. bassiana*	*Ceratitis capitata*	Konstantopoulou and Mazomenos (2005)
Nomuraea rileyi, *Mucor hiemalis*, and *Penicillium chrysogenum*	*H. armigera*, *Ceratitis capitata*, and *Bactrocera oleae*	Vimala Devi (2001)
B. bassiana and *Clonostachys rosea*	Coffee berry borer	Vega et al. (2008)
Verticillium lecanii	*Macrosiphum euphorbiae*	Askary et al. (1998)
Lecanicillium muscarium	*Macrosiphum euphorbiae* and *Aphidius nigripes*	Askary and Yarmand (2007)
L. longisporum	*Myzus persicae* and *Aphis gossypii*	Kim et al. (2007, 2008)
L. lecanii	*Coccus veridis*	Vandermeer et al. (2009)
Aspergillus flavus	*Culex quinquefasciatus*	Govindarajan et al. (2005)
A. niger	*Anopheles aegypti*, *Culex quinquefasciatus*	Seleena and Lee (1994)
Chrysosporium tropicum	*Anopheles stephensi*	Srivastava and Prakash (2001)

63 bacterial isolates out of 220 bacterial isolates obtained from the soil collected from termite mounds (along with 8 reference strains) killed the termites under Petri plate conditions at 2 days of observation. Killing frequency of different bacterial isolates was found to vary from 5 to 90%. Six bacterial isolates, i.e., PPM119, PPM123, PPM167, PPM194, PPM199, and PPM203, caused even 100% killing at 5 days of observation. Forty-eight bacterial isolates caused 90 to 100% killing of termites at 10 days of incubation.

9.2.2 Fungi

Fungi also play a prominent role in insect control, and over 700 species have been recorded as insect pathogens (Table 9.1.). Fungi invade directly through the cuticle and could be used for the control of all insects including sucking insects. Products based on *Beauveria bassiana* (Li et al. 2001), *Metarhizium anisopliae*, *Isaria fumosorosea* and *B. brongniartii*, *Verticillium lecanii*, *Nomuraea rileyi*, and *Paecilomyces fumosoroseus* are the most common fungal species among the 171 products currently used for insect control (de Faria and Wraight 2001; Lacey and Neven 2006). Some of these fungi are obligate for insects; for example, *Aschersonia aleyrodes* infects only scale insects and whiteflies, while other fungal

species are facultative with individual isolates being more specific to target pests. The fungus *B. bassiana* is being developed as a biocontrol agent against soil-dwelling pests such as scarabs and weevils (Klingen et al. 1998; Keller 2000) with no effect on the nontargeted insects (Goettel and Hajek 2001).

Colorado potato beetle, the codling moth, several genera of termites, and American bollworm *H. armigera* are the hosts of entomopathogenic fungi of agricultural and forest significance (Thakur and Sandhu 2010). *Hyblaeapara* and *Eutectona machaeralis*, *Ostrinia nubilalis*, pine caterpillars *Dendrolimus* sp. and green leafhoppers *Nephotettix* sp., *Lecanicillium* (*Verticillium*) *lecanii*, and *Paecilomyces fumosoroseus* fungi mainly attack sucking pests such as aphids and whiteflies (Kim et al. 2002; Nunez et al. 2008). *Isaria* (*Paecilomyces*) *fumosoroseus* has strong epizootic potential against *Bemisia* and *Trialeurodes* sp. in both greenhouse and open-field environments (de Faria and Wraight 2001). *Metarhizium* sp. has been found effective in controlling the several economically important insect pests of global importance, viz., *H. armigera* and *S. litura* that attack crops such as groundnut, soybean, sunflower, cotton, and tomato (Revathi et al. 2011). *M. anisopliae* has been tested on teak skeletonizer, *Eutectona machaeralis*, and found to be a potential myco-biocontrol agent of teak pest (Sandhu et al. 2000).

Some of these entomopathogenic fungi, viz., *B. bassiana*, *M. anisopliae*, and *Lecanicillium lecanii*, have been found to colonize plant tissues as symptomless endophytes (Rodriguez et al. 2009; Vidal and Jaber 2015). Plants harboring these fungi as endophytes have shown detrimental effects against herbivorous insects (Azevedo et al. 2000) or plant parasitic nematodes (Waweru et al. 2014). The endophytic growth of *B. bassiana* Vuillemin (BB) is a common feature in corn cropping systems in the USA, and the natural colonization of corn stalks ranged from 0 to more than 60% of plants sampled in different US federal states (Arnold and Lewis 2005). Plant species harboring BB include coffee (Posada et al. 2007), banana (Akello et al. 2008), sorghum (Tefera and Vidal 2009), cotton, pumpkin and wheat (Gurulingappa et al. 2010), jute (Biswas et al. 2013), and common bean (Parsa et al. 2013). *M. anisopliae* and *B. bassiana* and their related species are also used as biological pesticides to control a number of pests such as termites (Maniania et al. 2002), thrips (Ekesi et al. 2012), locusts (Ouedraogo et al. 2003), and hazelnut weevil (Cheng et al. 2016). *M. anisopliae* and *B. bassiana* do not infect humans or other animals and are, therefore, considered safe for use as pesticide.

Twenty-two fungal species have been reported as obligate ectoparasites of termites (Blackwell and Rossi 1986). Leong (1966) demonstrated high pathogenicity of *M. anisopliae* to *Coptotermes formosanus*. Mortality exceeded 86% with short exposures (e.g., 5–35 min) and termites succumbed within 3–6 days posttreatment. Exposure over 40 min caused 100% mortality and longer exposure times caused death within 24 h. No significant difference between the pathogenicities of *B. bassiana* and *M. anisopliae* was found. Grace (1991) found that *M. anisopliae* caused greater and rapid mortality than *B. bassiana* in *C. formosanus* to low conidial concentrations. Wells et al. (1995) concluded that

one isolate each of *B. bassiana* and *M. anisopliae* showed the greatest potential for control of *C. formosanus* populations, based on LD_{50} (median lethal dose, as conidia per insect), time to death, and conidial production. Similarly, different isolates of *Conidiobolus coronatus* were found pathogenic to *C. formosanus*, *R. flavipes*, and *Nasutitermes exitiosus* (Wells et al. 1995). Milner et al. (1998) tested 93 isolates of *M. anisopliae* (Metschnikoff) obtained from two species of termites. The direct inoculation of the most effective isolate FI-610 by applying 3 x 10^{11} conidia into termite mounds resulted in successful control of *Coptotermes acinaciformis* (Froggatt) in Australia. Wright et al. (2003) patented *Paecilomyces* sp. for controlling subterranean termites, and these *Paecilomyces* strains were transferred among termites and caused rapid mortality.

Grace and Zoberi (1992) found that living *R. flavipes* workers exposed to sporulating *B. bassiana* cultures effectively spread infection to unexposed nestmates, whereas introduction of fungus-killed workers did not result in sufficient spore transfer or mycelial growth to cause significant mortality. However, the level of mortality achieved in the laboratory was not considered sufficient to control a termite infestation in the field. Rath and Tidbury (1996) found that *Coptotermes acinaciformis* (Froggatt) (Rhinotermitidae) and *N. exitiosus* were equally susceptible to direct conidial applications of both Australian and American strains of *M. anisopliae*. The injection of large quantities of conidia directly into the termite nest had the greatest success in the field studies (Milner et al. 1996). Sun et al. (2002) quantified the sporulation of 22 isolates of *M. anisopliae* and *B. bassiana* on cadavers of the Formosan subterranean termite, *C. formosanus*. Conidial production increased significantly over 11 days post-death. Effects of *M. anisopliae* and *B. bassiana* isolates on in vivo sporulation were significant, and it differed by as much as 89x and 232x among the selected isolates of *M. anisopliae* and *B. bassiana*, respectively.

Wright et al. (2005) reported that *M. anisopliae* (Metschnikoff) isolate, C4-B, caused rapid mortality on Formosan subterranean termite alates. In initial experiments, C4-B was more lethal to both alates and workers as compared with *M. anisopliae* strains ESC 1, previously marketed as the termite biocontrol agent, BioBlast. Dose-response assays to a known concentration of C4-B spores revealed that 10^6 spores/µl killed 100% of the Formosan subterranean termite alates in 3 days, both 10^5 and 10^4 spores/µl in 6 days, 10^3 spores/µl in 9 days, and 10^2 spores/µl in 12 days. When the transfer of inoculum from infected workers to uninfected nestmates was tested, 62.8% of the workers died in 21 days when only 20% of the workers had been inoculated. Mortality of alates caused by *M. anisopliae* (Metschnikoff) isolate C4-B was studied at two field sites by dispersing fungal spores on grassy lawns and collecting the alates from the treated areas. Infected alates showed 100% mortality by day 5, whereas only 64.8% of untreated control alates from the same collection area were dead on that day. Thus, fungi have the potential for termite control of all the pathogens tested (Milner and Staples 1996).

9.2.3 Actinobacteria

Actinobacteria are important part of the microbial community in the rhizosphere soil and produce many secondary metabolites with different disease suppression effects. More than a 1000 secondary metabolites are produced by actinomycetes, which makes 45% of total microbial metabolites. Actinomycetes have been reported as biocontrol agents effective against numerous plant pathogens and insects (Snyder et al. 2007; Prapagdee et al. 2008; de Oliveira et al. 2010; Laid et al. 2016). The potential use of actinomycetes as a biocontrol agent has been reviewed recently (Sabaratnam and Traquair 2015), where inoculation with some of these microorganisms also promoted the growth of plants.

Actinomycetes were found effective against the housefly *Musca domestica* (Hussain et al. 2002), mosquito larvae (Dhanasekaran et al. 2010), and *Drosophila melanogaster* (Gadelhak et al. 2005). The mortality of insects by actinomycetes may be due to secretion of bioactive materials, which stimulate the gamma-aminobutyric acid (GABA) system or disruption of nicotinic acetylcholine receptors (Herbert 2010). Strains of *Streptomyces* inhibited the growth of *S. exigua*, *Dendrolimus punctatus*, *Plutella xylostella*, *Aphis glycines*, and *Culex pipiens* (Huamei et al. 2008). Spinosad is a novel insecticide produced from fermentation of the actinomycetes *Saccharopolyspora spinosa* (Snyder et al. 2007), and it has been accepted for application in organic farming. It is particularly toxic to lepidoptera and diptera insects. Avermectins (a series of 16-membered macrocyclic lactone derivatives) were obtained from the fermentation products by *S. avermitilis* that showed potent anthelmintic and insecticidal properties (Pitterna et al. 2009). Cholesterol oxidase derived from *Streptomyces* broth showed selective and high potency against cotton boll weevil and stunting effect on *H. virescens*, *H. zea*, and *Pectinophora gossypiella*, which might be due to disruption of the midgut epithelial membrane (Purcell et al. 1993).

9.2.4 Protozoa

Protozoan diseases of insects are ubiquitous and play an important regulatory role in insect populations (Brooks 1988). They are generally host specific and slow acting, most often producing chronic infections. Entomopathogenic protozoa develop only in living hosts, and many species require an intermediate host. Their main advantages are persistence and recycling in host populations and their debilitating effect on reproduction and overall fitness of target insects. Of the four groups of protozoa containing species parasitic to insects, the phylum Microspora includes species that are potentially most useful in applied insect control (Henry 1990). Desportes (1963) described a gregarine (phylum Apicomplexa) from the hemocoel of the damp-wood termite *Zootermopsis nevadensis* (Hagen) (Termopsidae). Microsporidians were found in the body cavity and proventriculus

of *Microcerotermes championi* collected from the roots of *Saccharum munja* Roxburgh (*Poaceae*) (Jafri et al. 1976). The organisms attacked fat body tissues in the midgut after ingestion with food and caused death. Although protozoa are important biocontrol agents for many insects, they have not been used as soil-applied microbial insecticides because they tend to be slow acting and cause low levels of immediate mortality. Moreover, the protozoa populations are vulnerable to changes in environmental conditions (Klein 1988; Henry 1990).

9.2.5 Nematodes

A plethora of nematode species in more than 30 families is associated with insects and other invertebrates (Kaya and Gaugler 1993). They have been found parasitizing species in the orders Hemiptera, Diptera, Hymenoptera, Lepidoptera, Orthoptera, Coleoptera, Thysanoptera, Siphonaptera, as well as Isoptera (Nickle and Welch 1984). Four families of nematodes, i.e., the Mermithidae, Allantonematidae, Steinernematidae, and Heterorhabditidae, have found application in insect control programs (Popiel and Hominick 1992). After infection of the host by nematodes, symbiotic bacteria are released into the insect hemocoel, causing septicemia and death (Kaya and Gaugler 1993). These entomopathogenic nematodes have a number of characteristics that make them especially suitable for biological control and for commercial production as microbial insecticides. These characteristics include a broad host range, especially among soil-dwelling pests; an ease of production, easy to store, and easy in application; and a high degree of safety to vertebrates, plants, and other nontarget organisms and amenability to genetic selection (Kaya et al. 1993).

Fujii (1975) obtained 96% mortality in *C. formosanus* termites within 7 days of exposure to infective-stage *Steinernema carpocapsae* (Weiser) (Steinernematidae) in laboratory experiments. Mortality exceeding 95% was recorded by Georgis et al. (1982) for both *Zootermopsis* sp. and *Reticulitermes* sp. within 3 days after laboratory exposure to *S. carpocapsae*; termites were also found to carry infection back to their colonies. Under laboratory conditions, high rates of infection of *Nasutitermes costalis* and *R. flavipes* were reported with *S. carpocapsae* (Trudeau 1989). Yu et al. (2006) showed that different species of entomopathogenic nematodes, i.e., *Steinernema riobrave* Cabanillas, Poinar, and Raulston (355 strain), *Steinernema carpocapsae* (Weiser) (Mexican 33 strain), *Steinernema feltiae* (Filipjev) (UK76 strain), and *Heterorhabditis bacteriophora* Poinar (HP88 strain), were all capable of infecting and killing three termite species, *Heterotermes aureus* (Snyder), *Gnathamitermes perplexus* (Banks), and *Reticulitermes flavipes* (Kollar), in laboratory sand assays. *S. riobrave* and *S. feltiae* caused low levels of *Reticulitermes virginicus* (Banks) mortality under the same conditions. Nematode concentration and incubation time had significant effects on the mortality of worker *H. aureus*. *S. riobrave* consistently produced highest infection levels and mortality of *H. aureus* in sand assays.

Biocontrol efficacy of nematodes is usually inconsistent due to various abiotic and biotic factors and the complex interactions between these two after application. Cabanillas and Barker (1989) reported that *Paecilomyces lilacinus* was more effective in protecting tomato against *M. incognita* when it was delivered before transplanting or at transplanting stage than after plants were infected by nematodes. Similar results were obtained when tomato or banana plants were treated with the mutualistic endophyte *Fusarium oxysporum* Fo162 at transplanting (Vu 2005; Dababat and Sikora 2007). In comparison, post-planting application of biocontrol agents, especially in the case of endophytes, does not always lead to high levels of biocontrol since the establishment of a biocontrol agent in the rhizosphere is a prerequisite for the control of endoparasitic nematodes (Dababat and Sikora 2007).

9.2.6 Viruses

A large number of viruses offer potential as microbial control agents of plant pathogens and insects (Payne 1982). Baculoviruses are widely used as insect pest control agents (Payne 1982; Popham et al. 2016). More than 400 insect species, mostly in the Lepidoptera and Hymenoptera, have been reported to act as hosts for baculoviruses. These viruses are used extensively for control of insect pests in a diverse range of agricultural and forest habitats. Biotechnological techniques are being used for genetic enhancement of baculoviruses for improved insecticidal efficacy. Insects that feed openly on the foliage of host plants are most easily treated, and the most promising results have been obtained against pest of this type (e.g., caterpillars sawfly larvae) (Smith 1967). The efficacy, specificity, and production of secondary inoculum make baculoviruses attractive alternative to broad-spectrum insecticides and ideal components of integrated pest management (IPM) systems due to their lack of untoward effects on beneficial insects including other biological control organisms (Cunningham 1995).

Gibbs et al. (1970) isolated a virus infecting *Coptotermes lacteus* (Froggatt) (Rhinotermitidae), which was similar to acute paralysis virus of the honey bee *Apis mellifera* Linnaeus (Hymenoptera: Apidae). A nuclear polyhedrosis virus, obtained from caterpillars of *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae), was infective to a laboratory colony of *Kalotermes flavicollis* (Fabricius) (Kalotermitidae) (Al Fazairy and Hassan 1988). Termites died 2–10 days postinfection of viruses under laboratory conditions. The viral insecticide Elcar™ (*Heliothis zea* nuclear polyhedrosis virus, NPV) introduced during the 1980s provided control of cotton bollworm. Commercial preparations based on *Spodoptera* NPV were used to protect cotton, corn, and vegetables globally (Moscardi 1999; Kumari and Singh 2009). *Autographa californica* and *Anagrapha falcifera* NPVs with relatively broad host spectrum activity were used on a variety of crops infested with *Spodoptera* and *Helicoverpa*. *Amsacta moorei* entomopoxvirus has been reported to infect agriculturally important lepidopteran pests such as *Estigmene acrea* and *Lymantria dispar* (Muratoglu et al. 2010).

Insertion of insect-specific toxin genes such as juvenile hormone esterase, diuretic hormone, and prothoracicotropic hormone and genes encoding enzyme inhibitors, neuropeptides, or toxins improved the efficiency of viruses in killing of the insects. Application of recombinant baculoviruses, vAPcmIT2 and vAP10IT2, against two major pesticide-resistant pests *Plutella xylostella* and *S. exigua* resulted in shortening of the lethal time (Tuan et al. 2007). Seo et al. (2005) documented higher pathogenicity for recombinant baculovirus containing a fusion protein with polyhedrin and *Bt* toxin than wild-type strains. Two recombinant baculoviruses containing the ScathL gene from *Sarcophaga peregrina* (vSynScathL) and the keratinase gene from the fungus *Aspergillus fumigatus* (vSynKerat), against third-instar and neonate *S. frugiperda* larvae, showed protease activity in the hemolymph and reduced the time of insect killing (Gramkow et al. 2010).

9.3 Mechanisms Involved in Biocontrol

The mechanisms by which antagonistic microorganisms kill the insects or inhibit the growth of phytopathogenic microorganisms include (1) toxin production, (2) antibiotic production, (3) production of siderophores, (4) production of hydrolytic enzymes, (5) production of secondary metabolites and volatile organic compounds, and (6) phytoalexin production and induction of systemic resistance. Some of the microbial strains produced a wide range of secondary compounds that include siderophores, antibiotics, volatile metabolites, enzymes, etc. (Saraf et al. 2014). Mode of action of different allelochemicals and molecular mechanisms involved in plant-microbe-pathogen/insect interactions will contribute to better disease and insect management.

9.3.1 Toxin Production

Bacillus thuringiensis has been found to produce an array of virulence factors including insecticidal parasporal crystal (Cry) toxins, δ-endotoxin, vegetative insecticidal proteins, phospholipases, immune inhibitors, and antibiotics (de Maagd et al. 2003). There are about 200 registered Bt products in the USA, and worldwide sales of the Bt products amounted to about 100 million dollars (about 2% of the total global insecticide market) (Anonymous 1998). Most commercially available formulations are prepared using spore-crystal mixtures with effectiveness against different pest species. Ultraviolet (UV)-resistant mutant strains with high melanin, which absorb light of any wavelength, were used for large-scale production of light-stable insecticides (Liu et al. 2013). Most of the insecticidal activity of *Bacillus thuringiensis* is associated with the proteinaceous toxins located in the parasporal inclusion bodies, also known as parasporal crystals. The toxins found in the parasporal crystals are collectively referred to as

δ-endotoxins. The Cry1 proteins (protoxins) which are found in the crystals are biologically inactive. Following ingestion and solubilization in the alkaline midgut, cleavage of protoxins by gut proteases produces a 60- to 65-kDa activated protein that recognizes specific binding sites at the brush border membrane surface of epithelial columnar cells lining the gut lumen (van Rie et al. 1989). The activated protein subsequently causes pore formation, membrane transport disruption, and cell lysis leading to the death of the insect (Bravo et al. 2007).

Vegetative insecticidal proteins (Vips) produced by *B. cereus* and *B. thuringiensis* also showed similar activity to endotoxins. Vip1 and Vip2 are toxic to coleopteran insects, and Vip3 is toxic to lepidopteran insects (Zhu et al. 2006). VIPs have excellent activity against black cutworms and armyworms (Yu et al. 1997), *S. frugiperda* (Barreto et al. 1999), *S. litura* and *Plutella xylostella* (Bhalla et al. 2005), and *Heliothis zea*, *Trichoplusia* sp., and *Ostrinia nubilalis* (Fang et al. 2011; Sellami et al. 2011). The pathogenic action of the *B. cereus* bacterium normally occurs after ingestion of spores and crystalline inclusions containing insecticidal δ-endotoxins specifically interact with receptors in the insect midgut epithelial cells (Pigott and Ellar 2007). *Lysinibacillus sphaericus* (*B. sphaericus*) produced insecticidal toxins during the vegetative phase of growth, and mosquitoes have been found to be the major targets of bacterium. Sphaericolysin, a toxin from the *L. sphaericus*, was found lethal to the common cutworm *S. litura* (Nishiwaki et al. 2007).

In addition to endotoxins showing insecticidal properties in *Bt*, the exotoxins of microbial origin from *Pseudomonas* sp. were found toxic to larvae of mosquitoes as well as lepidopteran insects (Murty et al. 1994). *P. aeruginosa* oxyR mutant revealed its ability to kill the insect *Drosophila melanogaster* (Lau et al. 2003). *P. aeruginosa* strain also conferred an efficient protection against *Galleria mellonella* and *Bactrocera oleae* (Mostakim et al. 2012), and the potency was due to the presence of quantitatively as well as qualitatively different proportions of biosurfactants in the crude glycolipids (Desai and Banat 1997). *B. subtilis*, *B. amyloliquefaciens*, *B. megaterium*, and *Pseudomonas* sp. showed more than 50% mortality in *S. litura* and *H. armigera* (Gopalakrishnan et al. 2011).

Hurst et al. (2011) isolated *Yersinia entomophaga* from diseased larvae of the New Zealand grass-grub, *Costelytra zealandica* White (Coleoptera, Scarabaeidae). This bacterium secreted a multi-subunit toxin complex (Yen-Tc) that showed homology with toxin complexes produced by *Photorhabdus* sp. Tc-like proteins were also identified in other entomopathogenic bacteria such as *Serratia entomophila* and the nematode symbiont *Xenorhabdus nematophila* (Morgan et al. 2001). Histopathological studies on the effects caused by the ingestion of Yen-Tc revealed the progressive disorganization and deterioration of the midgut epithelium of *C. zealandica*. Recently, the insecticidal activity of formulations containing *Y. entomophaga* against the pasture pest porina (*Wiseana* sp. larvae) has been reported under the field conditions (Ferguson et al. 2012).

A mixture of soluble endotoxin, spores, and inclusion bodies of *Bacillus thuringiensis* (Berliner) caused greater than 95% mortality of the subterranean termite species, *Reticulitermes flavipes* (Kollar) and *R. hesperus* Banks

(Rhinotermitidae), after 6 days of exposure of the laboratory colonies (Smythe and Coppel 1965). Khan et al. (1978, 1985) employed a commercial preparation of Bt (Thuricide-HP concentrate) which exhibited 100% mortality of *H. indicola*, *M. championi*, and *Bifiditermes beesoni* (Gardner) (Kalotermitidae) within 6 days of exposure. Grace and Ewart (1996) constructed recombinant cells of the bacterium *Pseudomonas fluorescens* that expressed the δ-endotoxin genes of *Bacillus thuringiensis* (Bt). Two commercial agricultural formulations prepared by the CellCap process were evaluated for palatability to the termite *C. formosanus*. The MVP formulation, active against Lepidoptera, contained the *P. fluorescens* encapsulated δ-endotoxin of Bt var. *kurstaki*. Similarly, the M-Trak™ formulation, active against Coleoptera, contained the δ-endotoxin of Bt var. *san diego*. The palatability of the CellCap formulations indicated that the host bacterium, *P. fluorescens*, is a suitable delivery system for genetically engineered termiticides. Gunner et al. (1994) reported that the spores of entomopathogenic fungi may contain toxins which may kill the termite host when ingested. Insecticidal cyclic depsipeptides were found to be produced by entomopathogenic fungi including the destruxins from *M. anisopliae* var. *major* (Kaijiang and Roberts 1986) and *Aschersonia* sp. (Krasnoff and Gibson 1996) and the beauvericins from *B. bassiana* (Jegorov et al. 1989). It has been suggested that depsipeptides are localized on the surface of *Beauveria* sp. spores (Jegorov et al. 1989), whereas *Metarhizium* destruxins are generally associated with in vivo or in vitro mycelial growth (Chen et al. 1999).

9.3.2 Production of Antibiotics

Antibiotic production by microrganisms is one of the major mechanisms postulated for disease control. These antimicrobial compounds may act on plant pathogenic fungi by inducing fungistasis, inhibition of spore germination, and lysis of fungal mycelia or by exerting fungicidal effects. A large number of antibiotics including diacetylphloroglucinol, oomycin A, phenazines, pyocyanin, pyrroles, pyoluteorin, pyrrolnitrin, etc. are produced by rhizobacteria (Bender et al. 1999), which help in suppression of pathogen growth. Kido and Spyhalski (1950) isolated antimycin A from cultures of an unidentified species of *Streptomyces*. The initial tests showed that the antibiotic caused mortality to some insects which ingested the material. The toxicity of antimycin A was not confined to members of the Insecta, as it showed efficacy for the control of the red spider mite, *Tetranychys* sp. Beck (1950) reported that antimycin A had insecticidal possibilities for some species of insects and mites. The antibiotic inhibited either the succinoxidase system or some other essential step in the oxidative metabolic cycle of cockroaches. Inhibition of the oxidative cycle readily explained the depression of oxygen consumption by the poisoned insects. *Photorhabdus* is a virulent pathogen that kills its insect host by overcoming immune responses (Eleftherianos et al. 2007). *Photorhabdus* produces a small-molecule antibiotic (*E*)-1,3-dihydroxy-2-(isopropyl)-5- (2 phenylethenyl)benzene (ST) that also acts as an inhibitor of phenoloxidase (PO) in the insect host *Manduca*

sexta. The bacterium inhibits two key immune defenses of the insect: activity of the antimicrobial enzyme PO and formation of melanotic nodules. Due to production of antibiotics, different *Brevibacillus laterosporus* strains showed insecticidal action in different orders, including Coleoptera, Lepidoptera, and Diptera (Ruiu 2013).

9.3.3 Siderophore Production

Many microorganisms synthesize extracellular siderophores, in response to iron stress (Neilands 1981), which are involved in disease suppression and plant growth promotion. Extracellular siderophores of the brown-rot wood decay fungus *Gloeophyllum arabeum* (Persoon: Fries) Murnill (Polyporaceae) were found to inhibit feeding by *C. formosanus* termites (Grace et al. 1992). Siderophore-treated filter paper disks showed negligible feeding, whereas untreated disks were almost completely consumed over a 3-day test period.

9.3.4 Production of Extracellular Enzymes

Different extracellular enzymes are produced by rhizosphere microorganisms which contribute to killing of the pathogens and insects. Fungal isolates that produce extracellular enzymes to degrade the host cuticle have large scope in pest management. For example, *M. anisopliae* grown in optimum fermentation conditions produced host-degrading enzymes such as acid phosphatase and phosphatase isoenzymes (Strasser et al. 2000; Li et al. 2007). *Trichoderma* produced protease (31 kDa) and chitinase (44 kDa) during the growth phase (Shakeri and Foster 2007), and it also produced a number of antibiotics, such as trichodermin, trichodermol, harzianum A, harzianolide, and peptaibols (Hoell et al. 2005). The crude *Alternaria alternata* chitinase showed 82% mortality against fruitfly (Sharaf 2005). Quesada-Moraga et al. (2006) used the crude protein extracts of *M. anisopliae* for the control of *S. littoralis*. *Tolypocladium* and *Isaria fumosorosea* were found toxic to *Plutella xylostella* (Freed et al. 2012).

Different microorganisms including bacteria, fungi, and actinomycetes were found to produce proteases from various types of natural resources. Lysenko and Kucera (1971) showed that *Serratia marcescens* produced extracellular proteases that could be a mode of pathogenicity of these bacteria in termites. Osbrink et al. (2001) examined 15 bacteria and one fungus associated with dead termites as possible biological control agents against Formosan subterranean termites, *Coptotermes formosanus* Shiraki. Bacterial isolates obtained from dead termites were primarily *Serratia marcescens* Bizio that caused septicemia in *C. formosanus* and found to contain proteolytic enzymes. Singh (2007) reported chitinolytic activity in some of the bacterial isolates that killed the termites. Bahar et al. (2011) identified chitinase producing *Serratia marcescens* which were found

effective in killing the coleopteran insects with more chitin in their exoskeleton. Jafri et al. (1976) found microsporidians in the body cavity and proventriculus of *Microcerotermes championi* collected from the roots of *Saccharum munja*. These organisms passed into the midgut after ingestion with the food, attacked fat body tissues, and caused death of termites, indicating the role of lipolytic enzymes in termite killing. Rakshiya et al. (2016) reported that some of the bacterial isolates were found effective in termite killing and possessed all the three enzyme activities, i.e., lipase, protease, and chitinolytic activity. Lack of correlation between enzyme activities and termite killing indicated that besides the production of three enzymes, some other metabolites (toxin or siderophore) could also be contributing to the killing of termites.

9.3.5 *Production of Secondary Metabolites and Volatile Organic Compounds*

A large number of secondary metabolites are produced by rhizosphere bacteria, which play important roles in disease control and plant growth promotion. Hydrogen cyanide (HCN) is known to be produced by many rhizosphere bacteria and has been demonstrated to play a role in the biological control of the pathogens and pests. HCN-producing *P. aeruginosa* was found to have lethal effects on nematodes (Darby et al. 1999; Gallagher and Manoil 2001). Devi et al. (2007) tested three different species of HCN-producing rhizobacteria for their potential to kill subterranean termite *O. obesus*. The three bacterial species, *Rhizobium radiobacter*, *Alcaligenes latus*, and *Aeromonas caviae*, were found effective in killing the termites under in vitro conditions. *R. radiobacter* and *A. latus* caused 100% mortality of the termites following 1-h incubation. *A. caviae*, which produced significantly lower amounts of HCN, caused only 70% mortality. Termites exposed to exogenous HCN showed 80% mortality at cyanide concentrations of up to 2 μg/ml. The observed HCN toxicity in termites could be correlated with the inhibition of the respiratory enzymes.

Daisy et al. (2002) showed that naphthalene, an insect repellent, is produced by a fungus, *Muscodor vitigenus*. Three species of *Muscodor* and one *Gliocladium* sp. that produce volatile organic compounds with biocidal activity were isolated from several host plants in geographically diverse areas. A large number of metabolites are produced by different fungi (Table 9.2), which showed adverse effects on the insects (Boonphong et al. 2001; Shakeri and Foster 2007). For example, bassianin, beauvericin, bassianolide, and bassiacridin were produced by *Beauveria* sp. that controlled the *Culex pipiens*, *Aedes aegypti*, *Calliphora erythrocephala*, and *H. zea* (Quesada-Moraga and Alain 2004). Similarly, *Trichoderma* sp. produced trichodermin, trichodermol, harzianum A, harzianolide, and peptaibol metabolites having deleterious effects on *Tenebrio molitor* (Shakeri and Foster 2007).

Table 9.2 Secondary metabolites of fungi effective against insects

Organism	Metabolites	Insects controlled	References
Beauveria sp.	Bassianin, beauvericin, bassianolide, bassiacridin, oosporein, and tenellin	*Culex pipiens*, *Aedes aegypti*, *Calliphora erythrocephala*, *H. zea*	Quesada-Moraga and Alain (2004)
Paecilomyces fumosoroseus	Pecilomicine-B	*Trialeurodes vaporariorum*	Yankouskaya (2009)
Hirsutella thompsonii	Hirsutellin A, hirsutellin B, phomalatone	Mites	Mazet et al. (1995)
Aschersonia aleyrodis and *A. tubulata*	Destruxins, dustatin, and homodestruxins	Whitefly	Boonphong et al. (2001)
Trichoderma sp.	Trichodermin, trichodermol, harzianum A, harzianolide, and peptaibols	*Tenebrio molitor*	Shakeri and Foster (2007)

9.3.6 Induction of Systemic Resistance

Salicylic acid (SA) and jasmonic acid (JA) hormones control defense responses to different types of microbes, and they orchestrate a different and complex transcriptional reprogramming that eventually leads to plant resistance. Evidences indicate that cyclic precursors of jasmonic acid (JA), the cyclopentenones, can also function as potent signals of plant defense responses (Farmer and Ryan 1992). Similarly, volatile derivatives of JA, such as methyl jasmonate (meJA) and cis-jasmone, can act as airborne signals stimulating plant defenses and repelling insects (Birkett et al. 2000).

The attack of insect herbivores on the plant roots and leaves imposes different selection pressures on plants, which in turn produces contrasting responses in terms of changes in biomass, gene expression, production of secondary metabolites, and wound hormones (Johnson et al. 2016). Different kinds of plant defenses are reported against root herbivores as compared with foliar herbivores (Johnson and Rasmann 2015). Following herbivore recognition, plants configure their metabolism through changes in the phytohormonal networks (Johnson et al. 2016). Jasmonates, which are widely viewed as the master regulators of plant responses to herbivores, are less inducible in the roots than the leaves (Erb et al. 2012; Lu et al. 2015). Salicylic acid signaling can buffer the jasmonic acid response aboveground (Gilardoni et al. 2011). Root herbivores attack induces different signal signature compared with leaf attack. For instance, attacked rice roots do not increase the biosynthesis of abscisic acid and ethylene (Lu et al. 2015), two important synergistic signals in the wound response of leaves. The difference may be explained by the fact that both hormones strongly influence root growth and architecture; plants may therefore be able to maintain root development under herbivore attack by maintaining abscisic acid and ethylene homeostasis. Thus, it is apparent that roots respond to pathogen or insect attack differently than shoots and regulate the defenses through modulating their phytohormonal networks in a tissue-specific manner.

Biological control of termites may also be facilitated if their highly evolved immune systems can be suppressed (Connick et al. 2001). Eicosanoids (C20 polyunsaturated acids) have been found to play an important role in protecting insects from bacterial infections (Miller et al. 1994). In laboratory experiments, the eicosanoid biosynthesis inhibitors dexamethasone, ibuprofen, and ibuprofen sodium salt were each provided along with a red-pigmented isolate of *Serratia marcescens* Bizio to the Formosan subterranean termite, *C. formosanus* Shiraki, by means of treated filter paper (Stanley-Samuelson et al. 1991). The increased mortality was observed with dexamethasone and ibuprofen suggesting that the termites' immune systems were suppressed by these compounds, making the insects more vulnerable to infection by *S. marcescens* (Connick et al. 2001). This effect on mortality was noted only at 3.4 x 10^{10} colony-forming units ml^{-1} treatment level. A significant amount of infection and subsequent mortality may have resulted from direct contact with the bacterium and the remainder from its ingestion.

9.4 Approaches to Increase the Efficiency of Biocontrol Agents

Various entomopathogenic microbial species are normally able to persist in the environment, multiply in the host, and spread to other susceptible hosts. These entomopathogens have developed different strategies to attack, enter, and kill the attacking insect. Mycopathogens enter through the cuticle, whereas virus, bacteria, and protozoa enter through the midgut. Connick et al. (2001) reported that *Serratia marcescens* isolate T8 was highly virulent to the *Coptotermes formosanus* and termite mortality was 24% by 2 days and 99% after 19 days of the exposure. Nematodes belonging to the two families, *Steinernematidae* and *Heterorhabditidae*, have shown promise for use in termite control programs (Kaya and Gaugler 1993; Yu et al. 2006). The limited numbers of field trials attempted on insect or pathogen control have failed largely in reducing pathogen densities below economically damaging levels. Another factor that can contribute to inconsistent performance of biocontrol agents is variable production or inactivation in situ of microbial metabolites responsible for killing of insect/pathogen. The inconsistency in performance is a major constraint to the widespread use of biocontrol agents in commercial agriculture. Future strategies are required to clone genes involved in the production of toxins, antibiotics, and other metabolites so that these cloned genes could be transferred into the microbial strains having good colonization potential (Grace and Ewart 1996). Biotechnological approaches used in manipulation of microbial traits could lead to improved biocontrol activity of pathogenic microorganisms in the control of insects. The development of more stable formulations, such as microencapsulation, would be necessary to ensure their long-term, residual action (Grace and Ewart 1996).

9.5 Constraints in Development and Application of Biocontrol Agents

Recently, various biocontrol agents have been tested under field conditions on different crops for controlling pests and diseases of crop plants, and some of the antagonistic bacterial strains have led to the development of commercial biocontrol products. The major disadvantages in using microbes as a biocontrol agent include variability of field performance and the necessity to ensure survival and delivery of the product. Moreover, the effectiveness of a given biocontrol agent may be restricted to a specific location due to the effects of soil and climate. Many soil edaphic factors including temperature, soil moisture, pH, clay content, and interactions of biological disease control microorganisms with other rhizosphere bacteria and with pathogens will also affect their viability and tolerance to adverse conditions once applied. During root colonization, introduced biocontrol agents have to compete with indigenous microflora for carbon source, mineral nutrients, and infection sites on the roots. Sometimes, this competition is so severe that introduced biocontrol agents fail to survive in the soil. Another factor that can contribute to inconsistent performance is variable production or inactivation in situ of bacterial metabolites responsible for killing of the pathogen/insect.

Biological control strategies are also emerging as promising alternatives to the use of synthetic pesticides in the preservation of fruits. Antagonists must survive after their exposure to both postharvest treatments and storage conditions. The limitations of these biocontrol products can be addressed by enhancing the biocontrol through manipulation of the environment, using mixtures of beneficial organisms, physiological and genetic enhancement of the biocontrol mechanisms, manipulation of formulations, and integration of biocontrol with other alternative methods that in combination with biocontrol agents may provide additive or synergistic effects for adequate protection.

9.6 Conclusion

The role of microbial pesticides in the integrated management of insect pests has been reviewed for agriculture, forestry, and public health. In most cases, no single microbial control agent provides sustainable control of an insect pest or complex pests. As components of an integrated approach in all agricultural practices, entomopathogens could provide significant and selective insect control without interfering with the effectiveness of other practices. In the near future, synergistic combination of microbial control agents with other technologies (in combination with semiochemicals, soft chemical pesticides, other natural enemies, resistant plants, remote sensing, etc.) may enhance the effectiveness and sustainability of integrated control strategies. Till now, the market for microbial insecticides hardly represents only 1% of the total crop protection market. In the near future, microbials

will face even stiffer competition from new pesticide chemistries and transgenic plants. However, several microbial control agents have good potential for use in integrated pest management (IPM) programs.

Complete elimination of chemical pesticides for controlling plant pests and diseases in modern agriculture may be impossible, but a logical reduction in their application is absolutely feasible. Biopesticide use in combination or rotation with synthetic pesticides is likely to be enhanced in the near future, but more research is needed to come up with innovative solutions that can really meet farmer and regulator needs in terms of effectiveness and environmental sustainability (Glare et al. 2012). To have a sustainable agricultural system with minimum contamination and risks to the environment, a combination of all available methods should be applied to manage pest problems, and this can be achieved by integrated pest management. Implementation of IPM strategies may be the safest solution for management of pest problems including insect infestation in every cropping system. Biological control could be one of the most important components of integrated pest management, which can lead us toward a safe, sustainable, and environmentally sound agricultural system in the future.

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Chapter 10
Soil Organic Matter and Microbial Role in Plant Productivity and Soil Fertility

Tapas Biswas and Subhas Chandra Kole

Abstract The organic materials that become a part of the soil matrix are soil organic matter (SOM) which is the mainstay of soil quality. The heterotrophic microorganisms are the key players for degradation of organic matters by different enzymes due to assimilation of substrate carbon and energy for their nutrition. The SOM holds a key for sustained food production in a number of ways such as increased nutrient and water-use efficiency, improved physical properties of soils and improved biological activity. The temperature, O_2 supply, rainfall, parent material, soil fertility, biological activity, nature of the substrate, land use pattern, etc. are the important factors controlling the rate of decomposition of SOM and thereby the status of soil organic carbon (SOC). Deterioration of soil quality, especially SOC and its associated nutrient supply to soil, is one of the major factors for yield decline or stagnation under intensive cropping system in most of the countries. Agricultural intensification has resulted in loss of carbon to atmosphere, and its contribution to green house is also a serious problem. The SOM is the most vital parameter that needs to take care for restoration and maintenance of soil health vis-a-vis soil fertility and crop productivity. This can be done mainly by adopting different technological options like C sequestration, balanced and integrated nutrient management (INM), improving quality and quantity of FYM, compost, vermicompost, green manure, mulch farming, incorporation of crop residues and recycling, production and promotion of bio-inoculants, increased forestation, choice of cropping system, etc.

Keywords SOM · SOC · Soil quality · Microbial degradation · Integrated nutrient management

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10.1 Introduction

Soil organic matter (SOM) is the organic component of soil, consisting of plant and animal residues at various stages of decomposition, cells and tissues of soil inhab itants and substances synthesized by soil organisms. Soils become dynamic living system and support all life forms only in the presence of organic matter. Organic matter contributes different ways in maintaining soil fertility and productivity. Incorporation of organic matter increases soil carbon which indirectly increases soil fauna, microbial activity and diversity. It increases aggregate stability and macroporosity – increase water infiltration, minimize soil erosion and land degradation and increase water holding capacity. It facilitates the increase of CEC in soil and availability of certain nutrient cations like Ca, K, Mg, etc. and the buffering capacity of the soil (Pal 2016). During microbial decomposition of organic matter, many organic acids are produced which along with CO_2 solubilize soil minerals and essential nutrients like Ca, K, etc. are released. Organic acids also form chelate with cations like Fe, Zn, Cu, Mn, etc. as stable organo-mineral compounds and preventing their precipitation in soil and thus increase their availability. In highly acid soils, such chelation with Fe and Al reduces the toxicity of these elements and on the other hand reduces phosphate fixation in these soils. In the process of organic matter decomposition, different growth-promoting substances like vitamins, auxins and gibberellins are produced by microorganisms which stimulate plant growth.

Important factors controlling SOM status include climate (especially temperature and rainfall), O_2 supply, parent material, soil fertility, biological activity, vegetation and land use. The soil organic carbon (SOC) content of soils of India is relatively low, ranging from 0.1% to 1% (Swarup et al. 2000), and this is primarily due to high temperature prevailing throughout the year. Carbon reserve in the form of organic matter is about 1500 Pg (Lal 2000). Conversion of land from its natural state to agriculture generally leads to loss of SOC. SOM is an important determinant of soil fertility, productivity and sustainability and is a useful indicator of soil quality (Doran and Parkin 1994). Deterioration of soil quality, especially SOC and its associated nutrient supply to soil, is one of the major factors for yield decline or stagnation under intensive cropping system in most of the countries (Dawe et al. 2000). Managing agroecosystem is an important strategy for buildup of SOC and thereby improvement of SOM vis-à-vis soil fertility and crop productivity.

10.2 Sources of Organic Matter in Soil

The organic component of soil consists of three primary parts including fresh plant residues and small living soil organisms, decomposing organic matter and stable organic matter, i.e. humus. Organic materials in soils can be categorized into following forms:

- Litter: macro-organic matter that lies on the soil surface, e.g. crop residues, leaf litter
- Light fraction: plant residues and their partial decomposed products that reside within soil
- Microbial biomass: cells of living microorganisms, notably bacteria, actinobacteria, fungi, algae and cyanobacteria
- Faunal biomass: tissues of animals, primarily invertebrates
- Belowground plant constituents: primarily roots and exudates
- Water soluble organics: organic substances dissolved in the soil solution
- Stable humus

Most of the SOC are of plant origin. The forest ecology in hills and mountains is conducive for higher accumulations of SOC. Natural forests accumulate leaf litter equivalent to 2% of their underground biomass (Brown and Lugo 1982). In a temperate forest, annual leaf fall may be several tons of dry matter per hectare. In the tropical regions, plantation forests often accumulate litters in a higher quantity than in natural forests. Cultivated soils in temperate regions have organic matter to the tune of 5–10% in surface soils. Similar soils in the tropics have one-fifth to one-sixth as much.

Animals are the secondary sources of organic matter. As they eat the original plant tissue, they contribute waste products and leave their own bodies as they die. Certain forms of animal life, particularly earthworm, termite, ant and dung beetle also play a role in the incorporation and transformation of organic residues.

In India, there is a vast potential of manurial resources and organic wastes available for recycling. Residual wastes from crops, agro-based industries and natural biomass are important sources. About 350 million tonnes of organic wastes come from agricultural sources in the country. Organic matter may be introduced into the soil system in two different ways:

1. Raising of cover crop, sods, pastures and hays or utilization of crop residues.
2. It can be brought in as manures, composts, organic fertilizers, biosolids, organic wastes, etc.

10.3 Crop Residues on Soil Organic Matter Dynamics

The higher plants on the land and algae in aquatic habitats convert approximately 120 Pg C/year from atmospheric CO_2 into organic moieties through photosynthesis. Some of these organic molecules which are temporarily stored in the standing vegetation and microbial biomass are ultimately added to the soil after their death. In agricultural land, major part of the total organic matter comes from crop residues. The total organic matter content of a soil is the sum total of diverse pools of soil organic matter, viz. active, slow and passive pools (Fig. 10.1). The pools of

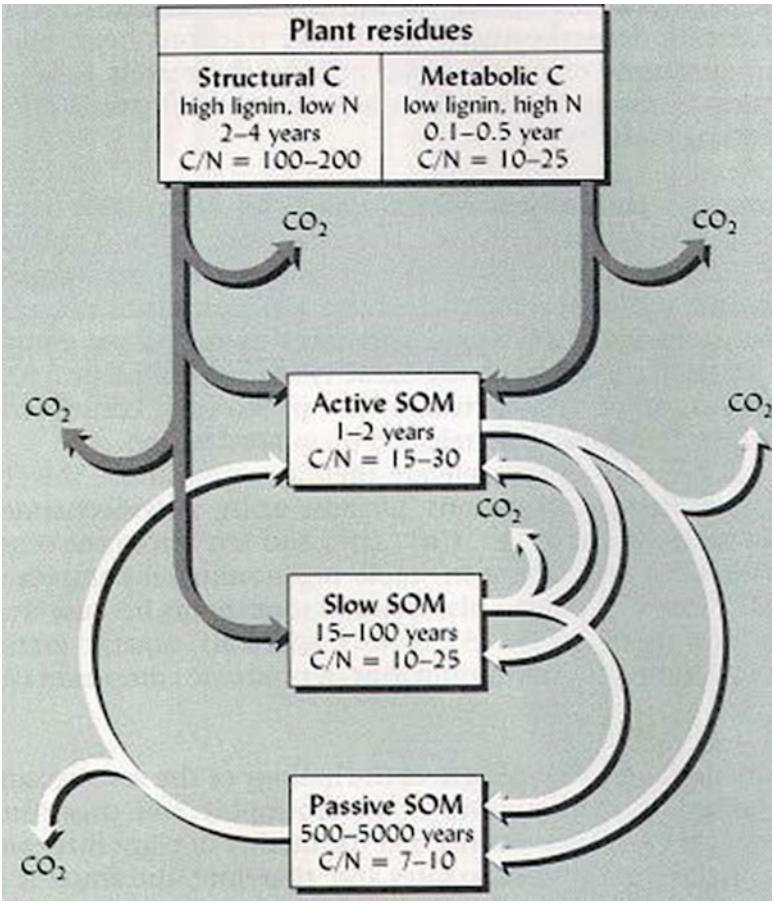

Fig. 10.1 Different pools of soil organic matter (Source: Brady and Weil 2002)

soil organic matter differ by their susceptibility to microbial metabolism. Microbial action can transfer organic carbon from one pool to another. Those metabolic changes result in some loss of carbon from the soil in the form of CO_2.

10.3.1 Active Fractions of Soil Organic Matter

The active fraction of SOM includes living biomass, fine particles of detritus (particulate organic matter), most of the polysaccharides, other non-humic substances like low molecular organic acids, some protein materials and some of the

more labile fulvic acids. The average C:N ratio of active fractions of SOM is 15–30. It remains in soil for a period of few months to a few years. These active fractions provide most of the readily mineralizable nitrogen (N). Its beneficial effect on soil structural stability leads to enhanced infiltration of water, resistance to erosion and ease to tillage. Incorporation of crop residues can readily increase the active portion which also is readily lost under intensive tillage. The active fractions comprise more than 10–20% of total organic matter.

10.3.2 Passive Fractions of Soil Organic Matter

The passive fraction of SOM includes very stable materials remaining in the soil from hundreds to thousands years. That means their changes in soil occur very slowly. It includes most of the humus physically protected in clay-humus complexes, most of the humin and much of the humic acids. The passive fractions in most of the soils account for 60–90% of the organic matter. It is closely associated with the colloidal properties of soil humus and is responsible for most of the CEC and water holding capacity of soil.

10.3.3 Slow Fractions of Soil Organic Matter

The slow fraction of organic matter is intermediate in properties between active and passive fractions. This fraction includes very finely degraded plant tissues, high in lignin, and other slowly decomposable and chemically stable components. The half-life of these materials may be over decades. The slow fractions are important source of mineralizable N and other plant nutrients. It is the food source for the steady metabolism of the autochthonous soil microorganisms.

10.3.4 Soil Organic Carbon Stocks

The status of organic carbon pools in soils of India and the world is presented in Table 10.1. The global pool of SOM is estimated at 1500 Pg of carbon up to 1 m depth from the surface. The geological C pool comprises of 4000 Pg as coal, 500 Pg as C gas and 500 Pg as oil (Lal 2000). The total organic pool of soils in India is 21 Pg till 30 cm depth and 63 Pg up to 150 cm depth. Total inorganic carbon pool in soils of India is 196 Pg up to 1 m depth.

Table 10.1 Organic carbon pools in soils of India and the world (Pg)

S. No.	Soil order	India (0–30) cm	World (0–30) cm
1	Alfisol	3.10	73
2	Andisol	–	38
3	Aridisol	0.77	57
4	Entisol	0.65	37
5	Histosol	–	26
6	Inceptisol	2.2	162
7	Mollisol	0.12	41
8	Oxisol	–	88
9	Spodosol	–	39
10	Ultisol	0.23	74
11	Vertisol	2.62	17
	Total	**9.77**	**652**

Source: Suri (2007)

10.4 Soil Organic Matter as Source of Plant Nutrients

Soil organic matter is a potential source of plant nutrients. On an average, plant, microflora and fauna contain 25% dry matter in which three elements – carbon, oxygen and hydrogen – collectively constitute 90–95% by weight and the rest 5–10% is constituted by different mineral elements, viz. nitrogen, phosphorous, potassium, sulphur, calcium, magnesium, iron, zinc, manganese and other essential nutrients. A major portion of N (95–99% of total), P (33–67% of total) and S (75% of total) in soil occurs in organic combinations. Microbial degradation of organic materials through mineralization releases these nutrients into plant available forms. The mineralization in most cases is slow and often cannot meet the full requirement of high-yielding varieties. However, it serves as a reservoir of plant nutrients. Crop residues including roots are the primary source of organic matter added to the soil. Decomposition of crop residues releases about 55–70% of the carbon to the atmosphere as CO_2, 5–15% is incorporated into microbial biomass and the remaining C (15–40%) is partially stabilized in soil as humus.

It is estimated that a gross quantity of 686 Mt of crop residues are available annually in India (Singh et al. 2015). In the country, total nutrient (NPK) potential of various organic resources was estimated at 14.85 Mt in 2000, which would become around 32.41 Mt by 2025. Among different crop residues, rice and wheat have the highest nutrient potential as well as highest residue-generating potential (Prasad 2015). Nutrient concentration (Brady and Weil 2002) of aboveground portion of some important crops is listed in Table 10.2.

Nitrogen availability from crop residues to subsequent crops depends on decomposition rate, residue quality and environmental conditions. The amount of N fixed from legume residues to cropping systems (about 60–70 kg ha^{-1}) depends on the

Table 10.2 Nutrient concentration of aboveground portion of some important crops

Crop	Botanical name	Nutrient concentration (%)			
		Nitrogen	Phosphorous	Potash	Sulphur
Rice	*Oryza sativa*	0.69	0.08	0.57	0.09
Wheat	*Triticum aestivum*	0.58	0.05	1.42	0.19
Barley	*Hordeum vulgare*	0.69	0.07	2.37	0.17
Corn	*Zea mays*	0.95	0.10	1.45	0.17
Sorghum	*Sorghum bicolor*	0.83	0.13	1.20	–
Oat	*Avena sativa*	0.70	0.06	2.57	0.23
Cotton	*Gossypium* spp.	0.90	0.15	1.45	–
Sunflower	*Helianthus annuus*	0.80	0.15	0.92	–
Groundnut	*Arachis hypogaea*	1.71	0.15	1.38	0.23
Soybean	*Glycine max*	0.83	0.47	0.93	0.30
Cowpea	*Vigna sinensis*	0.40	0.35	2.26	0.35
Alfalfa	*Medicago sativa*	2.09	0.18	1.78	0.25

Source: Brady and Weil (2002)

symbiotic activity. Nitrogen recovery by subsequent crops ranges from 10% to 35% of the N originally contained in the legume residues. Nitrogen mineralization and decomposition pattern of leguminous residues occur in two phases. In the first phase, rapidly mineralized N from easily decomposable plant materials appears that contains adequate N concentrations to meet the N demands for microbial community. The second pool of N has a slower mineralization rate, probably composed of ligno-proteins or lignin-protected N compounds that are less available to decomposing microorganisms.

Legume and nonlegume residues differ in chemical composition and, hence, differ in decomposition and N mineralization rates. Nonlegume residues have wider C:N ratio (low N content) that may need additional N for decomposition to proceed. Nonleguminous residues can affect soil N availability and subsequent crop production through microbial immobilization. Nutrient immobilization, particularly N immobilization, is usually favoured when residues with wider C:N ratios are added to soil. Residues from corn, sorghum, rice, wheat, etc. with wider C:N ratios result into initial N immobilization. Mineralization of N from such low N residues occurs only after 50–60% is decomposed or after the C:N ratio reaches below 30.

The tillage practice influences microbial dominance and diversity. A greater microbial biomass level near the soil surface in no-tillage system is related to greater immobilization of fertilizer N compared to conventional or shallow tillage practices. Conventional tillage is characterized by bacterial predominance resulting in faster rate of decomposition and nutrient mineralization, whereas no-tillage practice supports more fungal population resulting to slower decomposition and greater nutrient return.

10.5 Carbon Assimilation by Microbial Key Players

The most vital functions which the soil microorganisms carry out and have significant contribution to soil properties and plant growth are decomposition of organic matter and synthesis of humic substances, biological N fixation, microbial transformation of nutrients and nutrient recycling in soil. The microbial cells contain approximately 50% carbon of which heterotrophs obtain carbon from organic compounds and autotrophs from CO_2. The phenomenon of converting the substrate carbon to protoplasmic carbon is known as 'carbon assimilation'. The chemoheterotrophic microorganisms derive carbon and energy from organic compounds; therefore, carbon assimilation by them is concomitant with the decomposition of organic matter which can be grouped into three broad categories: (1) degradation of plant and animal residues by cellulase, ligninase and other microbial enzymes, (2) increase in the biomass of microorganisms of different groups by uptake of C and other elements at different stages and (3) accumulation or release of end products like CO_2, CH_4, organic acids, alcohols, humus, etc. The term mineralization is used to designate the conversion of organic complexes of an element to its inorganic state, which embodies the first process. The second process involves the microbial uptake of nutrients and increase in biomass of microorganisms which is opposite to mineralization and known as immobilization. The last of these processes provides an index of microbial activity in soil and is interlinked with humus formation (Subba Rao 2014).

About 30–35% of the substrate C is assimilated by the microorganisms. The rest is either released as CO_2 or accumulated as waste products, which means one-third C is assimilated and two-thirds C is released or accumulated. On an average, 5–10% substrate C is assimilated by bacteria, 15–30% by actinobacteria and 30–40% by fungi. Among the soil microorganisms, fungi release comparatively less CO_2 for each unit of its assimilation than that of other microbial groups. That means fungi are more efficient in their metabolism, and the per cent of cell carbon formed to the carbon source consumed is higher. Among the three primary soil microorganisms, fungi and actinobacteria show a greater efficiency than aerobic bacteria. Anaerobic bacteria leave behind a considerable amount of carbonaceous products because they are very inefficient in carbon utilization (Alexander 1977).

Decomposition of plant residues is accomplished by a complex web of microorganisms altering their composition during the process. The nature and composition of microbial flora vary with the chemical composition of the added substrates. Certain microbial groups predominate for a few days, while others maintain high population levels for longer periods. Each individual organism has a complex of enzymes, which permits it to oxidize specific compounds, but not others. If the proper substances are present in an accessible state, then the microorganisms will proliferate provided that it can cope with the competition of other microorganisms having similar enzymatic potentials. The microorganisms which proliferate and start to decompose the initial organic residues are the primary flora, and mostly they are the heterotrophic bacteria which utilize readily soluble source of energy from

sugar, starch, cellulose and proteins. The slow metabolizing autochthonous microorganism is overtaken by rapid metabolizing zymogenous population which were dormant earlier due to restricted availability of substrate. Faster metabolizing zymogenous organisms multiply at a faster rate than the autochthonous organisms (Pal 2016). The secondary flora of zymogenous organisms are then developed which grow upon compounds produced by the primary flora or growing upon the dead or living cells of the initial flora. Thereafter, a succeeding group of microorganisms develop with different biochemical makeup, which gradually narrow down the C:N ratio. Microbial population increase rapidly when succulent plant tissues are incorporated into the soil. Mature crop residues having a distinctly different chemical composition support a flora better adopted to utilize resistant carbonaceous compounds – this population is largely fungi and to a certain extent bacteria and actinobacteria (Alexander 1977). Microorganisms are involved in the degradation of different fractions of organic residues as reported by several researchers (Subba Rao 2014) (Table 10.3).

As carbon is assimilated for generation of new microbial cells, a simultaneous microbial uptake of N, P, K, S and other elements from soil as well as from residues occurs. The extent of availability of C, N, P, K, S and other elements also plays an important role in the decomposition of organic matter. As the plant and animal residues containing the said elements are available in a negligible amount, these are mainly assimilated by the microorganisms from the soil, particularly at the early stage of decomposition. Hence the status of inorganic substances has a great practical significance on nutrient assimilation by the microorganisms and ultimately towards the microbial immobilization of the C from organic waste or in other words microbial decomposition of organic waste. The magnitude of immobilization is proportional to the net quantity of the microbial biomass and related to C-assimilation by factors govern with the C:N, C:P and C:S ratios of newly generated cells (Alexander 1977). The ratio of C:N:P:S in different soils varies considerably. But, the average values for soils of different agroclimatic regions over the world are almost similar. Overall, the proportion of C:N:P:S in soil humus is around 140:10:1.3:1.3 (Stevenson and Cole 2015).

10.6 Role of C:N Ratio in Soil Organic Matter Decomposition

10.6.1 C:N Ratio of Microorganisms

C:N ratio for bacteria is 5:1, actinobacteria 5:1 and fungi 10:1. Hence, for decomposition of 100 units of substrate C, 1–2, 3–6 and 3–4 units of N are necessary for bacteria, actinobacteria and fungi, respectively (Alexander 1977). Considering the relative proportion of microbial groups and composition of their cells, the weighted average of C:N ratio of soil microbes is approximately 8:1.

Table 10.3 Microorganisms responsible for degradation of organic residues

Name of the substrate	Microbial genera responsible for degradation
Cellulose	**B**: *Bacillus*, *Cellulomonas*, *Cytophaga*, *Achromobacter*, *Clostridium*, *Angiococcus*, *Cellvibrio*, *Polyangium*, *Sporangium*, *Sporocytophaga*, *Vibrio*
	F: *Aspergillus*, *Alternaria*, *Penicillium*, *Chaetomium*, *Fomes*, *Fusarium*, *Polyporus*, *Rhizoctonia*, *Rhizopus*, *Trichoderma*, *Verticillium*, *Trichothecium*
	A: *Micromonospora*, *Nocardia*, *Streptomyces*, *Streptosporangium*
Hemicellulose	**B**: B*acillus*, *Pseudomonas*, *Achromobacter*, *Cytophaga*, *Sporocytophaga*, *Vibrio*, *Lactobacillus*
	F: *Alternaria*, *Fusarium*, *Aspergillus*, *Trichothecium*, *Rhizopus*, *Chaetomium*, *Helminthosporium*, *Penicillium*, *Coriolus*, *Fomes*, *Polyporus*
Lignin	**B**: *Pseudomonas*, *Flavobacterium*
	F: *Clavaria*, *Clitocybe*, *Lepiota*, *Collybia*, *Flammula*, *Hypholoma*, *Mycena*, *Pholiota*, *Arthrobotrys*, *Cephalosporium*, *Humicola*
Starch	**B**: *Bacillus*, *Cytophaga*, *Achromobacter*, *Chromobacterium*, *Clostridium*
	F: *Aspergillus*, *Fusarium*, *Fomes*, *Polyporus*, *Rhizopus*
	A: *Micromonospora*, *Nocardia*, *Streptomyces*
Pectin	**B**: *Bacillus*, *Pseudomonas*, *Clostridium*
	F: *Fusarium*, *Verticillium*
Chitin	**B**: *Cytophaga*, *Bacillus*, *Achromobacter*, *Micrococcus*, *Chromobacterium*, *Flavobacterium*, *Pseudomonas*
	F: *Fusarium*, *Mucor*, *Trichoderma*, *Aspergillus*, *Mortierella*, *Gliocladium*, *Thamnidium*, *Absidia*
	A: *Streptomyces*, *Nocardia*, *Micromonospora*
Proteins and nucleic acids	B: *Bacillus*, *Pseudomonas*, *Clostridium*, *Serratia*, *Micrococcus*
Tannins	**F**: *Aspergillus*, *Penicillium*
Humic acid	**F**: *Penicillium*, *Polystictus*
Fulvic acid	**F**: *Poria*

B bacteria, **A** actinobacteria, **F** fungi

10.6.2 C:N Ratio in Organic Residues

The C:N ratio in organic residues is important for its decomposition because of the fact that the ratio helps to determine the rate of decomposition and the rate at which N is mineralized and be available to plant. Crop residues with wider C:N ratio or low N content, e.g. stalks of corn (1.2%), wheat (0.5%), sorghum (0.87%), sugarcane trash (0.88%) and rice (0.7%), decay slowly. When residues having wider C:N ratio are added to soils, intense competition develops among microorganisms for available soil N. A typical plant dry matter contains about 42% carbon, whereas N content is much lower and varies widely (from <1 to >6%). The C:N ratio of plants ranges from 10:1 to 30:1 in legume residues or young green leaves to 600:1 in some

sawdusts. With gradual maturity of plants, the proportion of protein in their tissues declines and the proportions of lignin and cellulose increase, and thereby, C:N ratio becomes wider (Stevenson and Cole 2015). The N content or the C:N ratio of plant residues is a good indicator for predicting the rate of decomposition but is not the sole determinant. The succulent or young plant tissues undergo quick decomposition, and it is not for low C:N ratio, but for less lignin compounds, reverse being true for woody tissues.

10.6.3 C:N Ratio of Soil

The C:N ratio of cultivated surface soil horizon ranges from 8:1 to 15:1 with an average of 12:1. Forest soils commonly have higher C:N ratios ranging from 30:1 to 40:1. When forest soil is brought under cultivation, the enhanced decomposition brings down the C:N ratio to near 12:1 (Brady and Weil 2002).

10.6.4 Influence of C:N Ratio on Decomposition

Majority of soil microbes for their nutrient requirements degrade cells, extract energy and metabolize carbonaceous materials. However, they must also obtain sufficient N to synthesize N-containing cellular components, such as amino acids, enzymes and DNA. The active decomposition of organic matter triggers multiplication of microflora and simultaneous assimilation of N for its growth processes. Considering the average microbial C:N ratio of 8:1, soil microbes need to incorporate into their cells eight part carbon for every one part of N. However, only about one-third of C is lost as CO_2 through respiration. So, microbes must get 1 g N for every 24 g carbon in their substrate. If the C:N ratio of organic materials added to soil exceeds 25:1, the soil microbes for the purpose of fulfilling their demand will scavenge N from soil and, thereby, cause N deficiency to crops. Moreover, it causes delay in further decay of organic matter if sufficient N to support microbial growth is present neither in organic material added nor in soil solution. If the plant residues like straw contain 40% C and 0.5% N, then for each 100 parts of organic waste, 0.4–0.8, 1.2–2.4 and 1.2–1.6 parts of N will be needed for growth and activities of bacteria, actinobacteria and fungi, respectively. Hence, deficit of (−) 0.1–0.3, 0.7–1.9 and 0.7–1.1 units of N for bacteria, actinobacteria and fungi will develop, and this deficit of nutrients will be compensated by their uptake from soil environment – the resultant effect in the soil environment is the immobilization. A conventional means of expressing the deficit following the supplementation of wide C:N ratio is by N-factor. N-factor is defined as the number of units of inorganic N immobilized from soil environment for each 100 units of organic

materials undergoing decomposition or operationally the amount of N that must be added during decomposition of organic wastes in order to prevent net immobilization from soil environment.

Wider C:N ratio of organic wastes favours immobilization, and narrower C:N ratio favours mineralization in soil. Hence, fresh organic waste should not be incorporated in standing crops. The optimum levels of C:N ratio of 20–25 (1.4–1.7% N) are ideal for maximum decomposition because at this range of C:N ratio, equilibrium between mineralization and immobilization process develops. If C:N ratio is less than 20–25, mineralization will exceed immobilization leading to accumulation of ammonium and nitrate from N.

For transformation of organic material to a part of soil humus, their C:N ratio is very important. In the process of organic matter decomposition by soil microorganism, both mineralization and immobilization of C and N occur. As the time passes, the rate of carbon mineralization becomes nearly equal to that of N mineralization, and the C:N ratio stabilizes at 10–12:1 indicating the formation of almost stable product, i.e. soil humus. If the organic materials with a narrow C:N ratio are incorporated into the soil, mineralization exceeds immobilization (assimilation) and results into excess of nitrate formation over crop requirement. In extreme cases, it may cause nitrate toxicity or nitrate loss by leaching. The C:N ratio of soils with stable organic matter is between 10 and 12. But in humid temperate forest regions, carbon mineralization is low, and therefore, C:N ratio is also higher (Alexander 1977).

10.7 Microbial Decomposition in Aerobic and Anaerobic Soils

The organic constituents of crop residues are diverse in nature and are commonly grouped into five broad categories: (1) carbohydrates (simple sugars, starch, cellulose, hemicelluloses, pectins), (2) lignins, (3) proteins and amino acids, (4) ether and alcohol soluble constituents (fats, oils, waxes, resins, pigments) and (5) phenolic compounds (phenols, tannins, etc.) Plant residues generally contain 15–60% cellulose, 10–30% hemicellulose, 5–30% lignin, 2–15% protein and 10% sugars, amino acids, organic acids and others (Alexander 1977). The bulk of plant tissues accounts for cellulose, hemicelluloses and lignin. The composition, however, varies from plant to plant and also with the age of the plant. On an average, the elementary compositions are C 44%, O 40%, H 8% and others 8%.

Decomposition of organic matter in soil is influenced by a number of environmental, biological and chemical factors. The process is accomplished by a complex group of microorganisms – those altering the composition during different stages of decomposition. The rapidity of breakdown of the substrate depends upon its chemical compositions and the physical, chemical and biological composition of

the surrounding environment. The oxygen status of the soil is the most vital factor that determines the qualitative as well as quantitative aspects of microbially mediated biodegradation of soil organic residues. Temperature is also one of the important environmental conditions determining the rate of decomposition (Liao et al. 2015). It reaches optimum at 30–40 °C temperature. Organic matter decomposition is better at 60–80% WHC of the soil as well as the plant materials, and decomposition proceeds more rapidly in neutral soil than acid or alkaline soil. Along with the heterogeneous group of microorganisms and environmental factors, available minerals, C:N ratio of the plant residues, type, age and chemical composition of the plant materials, period and time are the important factors for decomposition (Alexander 1977).

Microbial decomposition proceeds most rapidly in the presence of abundant oxygen supply. As most of the organic matters decomposing microorganisms are aerobic in nature, the decomposition rate is faster under aerobic condition, slower in water logged condition and least under complete anaerobiosis. During the oxidation of organic compounds, oxygen acts as major electron accepter. When organic matters are incorporated into soil, there is an immediate and marked loss of O_2, and increase of CO_2 occurs. At the same time, the oxidation-reduction potential shifts to a more reduced condition. Initially a mixed flora develops which acts on chemically complex natural products. As a result, some components quickly disappear, while others are less susceptible to microbial enzymes and persist for a longer period. The metabolic carbon such as sugars, proteins and starches is readily metabolized by soil microbes, whereas structural carbon like celluloses, polysaccharides, lignins, waxes, etc. degrades comparatively at a slower rate.

During decomposition of cellulose, the microfibers are released by enzymatic action of cellulase. During this process linear anhydrous glucose chains are also released from microfibers. Endo-glucanase acts on the liner anhydrous glucose chain at random to release oligomers. During this reaction, some single glucose units may also be released. Exo-gluconase acts on non-reducing ends of long linear anhydrous glucose chains and also on oligomers to release cellobiose and glucose units by the enzyme cellobiohydrolase and β-glucosidase, respectively (Subba Rao 2014). All the reactions occur simultaneously and also synergistically on cellulose to release glucose units. The enzymes catalysing hemicellulose breakdown are broadly termed as hemicellulases which yield the polymers of hexoses, pentoses and sometimes uronic acids with commonly occurring monomers such as xylose and mannose. The oxidation of cellulose and hemicelluloses in decomposing plants proceeds more or less at parallel rates (Alexander 1977).

Three classes of enzymes are involved in the degradation of pectic carbohydrates. Protopectinase decomposes protopectin to soluble pectin, pectin methyl esterase hydrolyses the methyl ester linkage of pectin to yield pectic acid, and methanol and polygalactouronase destroy the linkages between galacturonic acid units of either pectin or pectic acid with the release of smaller chains and ultimately

free the galacturonic acid. Chitin is a common polysaccharide in nature which breaks down into glucosamine and acetic acid by the enzymes chitinase and chitobiase (Alexander 1977).

Since the organic fractions of plant residues are composed largely of carbohydrates, under aerobic conditions the enzymatic oxidation reaction can be expressed by the following equation:

$$\text{Carbohydrate} + 2O_2 \rightarrow CO_2 + H_2O + \text{energy} \quad \left(478\,\text{kj}\,\text{mol}^{-1}\text{C}\right)$$

The lignins are highly resistant and consequently become relatively more abundant in the residual decaying organic matter. The lignin, a polymer of aromatic nucleus which is more resistant to microbial degradation, is cleaved by the enzymes ligninase/lignase into low molecular weight aromatic substances like vanillin, vanillic acid, syringic acid, syringaldehyde, ferulic acids, coniferaldehyde, coniferyl alcohol, benzoic acid, guaiacylglycerol, etc. Certain components of plant tissue such as tannin and phenolic substances inhibit the decomposition. During decomposition of organic matter, various intermediate and end products as well as products of mineralization of organic N (ammonification, nitrification, denitrification) and mineralization of organic P, S, etc. are also produced. The OH^- content of the remaining residues declines gradually, while $COOH^-$ content and CEC rise as the decomposition progresses.

Apart from the transient products in the decomposition of organic residues, humus formation also takes an integral part in the organic matter complex in soil. Not only it takes the formation of new humus complex, but it also helps to degrade the earlier humus. Treatment of soil with plant residues enhances the humus degradation due to huge proliferation of microbes in such environment – the greater the addition rate, the greater the loss of humus.

Under anaerobic conditions, organic C is incompletely metabolized, and intermediate substances are accumulated. Production of abundant quantity of CH_4, organic acids and small amount of H_2 are the characteristic features of anaerobic C-transformation. The transformation is mediated by both mesophiles and thermophiles. Bacteria, particularly of the genus *Clostridium*, play more active role than fungi or actinobacteria in cellulose degradation. Initially the primary micro flora breaks down complex carbohydrates and proteins into organic acids and alcohols. At a later stage, the secondary substrates such as lactic, acetic, butyric acids, etc. are accumulated. The organic acids so produced serve as substrate for methanogenic bacteria which produce CH_4 in anaerobic habitats. The anaerobic methanogenic bacteria such as *Methanobacterium*, *Methanobacillus*, *Methanosarcina* and *Methanococcus* are involved in methane production (Subba Rao 2014).

Anaerobic decomposition releases relatively less energy for the microorganisms involved. Some products of anaerobic decomposition produce foul odours and inhibit plant growth. The reaction pertaining to methane emission by methanogenic bacteria is as follows:

$$4\,C_2H_5COOH + 2H_2O \xrightarrow{\text{Bacteria}} 4CH_3COOH + CO_2 + 3CH_4 \uparrow$$
$$CH_3COOH \xrightarrow{\text{Bacteria}} CO_2 + CH_4 \uparrow \quad \uparrow$$
$$CO_2 + 4H_2 \xrightarrow{\text{Bacteria}} 2H_2O + CH_4 \uparrow$$

Both aerobic and anaerobic micro-environments can coexist in close proximity within the soil. If the organic wastes enter into an anoxic environment, a large part of lignified material remains undegraded or is humified very slowly under such conditions.

Under permanent anaerobiosis (Eh −200 mV), about 90% of the carbon is transformed to gaseous forms (CH_4 and CO_2), whereas about only 10% is assimilated. Under conditions of temporary or less marked anaerobiosis (Eh between 0 and −200 mV), catabolism of organic substances leads to the formation and accumulation of metabolites like alcohol, organic acids, molecular hydrogen and CO_2. Under anaerobic conditions, sulphur containing organic compounds degrades to produce H_2S gas and volatile S-compound mercaptans, methane-thiol, ethane-thiol, dimethyl-di-sulphide, diethyl-di-sulphide, etc. In addition to these phytotoxic phenolic compounds, many amino acids and other unidentified products also accumulate (Gobat et al. 2004).

The magnitude of dry matter loss is reduced under anaerobiosis, but as that of aerobic environment the percentage of sugars, water-soluble constituents and cellulose decline with simultaneous rise in lignin percentage. The environment is then dominated by newly formed microbial cells and those plant fractions exhibiting greatest resistance to microbial attack such as aromatic compounds.

10.8 Management of Soil Organic Matter and Soil Fertility in India

The SOM holds a key for sustained production in a number of ways such as increased nutrient and water-use efficiency, improved physical properties of soils, improved biological activity and crop quality. Maintenance of SOC level is not only important from soil productivity point of view, but also it is related to global warming through greenhouse effect. The SOM is the most vital parameter that needs to be taken cared for restoration of soil health through addition of organic materials and adoption of different management practices.

10.8.1 Addition of Organic Materials

Maintenance of active fraction of SOM is very important from soil productivity point of view and can be achieved mainly through regular addition of different

organic matter in soil and minimizing its loss through soil management. The important sources of these materials are plant residues, compost, farm yard manure (FYM), crop residues, organic wastes and green manure.

Better plant growth ensures higher addition of organic matter to soil in the form of crop residues, even if the aboveground portion is removed during harvesting, through better root growth. Coarse organic matter of active factions is capable of loosening the soil. It helps to form macropores, channels, etc. which increase water infiltration, and consequently, the lesser the surface run-off, the lesser the water pollution. Simultaneously, there is increase in gas exchange that contributes to better aeration, and more oxygen reaches the roots. Thus, crop residues enhance water-use efficiency and overall productivity.

Raising of suitable green manuring crops can add about 3–4 tonnes of dry matter per hectare, which not only add adequate amount of carbon but also add a substantial amount of N to soil. Addition of organic matter to soil through value-added enriched compost, vermicompost, FYM and municipal compost helps in proper utilization of animal excreta, household wastes, crop residues, biodegradable city or municipal waste and return of organic carbon removed by harvest.

10.8.2 Management Practices

Intensive cultivation without addition of adequate quantity of organic materials and maintenance of SOC is mutually exclusive. The maintenance of adequate level of SOC can be done mainly by adopting different management options which are discussed hereunder.

10.8.2.1 Soil C Sequestration

Carbon sequestration is one of the primary means of organic carbon buildup in soil. Soil C sequestration implies removal of atmospheric CO_2 by plants and storage of fixed C as SOM. The technological options for reducing soil C loss include integrated nutrient management (INM), improving quality and quantity of FYM, compost, vermicompost, green manure, mulch farming, incorporation of crop residues and recycling, regulated grazing, increased forestation, scientific water use, soil and moisture conservation (Ravindra and Reddy 2011), amelioration of degraded lands and choice of cropping system.

10.8.2.2 Integrated Nutrient Management for Improving Soil Organic Carbon

Soil fertility maintenance and plant nutrient supply can be ensured through integrated nutrient management (INM), i.e. sustaining the desired productivity

through optimization of the benefits from all possible sources of organic, inorganic and biological components in an integrated manner (Chandrasekhar Rao 2012). The FYM is the most popular form of organic manure. It contains about 0.5% N, 0.2% P and 0.5% K besides all secondary and micronutrients. In addition, it improves soil moisture retention and soil health. Efficient crop residue management plays a vital role in replenishing soil fertility and improving soil physical and biological properties. Crop residues can be converted into high-value manure through enrichment such as phosphocompost, vermicompost, etc. (Basak et al. 2012). Biofertilizers such as *Rhizobium*, *Azotobacter*, *Azospirillum*, blue green algae, phosphate solubilizing organisms, VAM, cellulose decomposers, etc. can be important components particularly in dry areas where low levels of fertilizers are applied. Swarup (1998) reported that integrated nutrient management enhanced SOC concentration of rice soils from <5 g kg^{-1} in 1973 to about 8 g kg^{-1} in 1994. The long-term fertilizer experiments in India have shown that balanced fertilization resulted in an increase in status of SOC in the upper 42 cm soil by 8 t ha^{-1} at the rate of 0.25 t ha^{-1} $year^{-1}$.

10.8.2.3 Cropping Systems for Improving Soil Organic Carbon

Crop species and cropping systems play an important role in maintaining SOC stocks because both the quantity and quality of residues that are returned to the soil vary greatly affecting their residence time in soil. Significant improvement in organic carbon content (7.0 g kg^{-1}) was found in sunhemp-rice-rice cropping system which was comparable with greengram-rice-rice system (6.7 g kg^{-1}) among other rice-based cropping systems tested after 2 years of cropping in sandy loam soils of Anantapur (Table 10.4) in scarce rain fall zone (Bhargavi et al. 2007).

10.8.2.4 Land Use System for Improving Soil Organic Carbon

Agroforestry is a useful option as the vegetation adds good amount of carbon in soils. In India, average sequestration potential in agroforestry has been estimated at 25 t C ha^{-1} over 96 Mha, but there is considerable variation in different regions, depending on the biomass production. The agroforestry systems are promising management system to increase aboveground as well as soil carbon stocks to mitigate greenhouse gas emission (Chandrasekhar Rao 2012).

In horticulture crops, soil C loss can be minimized by reducing soil tillage, maximizing plant water-use efficiency and application of surface mulches. Inclusion of legumes can be effective in adding higher percentage of plant biomass C to belowground C sequestration. Vegetable biomass such as plant parts discarded after the harvest of the economic produce may be converted into biochars and incorporated into the soil so that the carbon may be retained for longer time in the soil (Ganeshamurthy 2011).

Table 10.4 Organic carbon (g kg^{-1}) in soil as influenced by different cropping systems

S. No.	Cropping system	SOC after 1st year crop cycle	SOC after 2nd year crop cycle
1	F-R-F	3.8	4.6
2	Sh-R-F	5.1	5.8
3	Sh-R-R	5.2	7.0
4	F-R-R	4.0	5.8
5	F-R-G	4.6	6.0
6	F-R-Sg	3.9	5.3
7	Se-R-R	4.3	6.2
8	Se-R-G	4.5	5.8
9	Se-R-R	3.9	5.5
10	Gg-R-R	4.9	6.7
11	Gg-R-G	4.8	6.3
12	Gg-R-Sf	4.8	6.2
S.E±		0.2	0.10
CD (P = 0.05)		0.6	0.40

Source: Bhargavi et al. (2007)
F fallow, *Sh* sunhemp, *Se* sesame, *Gg* greengram, *R* rice, *G* groundnut, *Sf* sunflower

10.8.2.5 On-Farm Strategies for Improving Soil Organic Carbon

Suitable on-farm strategies and land management practices can enhance the uptake of CO_2 and reduce its emission and have the potential for improving SOC. Site-specific nutrient management package for individual farmers' field can be developed based on crop grown and soil test data. Tillage accelerates loss of organic matter by increased rate of oxidation and erosion. Therefore, disturbance of soil by tillage should be restricted up to the level optimum for seeding, weed control and soil aeration. Conservation practices to minimize tillage slow down the rate of decomposition of crop residues left over the soil surface and reduce erosion losses (Pandey and Singh 2012).

10.9 Opportunities and Challenges

Majority of our natural resources such as soil, water and biodiversity have been overexploited and are in an advanced stage of degradation primarily due to loss of organic matter and depletion of nutrients from soils, overexploitation of groundwater, overgrazing, etc. Rapid degradation of land resources leads to a reduction in the use efficiency of fertilizer along with rising emission of pollutants and greenhouse gases. Modern agricultural practices also release toxic chemicals to the environment, contaminate food stuffs and create health hazards. 'Nanotechnology' can precisely detect and deliver the correct quantity of nutrients or other inputs required by crops that promote productivity while ensuring environmental safety.

It is, therefore, necessary to give priority to recycle crop residues for sustainable crop production and soil fertility management. There is a critical need for the development of the best management practices that enhance buildup of SOC, thus sequester more carbon which in turn has the potential to mitigate the increase in atmospheric CO_2. Optimum levels of SOC can be managed through crop rotation, proper tillage practices and fertility maintenance including use of judicious application of inorganic fertilizers and organic manures. Among these, addition of balanced and integrated nutrient management offers the greatest potential for increasing SOC in agricultural soils. The huge quantities of agricultural waste need to be recycled back into the soil by converting them into value-added manures through suitable techniques like vermin-composting, phosphocomposting, etc. This is a feasible, environment-friendly option having good potentiality of employment generation for rural youths.

Based on the information and knowledge achieved in soil health management in general and organic waste recycling in particular, the important challenges need to be addressed on priority basis in the future for more diversified agriculture:

- Sustainable productivity and soil quality improvement through proper soil organic matter management in an eco-friendly manner
- Sequestration of carbon in soil through different land use systems and cropping systems and in rain-fed agriculture along with all available organic resources
- Site-specific nutrient management and integrated nutrient management
- Production and promotion of value-added enriched compost, vermicompost, biochar, etc. from farm wastes and their use in agriculture
- Conservation agriculture (CA) practices like minimum soil disturbance, diverse crop rotation, cover crop, continuous crop residue cover, integration of crop and live-stock production, etc.

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