Dhananjaya Pratap Singh
Harikesh Bahadur Singh
Ratna Prabha *Editors*

Microbial Inoculants in Sustainable Agricultural Productivity

Vol. 1: Research Perspectives

Springer

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Foreword

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सचिव एवं महानिदेशक
Dr. S. AYYAPPAN
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Sustainable agriculture without bargaining environmental quality is among a global concern. In the era of hugely applied chemical inputs (fertilizers, nutrients and pesticides etc.) in farming systems, serious threats are being observed on the reduced crop productivity and nutritional quality, decline in soil fertility, resistance among pests and phytopathogens, contamination of agroecosystem with over and above health problems for humans and animals. Since last few decades, viable biological options based on the basic principles of environmental protection and ecological sustenance have been widely worked out to minimize the threats of huge chemicalization in agricultural systems. Agriculturally important microorganisms have been found to have vast potential to minimize the ecological threats arising due to chemical inputs in soils and crops. Inoculation of a number of microbial strains in agriculture as soil or seed treatment have been shown proven benefits to the crop plants as well as the soils making both of them healthier, safer and sustainable. This is why the research in exploring microbial population with higher impacts of plant growth promotion, biological control of pests and diseases and soil fertility increased exponentially in the last few decades and many microbes have been identified, characterized and their multifarious mode of action benefitting plants and soils have been established. The prospects of manipulating soil biology and plant root rhizosphere with microbial population by inoculating beneficial microbes (bacteria, actinobacteria, cyanobacteria, fungi, mycorrhizal fungi etc.) have been well documented on the growth and development of plants, enhancement of intrinsic resistance against biotic and abiotic factors, tolerance against diseases and pests and improvement in the soil fertility status. This eco-friendly approach will lead to the reduction in dependence on chemicals. Moreover, recent progress in understanding of the biological interactions of microbes within their communities, with hosts, biotic and abi-

otic stresses in the rhizosphere, delivery system, viability issues and technological reliability has led to the development of practical requirements for microbial inoculant formulation development and commercialization.

This edited volume, '*Microbial Inoculants in Sustainable Agricultural Productivity Vol. I – Research Perspectives*' is a comprehensive effort concerning research perspectives on the identification, characterization, functional community analysis, mode of interactions, delivery models and formulations and benefits of inoculating beneficial microbes in the agricultural system as inoculants. The efforts of the editors is commendable, and the book would be useful for the entine-scientific community.

New Delhi
27 July 2015

(S. Ayyappan)

Preface

Microbial communities have potential to play a vital role in solving many if not all problems of present-day agriculture and environment and can be equally beneficial for crop production and protection management, food security, public health, and societal well-being. Microbes are the key living components crucial for the ecological harmony, ecosystem function, agricultural sustainability, environmental wellness, and human and livestock health. They are the most important components of soil biodiversity contributing to the valued agroecological services with their vast functional gene pool and metabolic capabilities. In the era of huge chemicalization and industrialization of agricultural ecosystems, microbes are fundamentally important for natural ecological functioning and balance, biotic and abiotic stress management, mineralization and nutrient recycling, bioconversion of complex animal and plant residues and bioremediation of soil contaminants, and, therefore, support of plant growth and development. Very close interactive mechanisms have been observed within the root rhizosphere of plants with microbial communities that survive on root exudates and strengthen plants in terms of growth, immunity, and resistance against abiotic and biotic stresses. This is why the task of identification, characterization, judicious exploitation of microbes and their communities, and finally utilization of an array of their functional characteristics has been taken at priority in the past several decades. The whole exercise is to come up with such efficient microbial systems that can offer their services at the farming level. Such microbial systems can be termed as "microbial inoculants" that provide beneficial agricultural services like plant growth promotion, nutrient use efficiency, bioremediation, and control of pests/phytopathogens.

Our understanding of the microbial communities, their specific functions, responses of plants and soils to such communities, and ecological impacts of such communities on other biotic and abiotic mechanisms has increased in the past to a greater extent. With the advent of technological advancements in the area of molecular biology and biotechnology, new avenues have been established to identify and characterize microbes and their communities and in assigning functions to them. Cumulatively, all these studies have led to the identification of several microbial species that were proved potential candidates for offering plant growth promotion, soil fertility management, biological control of pests and diseases, and bioremediation of environmental pollutants. The book *Microbial Inoculants in Sustainable Agricultural*

Productivity Vol. I Research Perspectives presents a holistic view of analyzing microbes and their communities and describing their functional role during the endeavor of developing microbial inoculants for the benefit of agricultural productivity. While going through the book, readers can find a detailed account of all such aspects that are required for making a microbe "microbial inoculant." The views of the authors are thorough and authoritative and based on their long research experience in the subject area. We are thankful to all the contributing authors for making their efforts to provide their valuable inputs in this volume. We hope that this Volume of the book will be very useful for all those who are actively involved in the endeavor of developing microbial inoculants for reaping their benefits in sustainable agricultural productivity.

Maunath Bhanjan, Uttar Pradesh, India — Dhananjaya Pratap Singh
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Strategies for Characterization of Agriculturally Important Bacteria

1

V.J. Szilagyi-Zecchin, Á.F. Mógor, and G.G.O. Figueiredo

Abstract

The technology of plant production always faced fast-growing food and energy demands, but driven by a new approach, the answer for those demands must be socially and environmentally conscious. In this way we have a very powerful tool, bacteria, that benefit the plants. Therefore, to use that natural resource, some aspects must be observed, such as carrying out isolation of strains directed to the use (when possible) and correctly identify the strain used, not only by morphological techniques but also by molecular techniques, looking for the necessary biosafety for those who will use the developed technology. The characterization of strains will define the potential use that we want to follow: biofertilizer, phytostimulators, or biocontrol agents. After identifying the main characteristics of bacteria, there is a universe of possibilities regarding the plant interaction and bacteria, such as the signal recognition, penetration, and establishment, and whether the bacteria are endophytic, epiphytic, or rhizospheric. Before the immersion on the complexity of the issue, our aim was to contribute for the characterization of agricultural interest of bacteria, with attention to the desired characteristics, and discuss the mechanisms within each line of action – biofertilizer, phytostimulators, or biocontrol agents.

Keywords

PGPB • Biofertilizer • Phytostimulant • Biocontrol agents • Characterization

1.1 Introduction

Due the necessity to reduce chemical products (chemical fertilizers, pesticides, and supplements), aiming sustainable agriculture and protecting the environment, the use and research of microorganisms have been focused in the whole world (Vale et al. 2010). An alternative to reduce chemicals,

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intent to greater productivity, and the plant growth-promoting bacteria (PGPB) are showing a promising and viable "noble tool" (Bevivino et al. 2000; Harthmann et al. 2009; Hungria et al. 2010). This occurs because there is a continuous presence of bacteria in the rhizosphere soil, rhizoplane, and internal plant tissues (Hallmann et al. 1997).

The preferential site of the bacteria colonization may diversify plant by plant or among different growth-promoting bacteria, but all of them bring benefits to the host plant, for example, the rhizosphere bacterial group; these communities have efficient systems for uptake and catabolism of organic compounds present in root exudates (Barraquio et al. 2000). Several bacteria may also help to derive maximum benefit from root exudates by their ability to attach to the root surfaces (rhizoplane) (Compant et al. 2005), or endophytic microorganisms which have advantages, by the developing inside of plant tissues, suffering smaller population losses, due to environmental interaction (Sharma and Nowak 1998).

Although there is the importance of bacteria type and colonization site, selecting the right bacteria is primal, with characteristics of interest, according to the target, whether, e.g., they are phytostimulators, biofertilizer, or biocontrol agents (Pal et al. 2001; Rana et al. 2011). Indeed, it is required to understand how the characteristics of the bacteria act in plants and promote effects; normally there is a lot of characteristics expressed by each bacteria in relation with plants.

1.2 Bioproducts, Biofertilizer, and Biopesticides

In facing the challenge to feed humankind on an ecologically friendly way, a new agriculture comes driven by a critical consciousness, knowledge, and technology, having bioproducts as an effective tool. Biofertilizers are a class that aggregates a range of bioproducts related to their bioactivity and to improving biological processes.

Biofertilizers and biopesticides hold the potential to increase agricultural productivity with a sustainable approach. A number of countries such as Argentina, Canada, South Africa, India, Australia, the Philippines, the United States, and Brazil, among others, have embraced these technologies (Simiyu et al. 2013).

Biofertilizers are related commonly to plant growth promotion and responses to abiotic stresses, induced by a pool of bioactive compounds from a great diversity of environment-friendly sources. The beneficial bacteria can produce phytohormones and other compounds (Borriss 2011), biomasses and their extracts, e.g., algae (Jannin et al. 2013) and yeast (Lonhienne et al. 2014), or by mycorrhizal fungi (Bettoni et al. 2014), even products obtained by fermentation as amino acid sources (Civiero et al. 2013), among a huge diversity of sources that nature and the biotechnology can offer.

Under the same concept, the biopesticides defined by the US Environmental Protection Agency (EPA) as pesticides derived from natural materials (Borriss 2011), which in general are no pathogenic microorganism strains (Vinale 2014) or plant extracts (Kasiotis 2013) with effect against pests or diseases, or the bio-inoculants related to biologic nitrogen uptake, are called sometimes as biofertilizers too.

The biofertilizers definition on regulatory affairs not exactly specify the sources, but, for example, Brazilian regulation determines the bioactivity as a main effect: "Biofertilizer is a product that contains active ingredient or organic agent, free for agrochemicals, capable of act directly or indirectly on all or part of cultivated plants, raising the productivity, without taking into account their hormonal or stimulating value" (Brasil 2004). On Brazilian regulation of organic production, biofertilizer is defined as a "product containing active components or biological agents capable to acting, directly or indirectly, on the whole or part of cultivated plants, improving the performance of the production system and that been free from substances prohibited by the rules of organic production" (Brasil 2008). In both regulations, the bioactivity and/or some active ingredients is needed to characterize a biofertilizer.

According to Balachandar (2012) even though hundreds of bacteria and fungi were identified for enhancing plant growth, only few have been commercially exploited as biofertilizers. In the same way, many natural compounds could be classified as biofertilizer with proven bioactivity, such as fulvic acid, amino acids, and kelp extracts. These compounds are sold as common mixed fertilizer with mineral nutrients, and their bioactivity is not observed.

To stimulate researchers and companies for finding new biofertilizer sources and deliver them according to regulations to the market as a sustainable tool to the growers, the characterization of the plant growth promotion (PGP) and distinction from biopesticides, bio-inoculants, mineral fertilizers, and biostimulants is desirable.

The establishment of simple bioassays to find and characterize the PGP effect before the field trials could be an efficient tool on biofertilizers research. The bioassays developed from the 1960s to 1990s, following the development of plant hormones and plant growth regulators knowledge, could be very useful in screenings to find PGP bioactivity on potential biofertilizer sources, as the classical bioassay described by Zhao et al. (1992), which uses cucumber (*Cucumis sativus*) hypocotyl and cotyledons evaluating expansion after excision of whole seedlings used to find growth effect by action of the tested substances. In the same way, Stirk et al. (2002) got results with cucumber cotyledon root formation using Cyanophyta and microalgae extracts, and Sharma et al. (2012) show the bioactivity of brown seaweed species with bioassay of extracts using mung bean (*Vigna radiata*) and pak choi (*Brassica rapa chinensis*).

The clear characterization of biofertilizer related to their bioactivity, and the consolidation of nomenclature of biofertilizer in both scientific and regulatory literature as a class of natural source bioactive products, could consolidate this eco-friendly technology to the new agriculture. Focused in search and characterization of bacteria to potential use in agriculture, showing PGP effect or as biofertilizer, bio-inoculant, or even biopesticide, some strategies are discussed forward.

1.3 Strain Identification

The correct identification of microorganism is the major importance to establish control measures, to prevent pathogenic dissemination, or to reference strains with biotechnological interest. Nevertheless, the fast and sensitive techniques that provide reliable results to the correct identification of microorganisms are essential, seeking the protocol optimization and diversification of the research methods (Atkins and Clark 2004). The phenotypic classic methods utilized for bacteria identification are important, as well as morphology, biochemical and serological tests, fatty acids and exopolysaccharides (EPSs) profile, and enzymatic standard, though these methods are limited and insufficient for accurate discrimination of species and strains (Oliveira et al. 1999).

However, when these classic methods are associated to molecular biology, it can become together, as powerful tools in the characterization and identification of microbial germplasm (Gütler and Stanisich 1996; Oliveira et al. 1999). Woese and Fox (1977) conducted studies that indicated the ribosomal RNA utilization, more specifically the small ribosome (16S for prokaryotes) subunit, as phylogenetic marker. The rRNA sequence is present in all organisms and evolves at a relatively low rate, allowing kin detection between very distant species (Harris et al. 2003). The utilization of 16S rRNA revolutionized microbial ecology, enabling to investigate and to determine phylogenetic position of bacterial communities of the environment (Hentschel et al. 2002; Barreto et al. 2008) and associated with plants (Rijavec et al. 2007; Fürnkranz et al. 2009; Ikeda et al. 2013).

Among closely related species, the only use of 16S rRNA does not allow to identify their differences (Martens et al. 2007); this fact was proved by some studies, which demonstrated that genetic recombination and horizontal transference could occur with 16S rRNA, implying the introduction of other markers to complement the use of 16S rRNA (van Berkum et al. 2003; Gevers et al. 2005).

Bacterial species may be defined as a group of similar strains genomically, where they share high

similarity in many independent characteristics (Rosselló-Mora and Amann 2001). The similarity between prokaryotic to be considered a species, must be greater than 97 % of the 16S ribosomal gene sequence compared to the type strain, allowing microbiologists to rapidly identify new species (Vandamme et al. 1996; Gevers et al. 2005).

Therefore, other genes have been proposed to realize phylogenetic analysis, where generally genes with higher evolution rate than 16S rRNA are utilized, though they are preserved to maintain genetic information to be classified taxonomically (Silva et al. 2005; Martens et al. 2007). A few examples of those genes are *rec*A, *dna*K, *glt*A, *gln*II, *rpo*A, and *atp*A (Naser et al. 2005; Ribeiro et al. 2009).

Among the requirements in the selection of the sequences, the wide distribution in a taxon should be considered, as well as being present in a single copy of the genome (Gevers et al. 2005; Thompson et al. 2005); howsoever, to realize phylogenetic analysis, multilocus sequence analysis (MLSA) has been used for some genus, like *Burkholderia*, *Bacillus*, *Vibrio*, *Mycobacterium*, *Ensifer*, *Rhizobium tropici*, *R. leucaenae*, *R. freirei*, and *Mesorhizobium* (Gevers et al. 2005; Thompson et al. 2005; Martens et al. 2007; Ribeiro et al. 2009; Laranjo et al. 2012; Dall'Agnol et al. 2013), reducing ambiguous possibilities caused by genetic recombination and specific selection.

1.4 Biofertilizers' Characteristics

Biofertilizers' characteristics are known for their ability to provide the plant root with nutrients such as nitrogen, phosphorus, and iron.

1.4.1 Biological Nitrogen Fixation

Nearly 78 % of atmosphere gas constituents are represented by nitrogen in molecular form (N_2). In this form, plants could not absorb nitrogen because the mainly absorbed forms are nitrate (NO_3^-) and ammonium (NH_4^+) (Taiz and Zeiger 2009). When there are low rates of nitrogen in plants, this nutrient turns the "first" limiting factor of vegetal growth, causing reduced productivity (Durães et al. 2004).

Some prokaryotic organisms are able to assimilate the N_2 from atmosphere and convert in absorbed form as NH_3^-, and this process is called biological nitrogen fixation (BNF) (Reis et al. 2006). The organisms that could be included at this process (N_2-fixing forms) are the symbiotic bacteria like *Rhizobium*, one of the obligate symbionts in leguminous plants, *Frankia* in nonleguminous, and the nonsymbiotic bacteria (free living, associative, or endophytic) such as *Azospirillum*, *Azotobacter*, and *Acetobacter* (Saharan and Nehra 2011).

The occurrence of BNF is linked by the action of enzymatic system of the nitrogenase or dinitrogenase, and this complex spends a lot of energy from the organism (Reis et al. 2006). The complex is formed by two components, one of them called component I which has been known, like dinitrogenase reductase (iron protein), that consists of a dimer of identical subunits (γ2), and the subunits are codified by *nif*H gene, while the component II (molybdenum–iron protein) is a tetramer α2ß2 which has two molybdenum atoms, 30 of iron, and 30 of sulfur. The subunity α is codified by *nif*D gene and ß *nif*K gene (Eady 1991). Thereat if utilized the *nif*H amplification, as a tool, may inform what kind of microorganisms are involved in the BNF (Zhan and Sun 2011) and confirm the gene presence of organism that expects to be a fixer (Beneduzi 2008).

The symbiotic bacteria like rhizobia have a very singular relationship with legume plants, involving a series of chemical signals to promote a nodule development, truly "nitrogen machine." Legumes start this exchange by secreting compounds from their roots, like flavonoids. The flavonoids bind to receptors in the plasma membranes of compatible soil rhizobia, therewith in response, the rhizobia secrete *Nod* factors (nodulation factors) allowing bacteria to enter roots via root hairs. In the next step, the root hair plasma membrane allows an influx of calcium ions, and this calcium changes by swelling at their tips and curling around the rhizobia. Then, these bacteria inject infection proteins into root

hairs, which the root hair cell wall degrades and the plasma membrane forms a tubular thread where the rhizobia enter. The bacteria move through this infection thread into the root cortex, and the tip of the infection thread fuses with the plasma membrane of a cortex cell. The rhizobia are then released into the cortex cell cytoplasm, enclosed by the host membrane (Hirsch 1992; Taiz and Zeiger 2009).

When plants send an undetermined signal after the bacteria reach the cortex cell cytoplasm, they start to enlarge and to differentiate into *Bacteroides*, which are endosymbiotic organelles to nitrogen fixing. Therefore, a nodule involves *Bacteroides*, important to avoid interference of oxygen and sequent reduction and the efficiency of fixation, where it has a vascular system to help exchange between nitrogen fixed by the bacteria and nutrients provided by the plant. The ammonia produced by *Bacteroides* is toxic, whence it is converted to organic forms before being transported to the vessels via xylem (Hirsch 1992; Taiz and Zeiger 2009).

The organic forms transported to plants depend on the composition of the xylem sap, divided by amide exporters or ureide exporters. The amides mainly asparagine and glutamine are the way to transport nitrogen by legumes from temperate region, e.g., pea (*Pisum*), clover (*Trifolium*), broad bean (*Vicia*), and lentil (*Lens*). In the tropical regions the preferred is ureide exporters by legumes, represented by, e.g., soybean (*Glycine*), kidney bean (*Phaseolus*), peanut (*Arachis*), and southern pea (*Vigna*) (Alves et al. 2000; Sprent 2001; Taiz and Zeiger 2009).

Examples of the contribution of BNF in crops can be seen, like soybean (*Glycine max*), in Brazil, which is a successful methodology applied in the field production. The success was resulted by the strain selection with affinity of the Brazilian cultivars, further to high efficiency of BNF and the adaptation along the Brazilian environments. The contribution of BNF to the total N accumulated in plants from established population of *Bradyrhizobium* ranges from 75 to 92 % (Hungria et al. 2006). And for other important legume, common bean (*Phaseolus vulgaris*), the *Rhizobium* spp. strains can fix about 66–78 % of total nitrogen used by plant (Hungria and Neves 1987; Franzini et al. 2013).

The interaction of free-living, associative, or endophytic bacteria with plants was demonstrated under sterile conditions followed by microscopic analysis (Roncato-Maccari et al. 2003; Fan et al. 2012; Quecine et al. 2012). According to Azevedo (1998), bacterial colonization mainly occurs due to injuries occasioned by secondary lateral root emergency, thus allowing microorganisms input in plant. They colonize the spaces at the junctions of the lateral roots and the intercellular spaces of the root epidermis with different patterns in different species, such as maize (*Zea mays*), sorghum (*Sorghum bicolor*), wheat (*Triticum aestivum)*, rice (*Oryza sativa)*, *Arabidopsis thaliana*, and *Lemna minor* (Roncato-Maccari et al. 2003; Fan et al. 2012), or penetrate deeply to enter the internal tissues of the roots and basal stem of rice and *L. minor* (James et al. 2000; Fan et al. 2012), even colonize the aerial parts by entering in the xylem tissues of the roots and stem of rice and sugarcane (*Saccharum* spp.) (James et al. 2000; Quecine et al. 2012).

The nitrogen provided by BNF to plants can vary between species, mainly in nonleguminous, according to some studies the amounts of nitrogen supplied are ranged from 4 to 70 % in sugarcane, about 50 % in maize, and could be until 36 % in cultivated rice (Yoneyama et al. 1997; Malarvizhi and Ladha 1999; Hungria et al. 2010).

It is believed that the variation among species in relation to the amount of fixed nitrogen depends on cultivar, plant stage, strain, inoculation method, and environmental conditions. The ability of a bacteria to fix atmospheric nitrogen within a host has been detected using different approaches: acetylene reduction assay and 15 N isotope experiments developed with rice and sugarcane plants (Iniguez et al. 2004), tetrazolium reduction staining technique (Patriquin and Döbereiner 1978), detection of the nitrate reductase ability of the bacteria to help in the incorporation of the nitrogen assimilated from soil by the plant (Ferreira et al. 1987), and gene amplification related to BNF, as *nifH* by means of degenerated primers (Zehr and Capone 1996). The ideal

approach is to combine different techniques working together and to aim a safety response, as to BNF capacity of strains (Rana et al. 2011; Szilagyi-Zecchin et al. 2014).

1.4.2 Phosphate Solubilization and Phytase Production

The second essential element in plants' necessity is the phosphorus (P), being only nitrogen's behind (Kucey 1988), and making up for about 0.2 % of a plant's dry weight. It is a component of key molecules such as nucleic acids, phospholipids, and ATP. P is also involved in controlling key enzyme reactions and in the regulation of metabolic pathways (Taiz and Zeiger 2009).

The amount of phosphorus in the soil is generally high (often between 400 and 1200 mg/kg of soil) (Khan et al. 2007), but due to its reactivity, most of this phosphorus is insoluble and therefore not available to support plant growth (López-Bucio et al. 2002). The insoluble phosphorus is present like inorganic mineral such as apatite or in an organic form including inositol phosphate (soil phytate), phosphomonesters, and phosphotriesters (Khan et al. 2007). Chemical fertilization usually is done with soluble inorganic phosphorus, but much of that is immobilized soon after it is applied and is wasted because it becomes unavailable to plants (Feng et al. 2004). To improve the phosphorus nutrition is achievable by "mobilization" of phosphorus as insoluble inorganic polyphosphates and phytate, which accounts for 20–50 % of the total soil organic phosphorus (Richardson et al. 2001a).

In the rhizosphere, the conversion of the insoluble forms of inorganic P to a form accessible by plants is achieved by phosphate-solubilizing bacteria (PSB) which release phosphates meanly by organic acids releasing (Richardson et al. 2001b) such as gluconic and citric acid, both of which are synthesized by various soil bacteria (Bnayahu 1991; Rodriguez et al. 2004). Notwithstanding, the phytates of an organic P form, occurring in great quantity in most soils, around 10–50 % of total P, might be mineralized by phytases, like myo-inositol hexakisphosphate phosphohydrolases, and in this way may the P be ready to use in plant nutrition. Thereat, bacteria with production of phytase and organic acids in same strains are interesting to agricultural uses (Richardson et al. 2001b; Tao et al. 2008).

Many studies have examined the abilities of different bacterial species to solubilize the compounds of inorganic P (Song et al. 2008; Chagas Junior et al. 2010) or even both activities, phytase positive and phosphorus solubilization, showing the capacity for accumulation of phosphorus in plants (Singh et al. 2014).

1.4.3 Siderophores

Iron is the fourth most abundant element on earth; in aerobic soils, iron is not readily assimilated by bacteria or plants. This element can exist in aqueous solution in two states: Fe^{2+} and Fe^{3+}. The Fe^{3+} forms are not utilizable by plants and microorganisms because they often form insoluble oxides or hydroxides limiting their bioavailability (Ma 2005; Zuo and Zhang 2011). The iron is an essential nutrient for plants, and its deficiency is exhibited in severe metabolic alterations, mainly because iron is present as a cofactor in many enzymes essential to physiological processes, such as respiration, photosynthesis, and nitrogen fixation (Taiz and Zeiger 2009).

Microorganisms and plants require a high level of iron and to obtain sufficient iron is even more complicated in the rhizosphere, because at this site, plant, bacteria, and fungi compete for nutrient; in this way the siderophores may act directly in the growth promotion and indirectly in biological control (Guerinot and Ying 1994; Hu and Xu 2011).

Plants can use two strategies to acquire iron: (i) acidification of the rhizosphere followed by reduction of Fe^{3+} ions by membrane-bound Fe(III)–chelate reductase and uptake of Fe^{2+} into root cells; (ii) plants secrete low-molecular-weight phytosiderophores for solubilization and bind iron which is then transported into root cells via membrane proteins (Altomare and Tringovska 2011). However, these strategies are often not efficient enough to

the necessity of plants growing especially in calcareous or alkaline soils. Therefore, in this case it is necessary providing plants accessible forms of iron (Zuo and Zhang 2011).

Microorganisms also secrete siderophores due to the low disponibility of Fe^{+3} in solution. The bacterial growth as well as siderophore production is stimulated by $(NH_4)_2SO_4$ (ammonium sulfate) and amino acids; however, the optimum siderophore yield is obtained with urea (Sayyed et al. 2005). Many siderophores may form complexes with some elements such as copper, aluminum, and molybdenum (Benite et al. 2002). These elements may act on the external side of cell membrane, binding iron molecules in solution with specifically membrane receptor, where they are absorbed, thereby making iron available for growth promotion in plant (Taiz and Zeiger 2009).

There are more than 500 known siderophores, and the chemical structures of 270 of these compounds have been determined (Hider and Kong 2010). Production of siderophores by bacteria is detected via the chrome azurol S assay, a general test, which is independent of siderophore structure. Siderophores are usually classified by the ligands used to chelate the ferric iron: catecholates (phenolates), hydroxamates, and carboxylates (e.g., derivatives of citric acid) (Taiz and Zeiger 2009).

Glick (2012) defined the benefits of bacterial siderophores to plants using examples of different experiments. The experiments cited including benefits in mung bean (*Vigna radiata*), peanut (*Arachis hypogaea*), and *Arabidopsis thaliana* plants. In mung bean plants the *Pseudomonas* produced the siderophore, growing in iron-limited condition, enhancing chlorophyll contents in plant, and reducing chlorotic aspect in leaves (Sharma et al. 2003). Either the species *Pseudomonas putida* also reduced chlorotic aspect in peanut, when iron deficiency was induced (Jurkevitch et al. 1992). Likewise, *Pseudomonas fluorescens* helped to better performance in *Arabidopsis thaliana*, raising iron contents in plant tissues, mediated by the bacterium Fe–pyoverdine complex inside plants (Vansuyt et al. 2007).

The provision of iron to plants by bacteria is even more important when the plants suffer an environmental stress (e.g., heavy metal pollution). In this situation, siderophores help to alleviate the stresses imposed on plants by high soil levels of heavy metals (Braud et al. 2006; Ines et al. 2012).

1.5 Phytostimulators' Characteristics

Plant hormones are a group of naturally occurring organic substances that influence physiological processes at low concentrations in response to the environment stimulus (Davies 2004). When these plant responses are not so effective, rhizosphere microorganisms may also produce or modulate phytohormones (Salamone et al. 2005) so that many bacteria can alter phytohormone levels and thereby affect the plant's hormonal balance and its response to environment (Glick et al. 2007).

1.5.1 Auxins

The indoleacetic acid (IAA) is the main auxin in natural occurrence of plants (Taiz and Zeiger 2009). IAA may act in many physiological processes in the plants; affects photosynthesis and pigment formation; mediates responses to light, gravity, and florescence; controls biosynthesis of various metabolites; modulates resistance to stressful conditions; controls processes of vegetative growth; and more specifically acts in cell division and differentiation, stimulates seed and tuber germination, increases the rate of xylem and root development, and initiates lateral and adventitious root formation (Spaepen and Vanderleyden 2011; Taiz and Zeiger 2009). Howsoever, countless bacteria are still able to synthesize IAA, such as *Azospirillum brasilense*, *A. lipoferum* (Kuss et al. 2007) species of *Bacillus* and *Paenibacillus* (Beneduzi et al. 2008), *Providencia* (Rana et al. 2011), and *Pseudomonas fluorescens* (Hernandez-Rodriguez et al. 2008). In general, there is a partnership between the

plant and the bacteria, as in the situation where the bacterial IAA increases root surface area and length, providing the plant greater access to soil nutrients. In addition, bacterial IAA loosens plant cell walls and as a result facilitates an increasing amount of root exudation, allowing more nutrients to support the growth of rhizosphere bacteria (Glick 2012).

The response of the plant to IAA varies with the type, degree of sensitivity, developmental stage of the plant, and according to the particular tissue involved, for example, in roots versus shoots (the optimal level of IAA for supporting plant growth is ~5 orders of magnitude lower for roots than for shoots) (Taiz and Zeiger 2009). However, the endogenous pool of plant IAA may be altered by the acquisition of bacterial IAA. The level of IAA synthesized by the plant is important in determining whether bacterial IAA stimulates or suppresses plant growth (Glick 2012).

For example, canola (*Brassica campestris*) seeds inoculated with wild-type *Pseudomonas putida* increased the length of roots compared with an IAA-deficient mutant and the control uninoculated (Xie et al. 1996), when the same strain was inoculated in mung bean (*Vigna radiata*) cuttings with a mutant which overproduces IAA, yielded a much greater number of shorter roots compared with controls (Mayak et al. 1999). Or even with the use of purified bacterial auxins of *B. subtilis* and *B. licheniformis* also has an influence on plant growth of red pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum*), displaying up to 20 % increased root, stem, and leaf growth (Lim and Kim 2009).

Root nodulation is also affected by IAA, most rhizobia strains that have been examined have been found to produce IAA (Badenoch-Jones et al. 1984; Boot et al. 1999; Datta and Basu 2000), and several studies have suggested that increases in auxin levels in the host plant are necessary for nodule formation (Mathesius et al. 1998; Pii et al. 2007; Mathesius 2008). Soybean plants inoculated with *Bradyrhizobium* spp. mutant that had a decreased level of IAA synthesis had a lower nodule mass and fixed less nitrogen per gram of nodule (Hunter 1987) and induced fewer nodules on soybean roots (Fukuhara et al. 1994) than did plants inoculated with wild-type bacteria, supporting the idea that part of the IAA found in nodules is of prokaryotic origin and that this IAA facilitates nodulation.

The bacterial IAA not only serves to manipulate host physiology but also acts as a bacterial signal (Spaepen et al. 2007). Interesting in this context is the stimulation by IAA of its own synthesis in *Azospirillum* species, analogous to a *quorum sensing* (QS) or autoactivation mechanism. This hypothesis has calling attention for the plant-associated bacteria that can actively destroy IAA and can be quite common on plant (Riviere and Berthier 1964), such as *Alcaligenes*, *Pseudomonas* (Libbert and Risch 1969), *Arthrobacter* (Mino 1970), and *Bradyrhizobium* (Egebo et al. 1991). Some, like *Pseudomonas putida*, can use IAA as a sole source of carbon, nitrogen, and energy (Leveau and Lindow 2005).

1.5.2 ACC Deaminase and Ethylene

The plant hormone ethylene has a wide range of biological activities and can affect plant growth and development in a large number of different ways, such as promoting root initiation, fruit ripening, flower wilting, leaf abscission; stimulating seed germination; activating the synthesis of other plant hormones; inhibiting root elongation, nodule formation, mycorrhizae–plant interaction; and responding to both biotic and abiotic stresses (Abeles et al. 1992; Taiz and Zeiger 2009).

The ethylene biosynthesis in higher plants uses methionine amino acid as biological precursor into two steps. The first reaction occurs when S-adenosyl-methionine (SAM) is converted to the 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthetase enzyme (ACCS) reaction. Then the ACC is metabolized by ACC oxidase (ACCO), which this reaction needs oxygen (O_2) and iron, wherein it is activated through CO_2 to produce ethylene (Yang and Hoffman 1984).

After the discovery of the enzyme 1-aminocyc lopropane-1-carboxylate (ACC) deaminase in bacteria, which is capable of degrading ACC to α-ketobutyric acid (Honma and Shimomura

1978), many studies indicated that this enzyme was common in plant growth-promoting bacteria (PGPB) (Belimov et al. 2001; Blaha et al. 2006; Sgroy et al. 2009). Therewith, the effects of plant growth promotion and the ACC deaminase enzyme are linked to the decrease of ethylene rates in plants, especially in environmental stress like (i) salt stress, *Brevibacterium iodinum*, *Bacillus licheniformis*, and *Zhihengliuella alba* improving red pepper plant growth (Siddikee et al. 2011) and *Pseudomonas fluorescens* and *Pseudomonas migulae* promoting tomato plant growth under two different levels of salt stress (Ali et al. 2014) and (ii) metal stress, *Ralstonia* sp., *Pantoea agglomerans*, and *Pseudomonas thivervalensis* providing a canola plant growth in copper-contaminated soil (Zhang et al. 2011). The main visible effect of seed or root inoculation with ACC deaminase-producing bacteria is the enhancement of plant root elongation in many kinds of plants, like *Arabidopsis* (Contesto et al. 2008), tomato (Xu et al. 2014), chickpea (Nascimento et al. 2012), and canola (Zhang et al. 2011).

The plant ethylene levels can rise following the infection of legumes by *Rhizobium* spp.; this increased ethylene concentration can inhibit subsequent rhizobial infection and nodulation (Ma et al. 2002). Some rhizobia limit the increase of ethylene and raise the number of nodules, producing a molecule called rhizobitoxine (Yuhashi et al. 2000), that chemically inhibits the functioning of the enzyme ACC synthase, one of the ethylene biosynthetic enzymes. Other rhizobial strains produce the enzyme ACC deaminase which removes some of the ACC (the immediate precursor to ethylene in plants) before it can be converted to ethylene (Ma et al. 2002).

This bacterial enzyme facilitates the growth of the plant, when the bacteria colonized roots or seeds, and thus in response to tryptophan either other small molecules from seed or root exudates, the bacteria synthesize and secrete IAA (Patten and Glick 1996, 2002). This bacterial IAA, together with endogenous plant IAA, can stimulate plant growth or induce the synthesis of the plant enzyme ACC synthase that converts the compound S-adenosyl methionine to ACC, the immediate precursor of ethylene in higher plants. A portion of the newly synthesized ACC is excluded from seeds or plant roots, such as that seen in canola (*Brassica napus*) plants inoculated with *Enterobacter cloacae* (Penrose and Glick 2001) taken up by the bacteria, and converted by the enzyme ACC deaminase to ammonia and α-ketobutyrate, compounds that are readily assimilated.

The degradation ACC from the direct precursor of ethylene creates an ACC concentration gradient between the interior and the exterior of the plant, favoring its exudation and reducing the internal ethylene level (Glick et al. 1998). This, in combination with auxins that may be produced by the same microorganism, causes root system development, because the bacterial ACC deaminase competes with the plant's ACC oxidase (Glick 1995; Glick et al. 1998). As a direct consequence of this enzyme's activity, the amount of ethylene produced by the plant is reduced. Therefore, root or seed colonization by bacteria that synthesize ACC deaminase prevents plant ethylene levels from becoming growth inhibitory (Glick 1995; Glick et al. 1998).

1.5.3 Cytokinins

Cytokinins are plant hormones that influence many physiological processes, like stimulate cell division, initiate shoot growth, retard senescence (Mok and Mok 1994), regulate chloroplast development and leaf expansion (Taiz and Zeiger 2009), and modulate nodulation (Frugier et al. 2008; Plet et al. 2011). Cytokinins are synthesized in root tips and developing seeds, and they are transported to the shoot. The zeatin is the major representative (Taiz and Zeiger 2009).

The cytokinin biosynthesis in plants and bacteria has some differences; most begin with the transfer of isopentenyl group from dimethylallyl diphosphate (DMAPP) to the N6-amino group of adenine by either adenylate isopentenyltransferase (AIPT) or tRNA–IPT. Plant AIPTs use ATP/ADP as an isopentenyl acceptor, and bacterial AIPTs prefer AMP, whereas tRNA–IPTs act on specific sites of tRNA (Sakakibara et al. 2005)

The spectrum of cytokinins produced by bacteria is similar to that produced by plants of which zeatin and zeatin riboside excreted by *Bacillus licheniformis*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* (Hussain and Hasnain 2009) and isopentenyl adenosine, trans-zeatin ribose, and dihydrozeatin riboside produced by *Pseudomonas fluorescens* (Garcia de Salamone et al. 2001) and *Bacillus subtilis* (Arkhipova et al. 2005) are most commonly found.

Some studies indicate that cytokinin produced by bacteria becomes part of the plant cytokinin pool and thus influences plant growth and development. Inoculation of lettuce (*Lactuca sativa*) plants with *Bacillus subtilis* increased the cytokinin content of both shoots and roots. Accumulation of zeatin and its riboside was greatest in roots when their content was ten times higher than in control. Accumulation of cytokinins in inoculated lettuce plants was associated with an increase in plant shoot and root weight of approximately 30 % (Arkhipova et al. 2005); significant correlation of cytokinin with shoot length, fresh weight, and dry weight in plants inoculated with *Pseudomonas*, *Bacillus*, and *Azospirillum* was reported producing zeatin, zeatin riboside, and dihydrozeatin riboside (Hussain and Hasnain 2011).

1.5.4 Gibberellins

Gibberellins (GAs) are a kind of hormones that consist a group of terpenoids with 20 carbon atoms, although active GAs only have 19 carbon atoms. GAs are mainly involved in cell division and elongation within the subapical meristem, thereby playing a key role in internode elongation and act in seed germination, pollen tube growth, and flowering in rosette plants (Stowe and Yamaki 1957; Taiz and Zeiger 2009).

The formation of Gibberellins occurs by many reactions over different enzymes in process, which according to Morrone et al. (2009) include diterpene cyclases, cytochromes P450, and in plants, 2-oxoglutarate-dependent dioxygenases (2ODDs), using the intermediate precursor geranylgeranyl diphosphate (GGPP) (Hedden et al. 2001). In higher plants, cyclization of GGPP into ent-copalyl diphosphate (ent-CPP) and then to ent-kaur-16-ene is catalyzed by two distinct enzymes, ent-copalyl diphosphate synthase (Sun and Kamiya 1994) and ent-kaurene synthase (Yamaguchi et al. 1996), respectively.

Evidence for bacterial biosynthesis is not so clear, but some studies indicate that in general, as in higher plants, early steps of the gibberellin biosynthetic pathway in the bacterium may be regulated by membrane-related cytochrome P450 monooxygenases (Tully et al. 1998; Cassán et al. 2003) and the late hydroxylative steps by soluble 2-oxoglutarate-dependent dioxygenases (Cassán et al. 2001). But *Bradyrhizobium japonicum* encodes separate ent-copalyl diphosphate and ent-kaurene synthases. Morrone et al. (2009) cited: "These are found in an operon whose enzymatic composition indicates that gibberellin biosynthesis in bacteria represents a third independently assembled pathway relative to plants and fungi."

The bacterial gibberellins can modify the hormonal balance in plants causing structural changes. Dobert et al. (1992) demonstrated that *Phaseolus lunatus* plants inoculated with *Bradyrhizobium* sp. strain increased the internode elongation. Measurement of gibberellins content using deuterated internal standards, and gas chromatography and mass spectrometry (GC–MS) analysis, showed that increased levels of GA_1, GA_{19}, GA_{20}, and GA_{44} in nodules formed by the bacterial strain that enhanced internode elongation. Dwarf phenotype induced in *Alnus glutinosa* seedlings by paclobutrazol (an inhibitor of gibberellin biosynthesis) was effectively reversed by applications of extracts from medium incubated with bacteria *Bacillus pumilus* and *Bacillus licheniformis* and also by exogenous GA3. GC-MS analysis of extracts of these bacteria showed the presence of GA_1, GA_3, GA_4, and GA_{20} (Gutiérrez-Manero et al. 2001). *Bacillus cereus*, *B. macroides*, and *B. pumilus* promote the growth of red pepper plug seedlings. Gibberellins (GAs) were detected in the culture broth of their bacteria. Among the GAs, the contents of GA_1, GA_3, GA_4, and GA_7, physiologically active GAs, were comparatively higher than those of others,

suggesting that the growth-promoting effect was originated from the GAs (Joo et al. 2004).

1.6 Biocontrol Characteristics

Microbial agents have emerged as a viable and effective alternative within the biocontrol, and they are considered safe to human health and the environment (Zucchi and Melo 2009). They are used instead of chemicals, whereas chemicals may cause environmental implications that could affect soils and food; in addition the chemicals promote the emergence of resistant pathogens and decrease the population of beneficial organisms (Silva et al. 2004).

The biocontrol ability of microorganisms can reside on different mechanisms, such as the production of harmful substances (M'Piga et al. 1997), competition for space and nutrients, or even acting indirectly in host resistance induction (van Loon et al. 1994).

1.6.1 Antibiosis

The antibiosis is the most common phenomenon whereby a microorganism inhibits the growth of others, by producing toxic compounds (antibiotics) (Cook and Baker 1989), where these compounds can be volatile and nonvolatile constitution. This inhibitory feature is often used to evaluate the potential action of the bacteria, mainly on pathogenic fungi, using in vitro methods to confront microorganisms. The most common methods are dual culture technique on agar medium (Rana et al. 2011; Barra et al. 2008), conidia germination inhibition, antibiosis by two medium agar layers (Barra et al. 2008), and antibiosis with use of the medium broth filtrate in which the microorganism has been grown (Lee et al. 1995).

The contribution of bacterial antibiotics to biological control of disease can be documented in some steps like (i) purification and chemical identification of the antibiotic compound, (ii) detection and quantification in the rhizosphere of the secondary metabolite, and (iii) identification of the regulatory genes that control the expression of the antibiotic compound (Haas and Keel 2003).

The synthesis of antifungal metabolites is extremely sensitive to environmental conditions and varies according to the contents, soil mineral, oxygen tension, osmotic conditions, carbon sources, as well as fungal, bacteria, and plant metabolites can all influence the expression of secondary metabolites (Haas and Keel 2003). Examples of well-characterized antibiotics with biocontrol properties produced by bacteria include phenazines and biosurfactants by *Pseudomonas aeruginosa* against *Pythium splendens* on bean (*Phaseolus vulgaris*) and *Pythium myriotylum* on cocoyam (*Xanthosoma sagittifolium*) (Perneel et al. 2008); cyclic lipopeptides from *Pseudomonas* spp. (Raaijmakers et al. 2006); 2-hydroxymethyl-chroman-4-one by *Burkholderia* sp. exhibited good activities against *Pythium ultimum*, *Phytophthora capsici*, and *Sclerotinia sclerotiorum* (Kang et al. 2004); and lantibiotic mersacidin by *Bacillus amyloliquefaciens* (Herzner et al. 2011). In terms of volatile compounds, we can find benzothiazole, cyclohexanol, n-decanal, dimethyl trisulfide, 2-ethyl 1-hexanol, and nonane by many species of *Pseudomonas* inhibited sclerotia and ascospore germination and mycelial growth of *Sclerotinia sclerotiorum*, in vitro and in soil tests (Fernando et al. 2005); and benzothiazole, benzaldehyde, undecanal, dodecanal, hexadecanal, 2-tridecanone, and phenol by *Paenibacillus polymyxa* were found to inhibit the growth of *Fusarium oxysporum* (Raza et al. 2015).

1.6.2 Hydrogen Cyanide

A secondary metabolite produced commonly by rhizosphere pseudomonads is hydrogen cyanide (HCN), a gas known to negatively affect root metabolism and root growth (Schippers et al. 1990), has inhibitory properties to the pathogens, and on the other hand may also directly promote plant growth by increasing root hairs (Luz 1996). Evidences that glycine is an HCN precursor for *Pseudomonas aeruginosa* were presented by

Castric (1977), but this process differs significantly from cyanogenesis in other bacteria because (i) other amino acids besides glycine stimulate HCN production, and (ii) both carbons of glycine are used as sources of cyanide carbon.

The production of HCN is a phenomenon that occurs in some bacterial genera, such as *Bacillus* (Deepa et al. 2010), *Chromobacterium* (Barreto et al. 2008), *Pseudomonas* (Zdor and Anderson 1991), and *Rhizobium* (Blumer and Haas 2000).

The cyanide is produced in many cases with reduction of oxygen concentration closing the exponential phase in cells, when it achieve density that promotes quorum-sensing activation, of which cyanogenesis not always controlled by quorum sensing. In*Chromobacterium violaceum* CV0 the disruption of quorum sensing to abolish cyanogenesis was reported (Throup et al. 1995), but in *Pseudomonas aeruginosa* PAO1, quorum-sensing systems (RhlI/R and LasI/R) are necessary for HCN production (Pessi and Haas 2000).

Certain bacteria produce HCN that inhibits the cytochrome oxidase of many organisms. The producer strains possess an alternate cyanide-resistant cytochrome oxidase and are relatively insensitive to HCN. Baker and Schippers (1987) and Schippers et al. (1987) demonstrated that the cytochrome respiratory pathway of potato roots was particularly sensitive to cyanide.

1.6.3 Competition for Nutrients and Niches

To successfully colonize the plant, a microbe must effectively compete for the available nutrients and niches. Competition for nutrients and niches (CNN) between pathogens and beneficial organisms is important for limiting disease incidence and severity (Kamilova et al. 2005). Although it is difficult to demonstrate directly, some indirect evidence indicates that competition between pathogens and nonpathogens is effective (Glick 2012). For example, abundant nonpathogenic soil microbes rapidly can colonize plants and use most of the available nutrients, making it difficult for pathogens to grow. Treatment of plants with the leaf bacterium *Sphingomonas* sp. prevented the bacterial pathogen *Pseudomonas syringae* pv. tomato from causing pathogenic symptoms (Innerebner et al. 2011). Two strains of *Pseudomonas* AVO110 and AVO73 were selected for their efficient colonization of avocado root tip. However, only AVO110 demonstrated significant protection against avocado (*Persea americana*) white root rot caused by *Rosellinia necatrix*. The difference was in the fact that both strains colonize different sites on the root: Biocontrol strain AVO110 was observed to colonize the root at preferential penetration sites for *R. necatrix* infection (intercellular crevices between neighboring plant root epidermal cells and root wounds), while AVO73 predominantly was found forming dispersed microcolonies over the root surface and in the proximity of lateral roots, areas not colonized by this pathogen (Pliego et al. 2007, 2008). These results strongly suggest that biocontrol bacteria acting through CNN must efficiently colonize the same mininiche as the pathogen.

Bacteria produce extracellular siderophores (microbial iron transport agents) (Neilands 1995) which efficiently complex iron from environment, making it less available to certain native microflora (Kloepper et al. 1980), mainly fungal siderophores that have lower affinity (Loper and Henkels 1999). The competition for ferric iron ions is a well-documented example of competition of biocontrol bacteria with pathogenic fungi for nutrients. The relevance of siderophore production as a mechanism of biological control of *Erwinia carotovora* by *P. fluorescens* strains was described by Kloepper et al. (1980); after that, Jurkevitch et al. (1992) studied the differential availabilities of the hydroxamate siderophores ferrioxamine B (FOB) and ferrichrome (FC) and the pseudobactin siderophores as sources of Fe for soil and rhizosphere bacteria and found that the ability of bacteria to utilize a large variety of siderophores confers an ecological advantage.

1.6.4 Lytic Enzymes

The plant pathogens require entry sites to gain access to the interior of the host. Therefore, the biological control organisms need to gather

characteristics in order to be able to compete effectively for these sites of infection. This can occur by the use of the nutrients available and effective inhibition of germination of spore or vegetative growth of phytopathogens (Punja and Utkhede 2003). Some bacteria have strategies to join skills to get ecological advantages; they are able to produce extracellular enzymes such as chitinases, 1,3-β-glucanases, lipases, cellulases, and proteases. Sometimes, these enzymes act synergistically with antibiotics playing an important role in the antagonistic effect on phytopathogenic fungi. *Pseudomonas syringae* pv. *syringae* showed biocontrol action against *Trichoderma atroviride* through the purified toxins and chitinolytic and glucanolytic enzymes purified from the same bacterial strain (Fogliano et al. 2002). Biocontrol ability of *Lysobacter antibioticus* against *Phytophthora* blight was mediated by 4-hydroxyphenylacetic acid, chitinase, 1,3-β-glucanases, lipase, and protease (Ko et al. 2009).

Several studies have investigated in bacteria the possibility of the occurrence of mechanisms of active penetration. This hypothesis has been supported by the detection of pectinolytic and cellulolytic enzymes produced by some species, such as *P. fluorescens*, *Enterobacter asburiae*, and *Bacillus* sp. (Quadt-Hallmann et al. 1997; Ratón et al. 2011).

The cell wall is a barrier that provides protection to the actions carried out by microorganisms, and it is considered the starting point of the interactions in antagonism process involving fungi. Fungi have cell wall that consists primarily of chitin, 1,6-β-glucans, and other polysaccharides (Bartinicki-Garcia 1968). Physically, chitin appears to be protected by β-glucans, which hinder the access of chitinase (Cherif and Benhamou 1990), so for that, these extracellular enzymes were considered as the main hydrolases involved in parasitism processes (Martin et al. 2007; Zeilinger and Omann 2007).

When chitinases degrade the cell wall of fungi, they release oligomers which induce the expression of other genes of hydrolytic enzymes and thereby accentuating the attack on the host (Viterbo et al. 2002). The β-glucans are a group of abundant polysaccharide in nature, and its main function is to be a structural polymer and may be degraded to be used as a nutritional source. They can also protect cells from dehydration, because it forms a mucilage which encapsulates the hyphae (Pitson et al. 1993; Martin et al. 2007).

Proteases are enzymes which cleave peptide bonds and can be classified according to hidrogenionics, the optimal conditions for its action (acidic, neutral, and alkaline), substrate specificity (collagenase, elastase, etc.), or similarity (pepsin, trypsin, casein, etc.) (Kubicek 1992), which act as biocontrol function degrading the cell wall of the host (Martin et al. 2007).

1.6.5 Induced Systemic Resistance

In plant pathology it is assumed that immunity is the rule and exception susceptibility. Otherwise, any pathogen would be able to infect any plant and short term in evolutionary terms; the vegetables would disappear from the earth (Romeiro 1985). This does not happen because the plant defense mechanisms against pathogens exist in multiplicity and are efficient (Romeiro 1995). The "induction of resistance" may be used to denote local protection in tissues receiving treatment with inducing agent or systemic manifesting the distance from where the inductor was applied (Moraes 1992). The protection induced is dependent on the time interval between the treatment with inducer and application of the pathogen (Pascholati and Leite 1995). This dependence indicates that specific changes in plant metabolism involving synthesis and/or accumulation of substances occurred, which is an important fact in induced resistance phenomenon (Taiz and Zeiger 2009).

Two acronyms – ISR (induced systemic resistance) and SAR (systemic acquired resistance) – are recognized almost as synonyms to designate the phenomenon through which plants, after exposure to an inducing agent, have enabled their defense mechanisms. Activated not only in the induction site but also in other distant sites (Sticher et al. 1997), the inducing agent may be a chemical activator such as benzothiadiazole derivatives and other compound (Benhamou and Belanger 1998) extracts of microbial cells

(Romeiro and Kimura 1997) or live microorganisms (Liu et al. 1995).

The authors agree that SAR and ISR are distinct phenomena, by the way in which they are induced and triggered. They are also governed by different biochemical mechanisms, but similarity in phenotypic end result is expressed as systemically induced resistance (Romeiro 1999).

In SAR induction occurs as hypersensitive response (HR), which is characterized by the programmed death of cells around the infection, acts against biotrophic pathogens and wherefore restricts access to water and nutrients. The HR is activated by salicylic acid signal (AS) (Glazebrook 2005). If a hypersensitivity response is successful, a small region of dead tissue remains at the site of pathogen attack, but the rest of the plant is not affected (Taiz and Zeiger 2009). The SAR involves the accumulation of PRP (pathogenesis-related proteins); a number of these proteins have antimicrobial activity, and it is believed to contribute to the plant reaching the state of SAR (Ward et al. 1991).

In the case of ISR, no accumulation of PRPs happens; the plant that has to bear induction of resistance does not display changes; the inducing agent is usually a nonpathogen and its induction is not dependent of salicylate; there appears to be another signaling pathway and further linked to jasmonates and ethylene (Pieterse et al. 2005).

A clear example of these different routes was verified by Ton et al. (2002) using *Arabidopsis* and different pathogens, which seems to show that SAR seems to be based on an increase in the dependent defenses, whereas ISR seems to be based on an increase in defenses dependent on jasmonic acid (AJ) and ethylene (ET). In addition, there may be simultaneous activation of ISR and SAR resulting in a higher level of protection induced, determined by van Wees et al. (2000) in *Arabidopsis thaliana*. This indicates that the ISR route dependent on AJ and ET and SAR dependent on AS act independently and in additive protection against that particular pathogen. So the combination of these two types of induced resistance could protect the plant against a complementary spectrum of pathogens and may even result in an induced protection against pathogens' additive level; the respective host resistance may occur through the dependent route AJ/ET and AS. These data offer great potential for integrating both forms of resistance-induced protection in future agronomic practices (Pieterse et al. 2005).

Bacteria can perform the IRS process, and several studies have shown that there is a range of bacteria with the ability for different types of phytopathogens. In *Arabidopsis* seedlings exposed to bacterial volatile blends from *Bacillus subtilis* and *B. amyloliquefaciens*, disease severity of *Erwinia carotovora* subsp. *carotovora* was reduced compared with seedlings not exposed to bacterial volatiles before pathogen inoculation. This bacterial volatile was sufficient to activate ISR in *Arabidopsis* seedlings (Ryu et al. 2004). Exogenous application of the *B. subtilis*-derived elicitor, acetoin (3-hydroxy-2-butanone), was found to trigger ISR and protects plants of *Arabidopsis* against *Pseudomonas syringae* pv. *tomato* (Rudrappa et al. 2010).

1.7 Conclusions

In this chapter we presented techniques to assist in the characterization of interesting bacterial strains for use on sustainable agriculture, since it is primal selecting the right bacteria, with characteristics of interest according to the target, looking to the use of bioproducts, biofertilizers, or biopesticides. We discussed the topics of strain identification, biological nitrogen fixation, phosphate solubilization, phytase production, siderophores, phytostimulation, and biocontrol characteristics. But before, the concepts, definitions, and regulation of the use of these environment-friendly sources were discussed.

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Microbial Inoculants as Agents of Growth Promotion and Abiotic Stress Tolerance in Plants

2

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Abstract

The use of external chemical inputs such as chemical fertilizers and pesticides undoubtedly resulted in huge increase in agricultural products in the past many decades. Such indiscriminate use of agrochemicals has however resulted in various ecological imbalances and environmental disasters in various parts of the world. The use of plant growth-promoting rhizobacteria (PGPR) as biofertilizers and/or as biocontrol agents to enhance plant growth, increase yield, and suppress diseases in a wide range of agricultural crops is gaining momentum. If PGPR inoculants are to replace agrochemicals in the near future, the search for effective strains must focus on isolation and screening of single or consortium of the bacterial strains that have multiple traits. Moreover, a better result in microbial inoculant development could be achieved by investigating the different modes of actions in disease suppression and plant growth promotion, detection of important genes and traits associated with these, bacterial-host plant interaction, as well as relationships between the bacteria and various environmental factors.

Keywords

Rhizosphere • PGPR • Siderophore • Biocontrol • Growth promotion • Abiotic stress • Antibiosis • BNF • ACC deaminase

2.1 Introduction

The world population, currently estimated around seven billion people, is predicted to increase to around 10 billion in the next 50 years which requires that agricultural productivity be increased within the next few decades to sufficiently feed all these individuals (Glick 2014). A

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typical feature of modern intensive agriculture worldwide is to increase agricultural productivity by the application of external chemical inputs including fertilizers, pesticides, fungicides, and herbicides. It was reported, for instance, that widespread use of chemical fertilizers during the past 50 years has become a major input to supply N and P and had substantially increased food production worldwide (Abd-Alla et al. 2014). This practice is however not sustainable and has several negative impacts both on human health and environmental safety (Franks et al. 2006; Glick 2014). From an environmental perspective, for example, only 30–50 % of applied N fertilizers and 10–45 % of P fertilizers are taken up by crops, and the majority of the remaining nitrogen and phosphorous are lost to the environment through various processes (Adesemoye and Kloepper 2009). Another drawback of the excessive application of chemical pesticides is that it contributes to the development of pest resistance which leads to higher chemical input use (Chavez et al. 2013). Potential alternatives to the use of chemical fertilizers and pesticides are microbial inoculants, environmentally friendly microbial formulations that act as phytostimulants, biofertilizers, and/or microbial biocontrol agents (Olubukola et al. 2012). Thus, nowadays, tremendous effort is being put on research to develop such microbial inoculants which have beneficial plant growth properties in environmentally friendly sustainable agriculture (Barriuso et al. 2008). Such beneficial properties of microbial inoculants could be manifested either by direct promotion of plant growth, by indirectly protecting plants from phytopathogens, or by fortifying certain abiotic stress tolerance in plants that grow in soils with non-optimal environmental factors including extremes of high and low temperature, salinity, drought, acidity, and presence of heavy metals (Kang et al. 2014; Penrose and Glick 2003; Kloepper and Schroth 1978). Microbial inoculants can also play an important role in the formation of soil aggregation which helps stabilize the soil (van Veen et al. 1997).

2.2 Direct Plant Growth Promotion by Microbial Inoculants

For the past several decades, research dedicated to improve crop yield and plant growth with microbial inoculants mainly focused on the symbiotic rhizobia which have been successfully used worldwide for the establishment of the nitrogen-fixing symbiosis with legumes (Reddy 2013; van Veen et al. 1997). These groups of bacteria which generally belong to the alpha proteobacteria are capable of inducing nitrogen-fixing nodules on the roots of several hundreds of leguminous plants (Lorenzo et al. 2000). They are thus involved in direct promotion of plant growth by fulfilling the nitrogen requirement of legumes using a process known as biological nitrogen fixation (BNF) which occurs in the root nodules. On the other hand, there are other groups of soil bacteria living freely in close proximity to the active region of the roots, commonly known as the rhizosphere. In the past, several large areas of arable land in different parts of the world have been inoculated with nonsymbiotic free-living bacteria such as *Azotobacter*, *Azospirillum*, *Bacillus*, *Klebsiella*, and *Pseudomonas* (van Veen et al. 1997). The major mechanisms by which these free-living bacteria promote plant growth include nitrogen fixation, improving plant nutrient uptake, enhancing the growth of the entire root system, and reduction of the membrane potential of the roots (Glick and Bashan 1997).

2.2.1 Free-Living Plant Growth-Promoting Rhizobacteria (PGPR)

Kloepper and Schroth (1978) first defined the term plant growth-promoting rhizobacteria (PGPR) to describe soil bacteria that colonize the roots of plants and enhance plant growth following inoculation onto seeds. These plant growth-promoting rhizobacteria are mainly present in the region around the roots, the rhizosphere, which is

relatively rich in nutrients as a result of loss of 40 % of the plant photosynthate from the roots (Lynch and Whipps 1991). Apart from the major role of enhancing plant growth, an ideal PGPR must be highly competent in the rhizosphere, must colonize the roots sufficiently, should be compatible with other rhizobacteria, must have broad spectrum of action, should be easily multiplied, and must be safe to the environment (Reddy 2013). A number of rhizosphere bacteria which fulfill the above criteria including members of the genera *Azospirillum*, *Pseudomonas*, *Bacillus, Azotobacter*, *Burkholderia*, and *Enterobacter* have been widely reported in the past (Glick and Bashan 1997). However, not all rhizosphere bacterial strains in a given genus or species have beneficial PGPR effect on plants (Penrose and Glick 2003; Glick 2014). It is therefore very essential to conduct reliable screening and selection of PGPR in order to develop efficient microbial inoculants that promote plant growth and yield increase.

2.2.1.1 Siderophore Production

After coining the term PGPR, Kloepper et al. (1980a, b) demonstrated that the best known rhizobacteria with PGPR activities belong to the group of fluorescent P*seudomonas* species. Direct plant growth promotion by the fluorescent *Pseudomonas* mainly comes from their involvement in improving plant iron nutrition using siderophore secretions. Siderophores are low molecular mass proteins (~400–1500 Da) which have an exceptionally high affinity for iron (Fe^{+3}). Under aerobic condition, most of the iron is only sparingly soluble and therefore not readily available to either bacteria or plants. To overcome this limited supply of iron, PGPR such as *Pseudomonas*, mainly belonging to the fluorescent species, synthesize siderophores (Glick 2012; Neilands 1981; Kloepper et al. 1980a, b). Bacterial siderophores have been demonstrated to have direct benefits to plant growth promotion by acting as a direct source of iron and making it available to plants (Yehunda et al. 1996; Vansuyt et al. 2007). Siderophore-producing microbial inoculants have been shown to have a direct plant growth-promoting effect in various crops in the past. To cite a few examples, inoculation of mung bean with siderophore-producing *Pseudomonas* strain GRP3 under iron-limited condition showed enhanced growth and chlorophyll level (Sharma et al. 2003). In another experiment, inoculation of *Arabidopsis thaliana* with *Pseudomonas fluorescens* resulted in the uptake of the Fe-pyoverdin complex synthesized by the bacteria leading to an increase in the iron level inside the plant tissue and improved plant growth (Vansuyt et al. 2007).

Many plants use microbial siderophores as iron source for growth. Evidence of iron uptake by plants from hydroxamate siderophores produced by *Pseudomonas* spp. has been widely documented (Crowley 2006). Iron (Fe^{+3}) and molybdenum (Mo) are very much required by the free-living and symbiotic nitrogen-fixing diazotrophic bacteria such as *Rhizobium* and *Azospirillum* not only for the electron shuttle reactions but also as a component of the nitrogenase complex. Siderophore production by such types of microorganisms is an added advantage as it helps the bacteria by incorporating the iron and molybdenum into the nitrogenase enzyme complex. Moreover, the bacterial siderophores have higher affinity to these metals than fungal siderophores and compete with the fungal siderophores (Fig. 2.1) (Benjamine and Bruce 2008). Rhizobium requires iron to grow in the rhizosphere and for optimum nodulation and development of the *Bacteroides*. This suggests that siderophores are required for effective nitrogen fixation by the symbiotic rhizobia (Tang et al. 1992). In one investigation to select best strains of *Bradyrhizobium japonicum* for high plant yield, inoculation with siderophore-producing strains resulted in higher yield as compared to inoculants that do not produce siderophores (Khandelwal et al. 2002).

2.2.1.2 Indole-3-Acetic Acid (IAA) Secretion

Another very important microbial metabolite involved in direct plant growth promotion by free-living PGPR is indole-3-acetic acid (IAA). Several free-living PGPR such as *Azospirillum* and fluorescent *Pseudomonas* secrete IAA involved in promoting root growth and development (Figueiredo et al. 2010). Apart from their

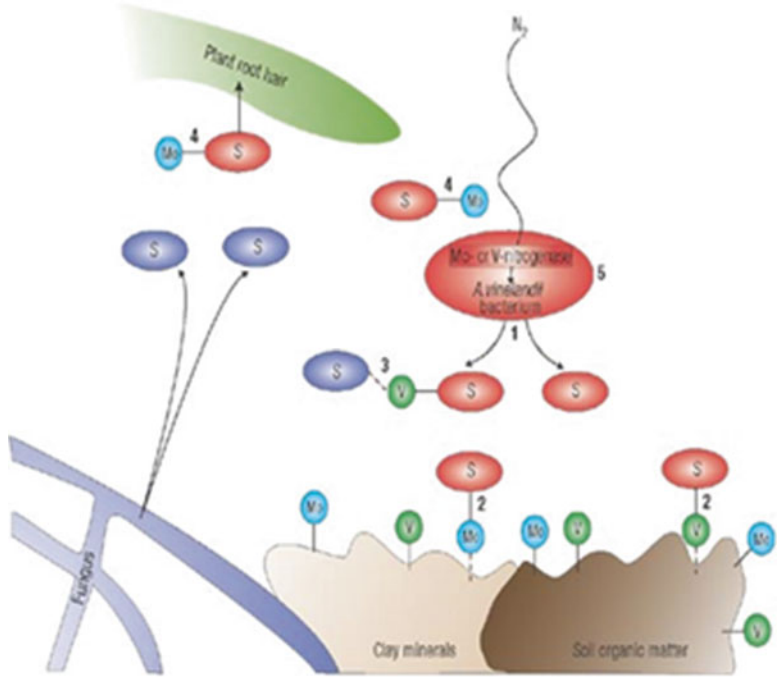

Fig. 2.1 Bacterial siderophores (*1*) scavenge the metals from unavailable complexes with clay, soil organic matter, or other elements (*2*). The siderophores compete with siderophores produced by fungi for these metals (*3*). The bacterium or plant roots readily take up the siderophore-metal complexes (*4*). Within the bacterium, the metal is incorporated into the enzyme nitrogenase (*5*), to allow the fixation of atmospheric nitrogen (N_2) that would otherwise be unusable to the bacterium (Adapted from: Benjamine and Bruce 2008)

capacity to fix atmospheric nitrogen under microaerophilic conditions, PGPR of the genus *Azospirillum* have long been considered the most important rhizobacteria for improvement of plant growth and crop yield because of their ability to colonize internal tissues of gramineous plants and promote growth by production of the phytohormone indole-3-acetic acid (Bashan et al. 2004; Perrig et al. 2007). Production of this phytohormone by *Azospirillum* species alters the metabolism and morphology of plant roots which result in a better absorption of mineral and water, producing larger and healthier roots (Bashan and de Bahsan 2010). The major outcomes of most inoculations with *Azospirillum* species are therefore changes in plant root architecture, while inoculation also promotes root elongation and development and branching of root hairs (Levanony and Bashan 1989; Okon and Kapulnik 1986). Many important plant microbe interactions are regulated by auxins, IAA being the major type of auxin produced by plants and several free-living PGPR including *Azospirillum*, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Burkholderia*, and the symbiotic rhizobia (Martinez-Viveros et al. 2010). In general, selection of PGPR isolates through screening for the production of IAA is one of the strategies in the development of microbial inoculants that stimulate seed germination, accelerate root growth, modify the architecture of the root system, increase root biomass, and ultimately enhance plant growth.

2.2.1.3 Phosphate Solubilization

Although most agricultural soils have large amounts of inorganic and organic phosphates, most of these are immobilized and unavailable to plants. Like Fe, phosphorous (P) is not readily available to plants due to its high reactivity with some metal complexes leading to precipitation or adsorption of 75–90 % of P into soil (Adesemoye and Kloepper 2009). In such soils, correcting P deficiency by applying P fertilizer is quite often unaffordable by most resource-poor farmers in the tropics and subtropics, particularly, in soils characterized by high P-fixing properties (Horst et al. 2001). Several PGPR strains such as *Pseudomonas*, *Bacillus*, *Burkholderia*, *Rhizobium*, and *Flavobacterium* have been reported to have the ability to solubilize such insoluble inorganic phosphate compounds. The use of these phosphate-solubilizing bacteria as

inoculants could increase the P uptake by plants and thus offers the benefit of direct plant growth promotion (Rodriguez and Fraga 1999; Bashan and de Bahsan 2010; Saharan and Nehra 2011).

2.2.2 Symbiotic PGPR (Rhizobium-Legume Symbiosis)

The air in the atmosphere is largely 78 % nitrogen gas (N_2), and yet it is ironic that nitrogen (N) has become one of the most limiting nutrients for crop production worldwide (Valentine et al. 2011). This is because atmospheric nitrogen (N_2) is very stable due to the strong triple bond between the two N atoms that require large amount of energy to break. Only few prokaryotic organisms called diazotrophs have the enzymatic machinery to break the strong bond that held the two N atoms. The most effective diazotrophic bacteria, the rhizobia, form a symbiotic interaction with legumes and reduce atmospheric N to a usable form of NH_3 by a process called biological nitrogen fixation (BNF). Symbiotic nitrogen fixation is one of the most important biological processes on the planet which provides the majority of the N requirement in agriculture (Howieson and McInnes 2001). The symbiosis between the root nodule rhizobia and legumes contributes at least 70 million metric tons of fixed nitrogen per year into terrestrial ecosystem which accounts for up to 40 % of the total N fixed on earth (Brockwell et al. 1995; McInnes and Haq 2007).

2.2.2.1 The Symbiotic Process

Nodulation and the associated legume-rhizobium symbiosis are complex processes involving the expression of both bacterial and plant genes which start by the production of a cocktail of phenolic molecules called flavonoids which can passively diffuse across the bacterial membrane (Smith and Wollum II 1989; Wang et al. 2012). As soon as the bacteria perceive the flavonoid signals, it results in the activation of the rhizobial nodulation (*nod*) genes that encode the enzymes required for the synthesis of bacterial Nod factors, a family of lipochitooligosaccharides essential for symbiotic development in most legumes. The Nod factors initiate most of the developmental changes in the legume roots during the early nodulation process such as root hair deformation, membrane depolarization, initiation of cell division in the root cortex, and formation of a meristem and nodule primordium (Abd-Alla et al. 2014).

2.2.2.2 Direct Plant Growth Promotion by Rhizobium Inoculation

Nitrogen fertilizer plays one of the decisive roles in the attainment of high yields from crop plants. Due to this, farmers often apply high amounts of nitrogen fertilizer which is not only very costly but also makes the environment hazardous when used indiscriminately (Abd-Alla et al. 2014). The best alternative to this could be provided by the process of biological nitrogen fixation (BNF) that occurs during the legume-rhizobium symbiotic interaction which plays a critical role in sustainable agriculture by reducing the need for exogenous nitrogen fertilizer (Wang et al. 2012). Therefore, inoculation of legumes with actively nodulating and nitrogen-fixing rhizobia significantly contributes to the N input of many agricultural systems. It provides a source of nitrogen not readily leached and is the most important route for sustainable nitrogen input into agroecosystems (Lindström et al. 2010). It has been experimentally proved that efficient and proper usage of legume inoculation using effective rhizobium inoculants significantly improves crop productivity and soil fertility in a wide range of legume-growing fields (Brockwell and Bottomlley 1995).

2.2.2.3 Rhizobium-PGPR Co-Inoculation

Recent exploitation of PGPR co-inoculation with *Rhizobium* constitutes an interesting alternative to improve nitrogen fixation. Nodulation and yield of several legume species including soybean, chickpea, pea, vetch, and clover have been increased as a result of co-inoculation of their respective rhizobium with the diazotrophic *Azospirillum* species. In a related report, co-inoculation of *Bradyrhizobium* and PGPR significantly improved soybean growth and yield as compared to the sole application of *Bradyrhizobium* (Masciarelli et al. 2014). Co-inoculation of rhizobia with the PGPR

Pseudomonas species has also been reported to enhance nodulation and nitrogen fixation by rhizobia (Perez-Montano et al. 2014). Although the mechanism in which the nonrhizobial PGPR is involved is poorly understood, it is believed that the role of the nonrhizobial PGPR such as *Azospirillum* is to increase the competitiveness of the rhizobial strains and to create additional infection sites which can be later occupied by the rhizobia (Antoun and Prevost 2005; Perez-Montano et al. 2014). In addition to their beneficial N_2-fixing activity, rhizobia can improve plant P nutrition by mobilizing organic and inorganic phosphates. Co-inoculation of rhizobia with phosphate-solubilizing bacteria revealed a synergistic effect on symbiotic parameters such as increasing nodule number and plant biomass which resulted in grain yield of legumes (Saharan and Nehra 2011). In another experiment, inoculation of groundnut with a consortium of PRPR comprising *Rhizobium* strain Tt 9 with the PGPR *Bacillus megaterium* var *phosphaticum* resulted in fulfilling about 50 % of the phosphatic fertilizer requirement of the groundnut thereby improving nodulation, plant growth, and yield (Kumar et al. 2011). In general, there is a promising trend of the practice of co-inoculation of rhizobia and PGPR in the development of sustainable agriculture in the future.

2.3 Microbial Inoculants as Biological Control Agents

Over the past few decades, pathogenic microorganisms that affect plant health have become a major threat to food production and to the stability of the ecosystem worldwide. This has resulted in more and more dependency on agrochemicals by food producers and farmers to protect their crops from potential pathogens (Compant et al. 2005). The increasing use of chemical pesticides in agricultural systems has several drawbacks. First, a large number of resource-poor communities in the developing world cannot afford the high cost of chemical pesticides. Second, chemical pesticides result in the development of pathogen resistance and negatively affect the ecosystem due to its nontarget environmental impact (Gerhardson 2002). As substitutes for chemical pesticides, the use of bacterial biocontrol agents against a wide variety of phytopathogens especially the root-associated soilborne pathogens has been extensively emphasized. This has led to the isolation and commercialization of numerous microbial inoculants for growth enhancement and as potential antagonists and disease management in various crops (Kakar et al. 2014).

2.3.1 Rhizosphere Competence

Among the major factors in the unsuccessful commercialization of microbial inoculants are the inconsistencies in field trial tests which raised concerns about the perspectives of the practical potentials offered by the microbial metabolites released into the soils (van Veen et al. 1997). For biocontrol agents to be effective once introduced into the soil, they should have a strong rhizosphere competence so that they colonize the root effectively and survive along with growing plant roots over a long period of time in the presence of indigenous microflora (Weller 1988; Lugtenberg and Deckers 1999). When introducing microbial inoculant strain into the soil, it is necessary that the strain should be inoculated at a density many times higher than the indigenous population. Additional approaches include using repeated inoculation as well as utilization of antibiotic-resistant bacteria simultaneously with antibiotic (Nautiyal 1997). Being an important first step in the interaction of an introduced microbial strain with plant roots, it is better to determine if the bacteria really have efficient root colonization capacity. Using molecular techniques such as the green fluorescent protein (gfp), it is possible to monitor the location of individual rhizobacteria on the root using confocal scanning microscopy (Bloemberg et al. 2000; Bloemberg and Lugtenberg 2001). Figure 2.2 represents one such study using confocal scanning laser microscopy to monitor the colonization of tomato root by a strain of *Bacillus simplex* KSB1F-3 in a glasshouse study (Hassen and Labuschagne 2010).

2.3.2 Antibiosis

Control of phytopathogens by applying chemical pesticides has resulted in the development of resistance to individual chemical controls over time, demanding a constant development of new pesticides. Moreover, there is a growing concern over environmental contamination (Martinez-Viveros et al. 2010). Microbial inoculants which are involved in indirect plant growth promotion are characterized by protecting plants from attack by phytopathogens. One of the mechanisms used by biocontrol PGPR to prevent plants from pathogen attack is by the production of antibiotics, low molecular weight compounds produced by microorganisms. Antibiosis plays an important role in disease suppression by PGPR and is often thought to act in concert with competition and parasitism (Reddy 2013). It is one of the most powerful and widely studied biocontrol mechanisms for combating phytopathogens.

Several different types of antibiotics produced by microbial inoculants with strong PGPR functions have been shown to be effective against a wide range of fungal pathogens. Over the past many years, contemporary *Pseudomonas* biocontrol research revealed the production of four classes of antibiotics by different strains of fluorescent pseudomonads: phenazine-1-carboxylic acid (PCA), 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (Prn), and pyoluteorin (Plt) (Weller 2007; Thomashow and Weller 1988). From a practical point of view, a PGPR strain of *Pseudomonas fluorescens* CHAO that produce DAPG sufficiently suppressed take-all of wheat and black root rot of tobacco. In addition, this strain produces Plt, Prn, IAA, and the siderophores pyochelin and pseudobactin (a pyoverdin siderophore) due to which it is considered as a PGPR strain with the highest biocontrol and growth-promoting potential (Weller 2007; Weller et al. 2012). Parallel to the discovery of such antibiotic-producing strains, several genes and traits have so far been detected. For example, the gene *phz*f detected in *Pseudomonas chlororaphis* can be used as a marker for the capacity of a PGPR to produce the antibiotic phenazine-1-carboxylic acid (PCA), a class of the broad-spectrum antibiotic suppressive to take-all and *Rhizoctonia* root rot and *Fusarium* wilt. Similarly, *phl*D is used as a key marker in the biosynthesis of 2,4-DAPG by *Pseudomonas* species (Wang et al. 2014). Other *Pseudomonas fluorescens* strains with potential biocontrol traits

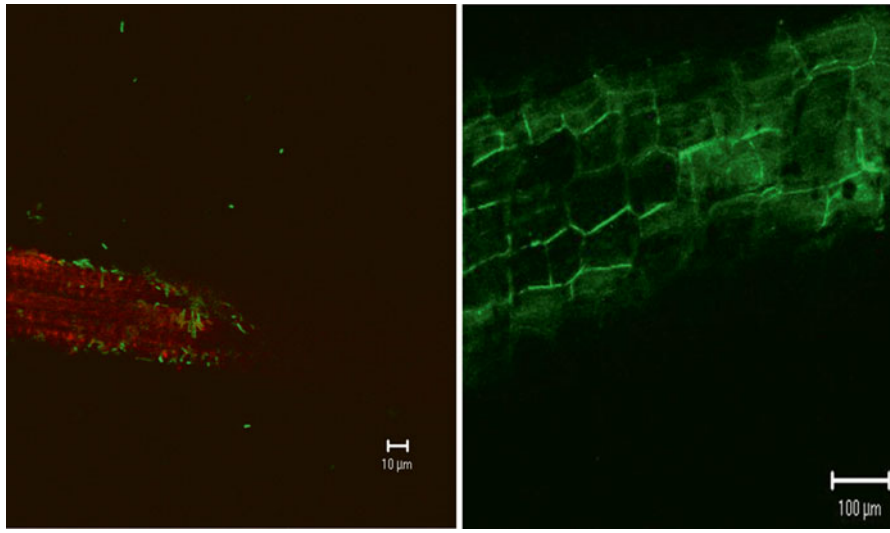

Fig. 2.2 Root colonization of *gfp*-tagged *Bacillus simplex* KBS1F-3 after inoculation of 2-week-old tomato seedlings with the tagged bacterial suspension (*left*). Plants treated with the wild-type strain show no fluorescence (*right*) and the *green color* of the root is due to auto fluorescence (Adapted from Hassen and Labuschagne 2010)

produce the antibiotics pyrrolnitrin and pyoluteorin encoded by the genes *prn*D and *plt*C and are highly active against *Pythium* and *Rhizoctonia* species (Loper et al. 2007; Glick and Bashan 1997).

2.3.3 Siderophore Production

Production of siderophores (pyoverdin and pseudobactin) by PGPR inoculants was identified as a new mechanism of biological control. Biocontrol strains of PGPR produce siderophores that have high affinity for iron so that fungal pathogens are unable to survive in the rhizosphere of the host plant due to lack of iron. Therefore, the major mode of action of siderophores as biocontrol agents is limiting the amount of iron available to the pathogens for growth (Kloepper et al. 1980a, b; Glick 2012). Production of a large amount of siderophores by *Pseudomonas* spp. in pure culture results in sequestering of all available iron leading to suppression of fungal pathogens. Previous field trial researches revealed that there are several direct evidences for the suppression of fungal pathogens in different crops by bacterial siderophores. *Pseudomonas fluorescens* WCS 358 is one of such potential examples of PGPR that inhibit *Fusarium* wilt of radish due to its siderophore mediated iron competition (Leeman et al. 1996a).

Siderophore production by certain *Pseudomonas* spp. also has a secondary effect by triggering systemic acquired resistance (SAR). To cite an example, the siderophore pseudobactin produced by strain WCS374 induced SAR to *Fusarium* wilt in radish (Leeman et al. 1996b). Biocontrol of wilt disease, damping off of cotton caused by *Pythium ultimum*, and *Pythium* root rot of wheat by siderophore-producing fluorescent pseudomonades are also very good examples of the role of siderophores in biocontrol of fungal pathogens. The rationale behind the effectiveness of bacterial siderophores against fungal pathogens which may also produce certain types of siderophores is that bacterial siderophores have higher affinity for iron than fungal siderophores due to which biocontrol PGPR outcompete fungal pathogens for the available iron in the rhizosphere (Glick and Bashan 1997) (Fig. 2.3).

2.3.4 Induced Systemic Resistance (ISR)

Plant growth-promoting rhizobacteria can trigger an induced systemic resistance (ISR) in plants which is phenotypically similar to the systemic acquired resistance (SAR) that occurs when plants activate their defense mechanism in response to infection by phytopathogens (Glick 2012). Unlike SAR, induced systemic resistance does not cause visible symptom on the host plant, but is effective against different types of pathogens (Compant et al. 2005). ISR by rhizobacteria was first demonstrated using *Pseudomonas* spp. and other gram-negative bacteria. However, a few effective cases of induced systemic resistance and promotion of plant growth have also been reported for the gram-positive *Bacillus* spp. Strains of the species *Bacillus subtilis*, *B. pumilus*, and *B. amyloliquefaciens* elicited significant reductions in the incidence of various diseases on greenhouse and field trials on tomato, sugar beet, watermelon, tobacco, and cucumber (Kloepper et al. 2004).

2.4 Abiotic Stress Tolerance in Plants by Microbial Inoculants

Many agricultural crops worldwide are exposed to several abiotic stresses such as extremely high or low temperature, salinity, drought, acidic soils, and metal toxicity. Depending on the type of crop, such abiotic stresses result in yield losses between 50 and 82 % (Kang et al. 2014). In response to such abiotic stress, plants undergo a variety of metabolic and physiological responses and typically stimulate the synthesis of 1-aminocyclopropane 1-carboxylic acid (ACC), which is a precursor to the synthesis of ethylene. Ethylene in turn helps to induce multiple physiological changes in the plants at molecular level (Saleem et al. 2007; Sharma et al. 2013). The

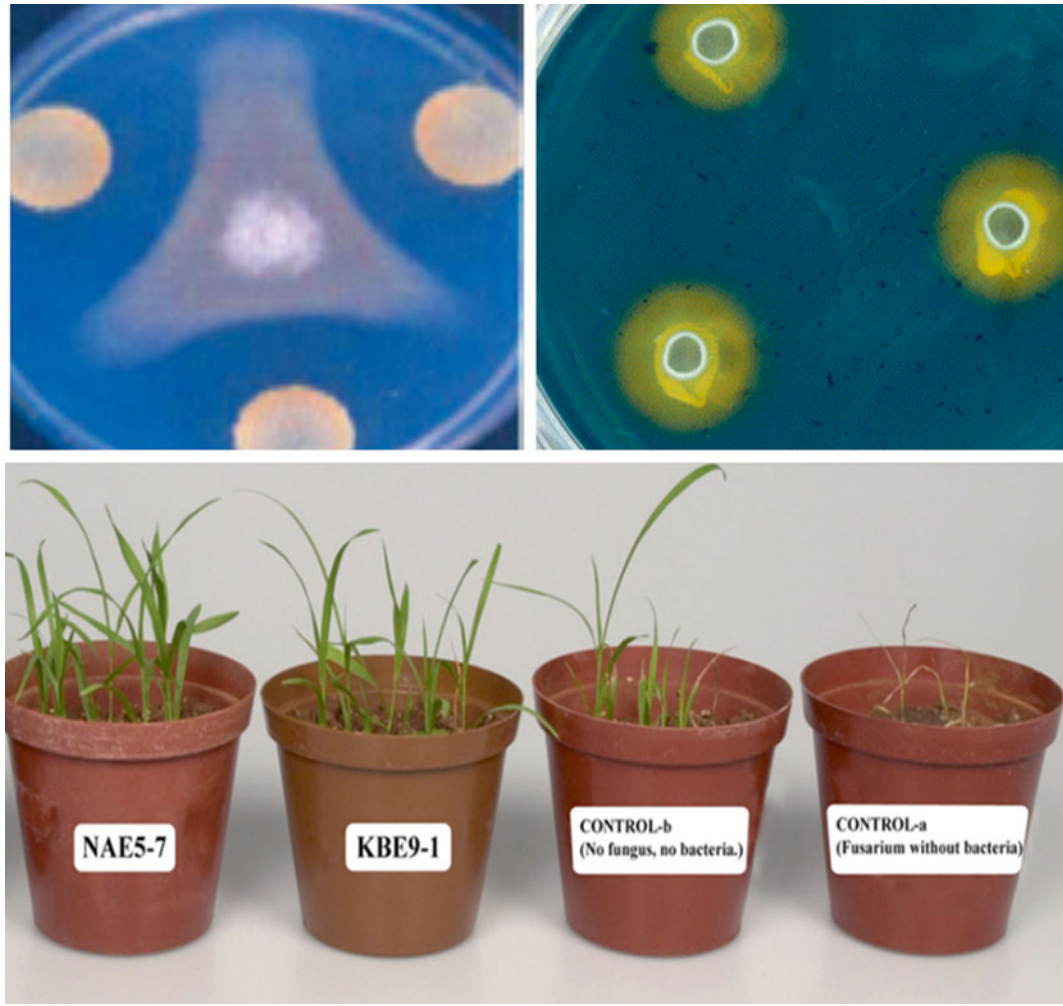

Fig. 2.3 *In vitro* antibiosis activity against *Fusarium oxysporum* (*top left*) and production of siderophore on CAS agar medium (*top right*) by some PGPR strains from sorghum rhizosphere. Glasshouse inhibition of *Fusarium oxysporum* root rot in sorghum after inoculation with rhizobacterial strains NAE5-7 and KBE9-1 (*bottom*). Control plants inoculated only with the pathogen and without the rhizobacteria are all infected and dead (*bottom right*) Source: Idris et al. (2007)

stress ethylene can trigger a senescence response in the plant leading to leaf or fruit abscission, disease development, prevention of enzyme and antibiotic and synthesis, and ultimately inhibition of growth (Glick and Bashan 1997).

2.4.1 ACC-Deaminase Activity

Although ethylene is required by many plants in the course of their growth, to break seed dormancy, high level of ethylene following germination is inhibitory for root elongation. A number of PGPR strains are able to produce the enzyme 1-amino-cyclopropane-1-carboxylate (ACC) deaminase, a pyridoxal 5′ phosphate (PLP)-dependent enzyme that cleaves the plant ethylene precursor ACC into ammonia and α-ketobutyrate thereby lowering the level of ethylene and the associated stress in plants (Penrose and Glick 2003; Blaha et al. 2006). ACC deaminase is produced by plant growth-promoting bacteria to effectively protect plants against a wide range of abiotic stresses such as drought, salinity, heat,

flooding or water logging, and heavy metal stress. Rhizobacteria belonging to the genera *Pseudomonas*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, and *Kluyvera* have so far been documented to have ACC deaminase activity (Saleem et al. 2007; Blaha et al. 2006).

Salinity stress inhibits plant growth as a result of inhibition of seed germination, seedling growth, vigor, and flowering due to the accumulation of stress ethylene. ACC deaminase-positive PGPR reduce the level of stress ethylene and confer salinity tolerance in these plants (Gontia-Mishra et al. 2014). Flooding is also another important abiotic stress that affects many plants as a result of lack of oxygen (anoxia). This results in various symptoms as a result of large quantities of ethylene and leads to yield reductions. Treatments of such plants abiotically stressed by flooding using ACC deaminase-positive PGPR strains could alleviate the stress (Barnawal et al. 2012). Drought stress affects plant-water relations both at cellular and whole plant level limiting crop productivity in most dry regions of the world. Selection and development of inoculants with drought-tolerant ACC deaminase-containing rhizobacteria could be the best strategy to protect plants growing in arid areas.

With the threat of the so-called global warming, heat stress is another threat to world agriculture as extremely high temperature results in hormonal imbalances in plants affecting their growth. ACC deaminase activity by the plant growth-promoting rhizobacteria *Burkholderia phytofirmans* helped potato plants to maintain normal growth under heat stress. In the other extreme of temperature stress, a psycho-tolerant ACC deaminase bacterium strain of *Pseudomonas putida* UW4 promoted canola growth at low temperature under salt stress (Saleem et al. 2007). In general, bacteria that express ACC deaminase activity are capable of lowering a wide range of abiotic stresses in plants. The *acdS* gene coding for the enzyme ACC deaminase can be a very useful candidate for the development of a microbial inoculants that can be used in the management of abiotic stress in plants (Ali et al. 2014). Apart from this role, there are several suggestions that the passion of *acdS* gene and the associated ACC deaminase activity by PGPR strains facilitates bacterial competitiveness and persistence in the rhizosphere (Glick 2014).

2.4.2 Other Stress Tolerance Traits

Certain PGPR such as *Pseudomonas* produce exopolysaccharides (EPS) which not only protect the bacteria from water stress, but they also play a vital role in the formation and stabilization of soil aggregates, regulation of plant nutrients, and water flow across plant roots through biofilm formation (Grover et al. 2011). Generally, salinity stress causes an imbalance in the ion flux in side plants, but inoculation with exopolysaccharides containing PGPR results in significantly decreased Na^+ and increased K^+ concentration and alleviates salt stress by potentially binding cations such as Na^+ and decreasing the level of Na^+ available for uptake (Nadeem et al. 2010; Kang et al. 2014). Gururani et al. (2013) reported that some free-living PGPR strains produce osmolytes which help plants to increase their osmotic potential within the cell thereby relieving the stress.

Abiotic stress in plants resulting from water deficiency and drought could be caused by the formation of reactive oxygen species (ROS) as a result of misdirection of electrons during photosystems. In one experiment, inoculating plants suffering from oxidative stress with *Azotobacter chrococcum* strain, that produce cytokinin and antioxidants, resulted in the accumulation of abscisic acid (ABA) that resulted in the degradation of ROS (Grover et al. 2011). In a related report, a significant increase in the activities of the antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT) was observed after treatment of stressed plant with the diazotrophic bacteria *Azospirillum* and *Azotobacter* (Karthikeyan et al. 2012).

Inoculating plants with PGPR increases proline biosynthesis that acts as reactive oxygen scavenger which can improve plant growth under stress. Proline accumulates in different legumes such as *Glycine max* and *Phaseolus vulgaris* as a characteristic response to prolonged severe water

stress, and it was shown that there is a direct correlation between proline accumulation and drought tolerance (Zahran 1999; Sharma et al. 2013). Proline production in *Zea mays* due to co-inoculation of *Rhizobium* and *Pseudomonas* resulted in salt tolerance as a result of maintenance of relative water content and selective uptake of K^+ ions (Bano and Fatima 2009). Enhanced uptake of nutrients and improving plant health under stress condition can be achieved by inoculating PGPR capable of producing IAA and gibberellins that result in increased root length, root surface area, and number of root tips (Egamverdieva and Kuchrova 2009)

2.4.3 Abiotic Stress Tolerance in Legume-Rhizobium Interaction

Abiotic stress is a common phenomenon in the legume-rhizobium symbiosis which greatly affects the nodulation process and thus that of nitrogen fixation. Legume nodules face abiotic stress including water stress, salinity, soil nitrate, temperature, acidity, and heavy metals (Walsh 1995). Inoculating legumes with mixed cultures of rhizobium and ACC deaminase-positive PGPR promotes nodulation through inhibition of ethylene biosynthesis thereby enhancing nodulation and nitrogen fixation. A few examples include early growth and promotion of nodulation in *Glycine max* by ACC deaminase rhizobacteria and enhanced nodulation in *Pisum sativum* by ACC deaminase *Rhizobium leguminosarum* bv. Viciae 128C53K (Cattelan et al. 1999).

Water stress caused by soil moisture deficiency has a serious negative effect on nodule initiation and thus on N_2 fixation. Since the sensitivity to moisture stress varies for a variety of rhizobial strains such as *R. leguminosarum* bv. trifoli, *Sinorhizobium meliloti*, cowpea *Bradyrhizobium*, and *B. japonicum* strains, it is possible to select the most stress-tolerant rhizobial strains within the range of their legume host (Zahran 1999). The above abiotic stresses added up together with aluminum (Al^+) toxicity and P deficiency hugely affect nodulation and nitrogen fixation. A number of *Rhozobium* strains have evolved some sort of adaptation to saline conditions by the accumulation of low molecular weight organic solutes called osmolytes which counteract the dehydration effect of low water activity (Zahran 1999). To summarize, with the increasing research on the beneficial aspects of plant-microbe interaction including the legume-rhizobium symbiosis, there exist tremendous perspectives of the development and application of rhizobium inoculants that can sustain high levels of N_2 fixation even in the presence of these adverse environmental factors.

2.5 Conclusion

Two major problems trigger the adoption of microbial inoculants for use in sustainable agriculture: (i) the prolonged and indiscriminate use of agrochemicals to improve plant growth and crop yield which leads to ecological imbalance and affects the environment negatively and (ii) environmental stresses that affect plant growth and productivity. The rhizosphere, with its high microbial diversity, is a vital source of beneficial plant growth-promoting rhizobacteria that could be screened and developed into potential microbial inoculants for sustainable agriculture. One of the most important problems however is the inconsistency in the field performance of PGPR inoculants that still warrants intensive research in the field. It is hence very essential to explore the soil microbial diversity and the various modes of actions involved in direct and indirect plant growth promotion and develop consortium of two or more PGPR to attain maximum benefits from microbial inoculation. With regard to developing microbial inoculants for biocontrol, isolation of bacteria from soils suppressive to a number of soilborne plant diseases where the disease development is minimal even in the presence of a virulent pathogen and susceptible host could be a strategy. In developing a PGPR strain into potential microbial inoculants, it is vital to elucidate new associations between different strains and/or species in the population and study

the various plant bacterial signal exchange. The choice of strains beneficial to both biocontrol and plant growth-promoting potentials is very essential which focuses on isolation and screening PGPR strains that exhibit various types of beneficial traits such as production of antibiotics, siderophores, indole-3-acetic acid, acc-deaminase activity, nodulation and nitrogen fixation and by detection of the genes involved in the regulation, and synthesis of these beneficial traits and compounds.

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A Renaissance in Plant Growth-Promoting and Biocontrol Agents by Endophytes

3

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Abstract

Endophytes are the microorganisms which colonize the internal tissue of host plants without causing any damage to the colonized plant. The beneficial role of endophytic organisms has dramatically documented worldwide in recent years. Endophytes promote plant growth and yield, remove contaminants from soil, and provide soil nutrients via phosphate solubilization/nitrogen fixation. The capacity of endophytes on abundant production of bioactive compounds against array of phytopathogens makes them a suitable platform for biocontrol explorations. Endophytes have unique interaction with their host plants and play an important role in induced systemic resistance or biological control of phytopathogens. This trait also benefits in promoting plant growth either directly or indirectly. Plant growth promotion and biocontrol are the two sturdy areas for sustainable agriculture where endophytes are the key players with their broad range of beneficial activities. The coexistence of endophytes and plants has been exploited recently in both of these arenas which are explored in this chapter.

Keywords

Endophytes • PGP • Biocontrol • *Bacillus* • *Piriformospora* • *Streptomyces*

3.1 Introduction

Plants have their life in soil and are required for soil development. They are naturally associated with microbes in various ways. They cannot live alone and hence they release signal to interact with microbes. Interaction can be of either beneficial

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or pathogenic. The pathogenic interaction where the bacteria inject the effector protein to suppress the host defense response leads to plant diseases. Agricultural productivity suffers a heavy loss due to this pathogenic interaction. There is an immediate need to find and establish an ideal strategy for sustainable agriculture and improvement in crop growth. Agriculture being the world's largest economic sector, the demand should be addressed seriously. Environmental pollution is the biggest problem and a public concern today, and that is caused either directly or indirectly by use of fertilizers, pesticides, and herbicides. This has turned to seek alternative for the established chemical strategy to facilitate plant growth in agriculture and horticulture (Glick et al. 2007a). Many approaches have been taken to control plant pathogens. Several investigations have aimed at improving the understanding of plant defense systems and plant pathogen interactions (Dodds and Rathjen 2010). For a sustainable agriculture, new ways are in line to develop either to control the plant diseases or to promote the plant growth. Plant growth-promoting rhizobacteria (PGPR) plays an important role in sustainable agriculture as it functions as both plant growth promotion and disease suppression (Shoebitz et al. 2009; Beneduzi et al. 2012).

3.2 Endophytes: The Origin and Dwelling

Symbiosis refers to "living together of dissimilar organism" (De Bary 1879). There are more life that lives in symbiotic relation based on macroscopic hosts and microscopic creatures. The plant root system mainly anchors in nutrient and water uptake. Apart from that, it mediates numerous underground interactions with beneficial microbes such as rhizobia, mycorrhiza, endophytes, and rhizobacteria. The word endophyte came from two Greek words, "endon" means within and "phyton" means plant. Endophytes are microorganisms that can asymptomatically grow within plant tissues without causing any damage or eliciting any disease to the host. Endophytic bacteria and fungi are ubiquitously found in all plant species and evolve with higher plants from the day they are derived. Since the endophyte may be of both beneficial and harmful, the changes in the environment might affect the host or be neutral to the plant (Lacava et al. 2004; Ardanov et al. 2012).

The plant and the endophytic microbes have symbiotic relationship where both species benefit from the interaction. The diversity of endophytes is surprising as each and every plant species harbors one or more endophytes and they are driven by symbiotic forces in the ecosystem (Faeth and Fagan 2002). Woody plants were found to have more than one hundred different species of endophytes (Saikkonen et al. 1998; Arnold et al. 2000). They are found to be a promising candidate to increase crop yields, remove contaminants, inhibit pathogens, and able to also produce novel metabolites and fixed nitrogen.

Endophytic colonization occurs in several ways in plants. The route of colonization seems to be the rhizosphere where the microbes reach by chemotaxis and attach to the plant tissues either by pili, lipopolysaccharide, or exopolysaccharide in their cell wall (Lugtenberg and Kamilova 2009; Malfanova et al. 2013). The endophytes which are rhizosphere colonizers attach to the cell elongation zone or root hair zone of the apical roots and enter through a crack or damage. Preferably the colonization takes place in differentiation zone and intercellular spaces in the epidermis (Raven et al. 2009). When bacteria enter the exodermal barrier, there are three places where they can reside, viz., the site of entry, deep inside the cortex, and at the intercellular space of the cortex. Only few penetrate the endodermal barrier and invade xylem vessels. They are influenced by abiotic and biotic factors. But comparative to rhizospheric microbes, the endophytes are more protected from the abiotic and biotic stresses (Seghers et al. 2004). The true endophytes should be isolated after surface sterilization and confirmed with tagged studies in microscope. The endophytes which are validated in microscope are named to be putative endophytes. Endophytes mediate plant defense by two ways: (i) the innate endophytic community that should contain resistance-

competent traits and (ii) reviving of innate endophytic bacterial subpopulations by an incoming bacterium (e.g., a biocontrol agent) (Podolich et al. 2014). Endophytes have attracted the attention of researchers to evaluate them to be a potential and more effective option for use as plant growth promotion (PGP)/biological control agents in agricultural system. Understanding the interactions among endophytic microbes and their plant hosts will hopefully prove them to be alternative control measures for diseases. Gaining knowledge of the way they enter their plant hosts, the interactions that occur, and the influence that can be made for biocontrol purposes all relate to control the agricultural diseases. This chapter walks in detail over the endophytes and its types which would give a new eye on PGP and biocontrol agents.

3.3 Beneficial Traits of Endophytes and Its Mechanism

On colonization of the microbe in the plant, they can positively influence the growth and disease resistance. Several groups report the mechanism of PGP and biocontrol to be similar as rhizobacteria, but only few mechanisms have been proven to occur *in planta*. Still this chapter will review on all the expected mechanism for PGP and biocontrol (Fig. 3.1).

3.3.1 Plant Growth Promotion

PGP can take place by two ways, viz., direct or indirect mechanism. Endophytic microbes can stimulate the PGP by providing the essential nutrients, directly producing phytohormones and growth regulators, or regulating phytohormone levels.

3.3.1.1 Nitrogen Fixation

Nitrogen is a major limiting nutrient for the growth of the plant. Plants uptake nitrogen from the atmosphere and make available by the help of symbionts in the root nodules of legumes, and the process is said to be biological nitrogen fixation. Rhizobia and nitrogen-fixing bacteria share *nod* and *nif* genes which encodes for nodulation and nitrogen fixation, respectively (Zehr and Turner 2001). Studies reveal that endophytes associate themselves in the same process in other agriculturally important crops. The nitrogen fixation is done by the nitrogenase enzyme produced by the bacteria (You et al. 2005). Nitrogen fixation is regulated by oxygen concentration and availability of nitrogen. Nitrogen-limited condition also interferes in plant hormone production, and hence some diazotrophs are able to produce phytohormones in addition to nitrogen fixation.

3.3.1.2 Phosphate Solubilization

Phosphorus is the next limited compound available for plants. They play a role in cell metabolism and signaling (Vance et al. 2003). Phosphorus in $H_2PO_4^-$ and HPO_4^{2-} can be absorbed by plants, but unfortunately they are present in bound form with organic or inorganic molecules which are unavailable to plants (Smyth 2011). Though phosphorus is used as a chemical fertilizer, excessive and unmanaged application has a negative impact on the environment. Endophytes are phosphate-solubilizing bacteria which solubilize the bound form thereby making available to plants. The production of organic acid like gluconic acid is a major factor in the release of phosphorus from a bound form (Rodriguez et al. 2006). In addition, enzymes including phosphonates, phytases, and C-P lyases also play a role in converting insoluble phosphorus to available phosphorus.

3.3.1.3 Siderophore Formation

Iron is a vital nutrient and occurs as Fe^{3+} in the aerobic environment and forms insoluble hydroxides and oxyhydroxides. These insoluble forms are not accessible to both plants and microbes. Generally, endophytes synthesize low molecular weight compounds termed as siderophores that sequester Fe^{3+} since they have high Fe^{3+} affinity constants and mobilize the irons present (Zhang et al. 2008; Vendan et al. 2010). Some endophytes produce hydroxamate type and other produce catecholate type of sidero-

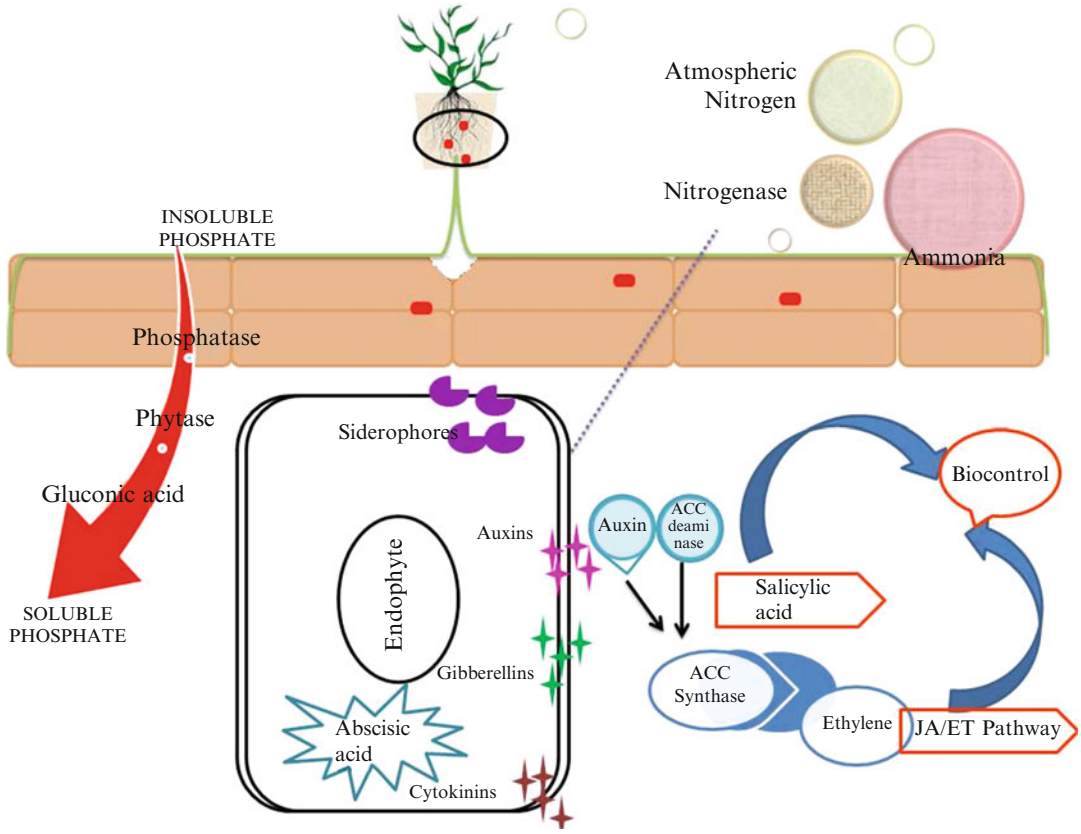

Fig. 3.1 A proposed schematic representation of PGP and defense response by endophytes

phores (Neilands and Nakamura 1991). The siderophores are water soluble and of two types, viz., extracellular and intracellular, i.e., secreted as iron-free siderophores for cellular iron uptake and located within the cell for intracellular iron storage, respectively (Johnson et al. 2013). Specific proteins are involved in transport of iron siderophore complex in iron-limited conditions. PGP and disease suppression are achieved by siderophore formation (Hayat et al. 2010). Many plant species absorb bacterial Fe^{3+} siderophore complexes, but the role of siderophores in PGP is yet to be proved.

3.3.1.4 Growth Regulators

Plants produce hormones such as auxins, cytokinins, gibberellins, ethylene, and abscisic acid. Endophytic microbes have the potent to produce these hormones which influence plant growth and development.

Auxins Auxin is the crucial plant hormone and fundamental component that modulates plant growth and development (Halliday et al. 2009; Grossmann 2010). Indole-3-acetic acid (IAA) is a member of auxin family produced by bacteria, fungi, and plants. IAA induces lateral root formation in dicots and adventitious root formation in monocots (McSteen 2010). IAA combines cambial growth and vascular development. Auxins promote secondary wall thickness and increase xylem cells (Uggla et al. 1996). They are transported via phloem by forming concentration gradients and accumulate in different tissues (Eklund et al. 2010; Tromas and Perrot-Rechenmann 2010). IAA concentrations vary depending on the tissues of the plant and organ (Reid et al. 2011). IAA pathway is a robust network which was identified by the enzymes that catalyze each reaction and the intermediates involved in each step (Lehmann et al. 2010). Several recent studies are

being proposed with IAA biosynthesis pathway. Detailed study of the IAA pathway is reviewed by Duca et al. (2014).

Cytokinins Zeatin is a member of cytokinin family. They play a role in division of plant cell in the presence of auxin. They involve in callus growth (Salome et al. 2001). Auxin and cytokinins help in root differentiation and shoot differentiation, respectively.

Gibberellins Terpenoid groups come under this category. They are mainly involved in cell division, cell elongation, and internode elongation. The mechanism by which plant growth is promoted through gibberellins is still unclear. Fulchieri et al. (1993) reported that they increase root hair density in root zones involved in uptake of nutrient and water.

Abscisic Acid It is a stress hormone which regulates the plant development and physiological process. They play an important role in seed germination, stromal closure, and abiotic stress tolerance (Lee and Luan 2012). It is an abiotic elicitor for plant biosynthesis of bioactive compounds (Sun et al. 2012).

ACC Deaminase Ethylene is produced from ACC synthase (Giovanelli et al. 1980) which inhibits primary root elongation and lateral root formation but promotes root hair formation (Dodd et al. 2010), thus having a positive and negative role. Ethylene increases at a higher rate when the plant is in stressed conditions (Glick 2005). Hence, it is also known as stress hormone. The enzyme ACC deaminase is produced by many endophytes which converts ACC into α-ketobutyrate and ammonia (Glick et al. 2007b). Reduction in ACC level reduces ethylene levels and thus decreases the plant stress.

3.3.2 Biocontrol

The use of agrochemicals to control plant diseases can be minimized by means of biological process such as the use of endophytes which inhibit or antagonize the phytopathogens. Though the chemical products kill the plant pathogen, workers and consumers are at high risk. Biocontrol agents communicate with other pathogens/organisms through a variety of signal molecules. These signal molecules play a role in the defense against disease. They include jasmonic acid, salicylic acid, abscisic acid, etc., which are induced during abiotic stress conditions.

Defense-related proteins and secondary metabolites are produced by induction of jasmonic acid (Brodersen et al. 2006; Balbi and Devoto 2008). Salicylic acid gets involved in flowering, growth and development, ethylene biosynthesis, stromal behavior, etc. Abscisic acid in defense signaling is found to promote seed dormancy (Asselbergh et al. 2008). Mechanisms of biocontrol by the endophytes may be either one of the following:

1. Antibiosis – many bacteria are potent in producing antibiotics which are the best known class of biocontrol agents. Limitation on using antibiotic-producing bacteria might be the cross-resistance, and also the genes encoding might be transferable (Zhang et al. 1993).
2. Predation and parasitism – control agents produce exoenzymes that can degrade the fungal cell and use them as food for their survival.
3. Induced systemic resistance (ISR) – ISR is the plant immune response that is activated by beneficial microbes (Kloepper et al. 2004, Van Wees et al. 2008). Upon immunization, the plant becomes more potent in producing infection-induced immune response which might result in enhanced protection. ISR is also a systemic response which is similar to systemic acquired resistance (SAR) and protects from many pathogens (Van Loon 2007). They induce innate immunity and use toll-like receptors (De Weert et al. 2007). The signal transduction pathway and the molecular basis underlying are different. In SAR, the signals include hypersensitive response, salicylic acid biosynthesis, or induction of pathogenesis-related proteins, whereas the hormone jasmonic acid and ethylene play a main role in ISR (Sena et al. 2013). Hence, in any of the

above means, the natural microbes, i.e., endophytes, can be potent in controlling diseases thereby reducing the usage of chemical products.

3.4 Bacterial Endophytes

The origination of bacterial endophytes is of 120 years older where they were initially identified from seeds and surrounding environment. Endophytic bacteria are reported to be present in roots, stems, leaves, seeds, fruits, tubers, ovules, and also inside legume nodules (Compant et al. 2011) in which more preferably in roots (Rosenblueth and Martinez-Romero 2006). The endophytic population varies depending on the bacteria and the host, host developmental stage, inoculum density, and environmental conditions (Tan et al. 2006). The endophytes that are dominating in the plants are intensively reviewed in many reports (Rosenblueth and Martinez-Romero 2006). Though, the community composition is non-determinable but can be determined by colonization process. Factors such as nature and stage of the host, physiological status, type of plant tissue, soil conditions, and agriculture practices determine colonization (Hardoim et al. 2008).

Endophytes are host specific, for example, a group of clostridia is found to be only in grass species, i.e., *Miscanthus sinensis*, but not in the soil (Miyamoto et al. 2004). Endophytic bacteria are seen in legume nodules as co-occupants (Benhizia et al. 2004). They are reported to be isolated from different vascular and nonvascular plants denoting the wide spectrum of endophytic bacteria (Hardoim et al. 2012; Rosenblueth and Martinez-Romero 2006). Metagenomic approach is the recent hot spots in endophytes due to the unculturable nature of certain groups of endophytes (Manter et al. 2010; Sessitsch et al. 2012; Bulgarelli et al. 2012; Bodenhausen et al. 2013). This approach exploits a deeper understanding of the functions of the endophytes and the mechanism used to reside inside the endosphere.

Based on the lifestyle, they are classified as obligate and facultative endophytes. Obligate endophytes depend on the host plant for their growth and survival and transmit to other vertical plants or through vectors, whereas facultative bacteria exist outside of the plant for a part of its lifetime, and for the rest, they dwell inside the plants. Bacterial phytopathogens also can be considered as facultative endophytes because they are present in avirulent forms. *Ralstonia solanacearum* can survive in water and occurs as an endophyte in tomato plants as avirulent bacteria (Van Overbeek et al. 2004). Endophytes include both Gram-positive and Gram-negative bacteria, and they are classified as *Alpha-*, *Beta-*, and *Gammaproteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* (Lodewyckx et al. 2002; Bacon and Hinton 2006). The higher percentage of rhizosphere community is *Acidobacteria* (31 %) and *Alphaproteobacteria* (30 %), whereas most endophytes were associated to *Gammaproteobacteria* (54 %) and *Alphaproteobacteria* (23 %) (Gottel et al. 2011).

3.4.1 Role in PGP and Biocontrol

PGP can be induced at higher rate by the bacteria. Most mechanistic pathway of either direct or induced PGP is more or less similar to rhizosphere bacteria. Direct PGP is caused by the inducing availability of nutrients or by hormone production. Indirect PGP might be taken place at three conditions: (1) in the presence of a pathogen, the beneficial bacteria inactivate/kill the pathogen; (2) when a remediation occurs, the bacterium inactivates a pollutant which stops the growth of the plant; (3) during stress conditions' excess of ethylene, heavy metal, drought, etc., ACC deaminase is produced which can tolerate stress conditions.

Bacterial endophytes are reported to produce auxins (Vendan et al. 2010, Shcherbakov et al. 2013) using tryptophan as a precursor (Rosenblueth and Martinez-Romero 2006), whereas gibberellins are reported to be produced by rhizosphere bacteria. IAA production by *P. putida* CR_3 and *Rahnella aquatilis* HC_2 stimulates growth in cereals and radish (Malfanova 2013). *Bacillus subtilis* HC-8 induced plant growth by

gibberellin production. Ethylene is a stress hormone for which ACC is the precursor. The bacteria convert ACC into α-ketobutyrate and ammonia which can tolerate the stress conditions caused by ethylene, salination, and heavy metals (Malfanova et al. 2011). A total of 174 endophytes isolated from interior tissues of tomato plants were collected from various countries in the world. The bacteria that are able to utilize ACC as sole carbon source were selected further and tested for IAA synthesis, siderophore formation, phosphate solubilization, optimal growth temperature, salt tolerance, and antibiotic sensitivity. Of the 174 endophytes, 25 isolates were potent in all the parameters tested, and they were found to be the genera of *Pseudomonas* spp., *Microbacterium* spp., *Agrobacterium* spp., *Bacillus* spp., and few unculturables (Rashid et al. 2012). Plants which prefer the endophytes with high ACC deaminase activity will confer benefits for both plant and bacteria (Hardoim et al. 2008).

Nitrogen fixation is involved in growth stimulation (Iniguez et al. 2004). Some endophytic bacteria are able to fix atmospheric nitrogen and convert them into ammonia which can be taken by the plant (Krause et al. 2006; Vendan et al. 2010; Shcherbakov et al. 2013). Endophytes such as *A. diazotrophicus* PA15 and *Herbaspirillum* sp. B5D when inoculated on sugarcane and rice, respectively, enhanced 0.6 % and 0.14 % total nitrogen in 24 h (Sevilla et al. 2001; Wu et al. 2009). Bacteria producing enzymes that can solubilize the phosphorus are agriculturally important. Some endophytic bacteria which cannot enter the interior layers of the plant cell are found to be potent in mobilizing the phosphorus (Sturz et al. 2000). Endophytic bacteria are potent antagonist in controlling the fungal pathogens. *Pseudomonas* species as an endophyte was reported to be an antagonist for different phytopathogens on various hosts (Adhikari et al. 2001; Grosch et al. 2005; Prieto et al. 2009). Similarly, plant defense mechanism is also activated by ISR. This ISR can be done by various metabolites, molecules, or volatiles produced by the bacteria inside plant tissues. For instance, *B. amyloliquefaciens*, *B. subtilis*, *P. fluorescens*, and *Serratia marcescens* were reported to induce ISR (Kloepper and Ryu 2006). Reiter et al. (2002) demonstrated many genera of endophytic bacteria such as *P. fluorescens*, *P. alcaligenes*, *P. putida*, *Flavobacterium* spp., and *B. megaterium* inhibiting plant pathogens. Other endophytes that inhibit pathogens include *Alcaligenes* spp., *Kluyvera* sp., *Microbacterium* sp., and *Curtobacterium* sp. (Zinniel et al. 2002). Ramesh et al. (2009) reported 28 isolates of endophytic bacteria inhibiting bacterial wilt pathogen *Ralstonia solanacearum*.

Endophytic bacteria might follow a predation and parasitism mechanism. This might be due to production of cell wall-degrading enzymes such as cellulase, chitinase, and glucanase (Krechel et al. 2002; Berg and Hallmann 2006). They are also potent in suppressing the proliferation of nematode in host plants (Sturz and Kimpinski 2004). *Curtobacterium flaccumfaciens*, an endophyte isolated from citrus plant, was reported to inhibit the pathogen *Xylella fastidiosa* (Araujo et al. 2002). Similarly, endophytes from potato act as antagonist against bacteria and fungi (Sessitsch et al. 2004; Berg et al. 2005). Recent interest is on genetically engineered endophytes. For instance, *Herbaspirillum seropedicae* and *Clavibacter xyli* are genetically engineered endophytes that produce endotoxin of *B. thuringiensis* in order to control insect pests (Downing et al. 2000). Another endophyte *Burkholderia cepacia* has modified to tolerate toluene (Barac et al. 2004). Hence, with the detailed study of the mechanism in colonization, these can be implemented in promoting plant growth and as biocontrol agents. Recently studied endophytes with plant host are tabulated (Table 3.1).

3.5 Fungal Endophytes

More than 100 years of research suggests that most, if not all, plants in natural ecosystem are symbiotic with mycorrhizal fungi. Among all endophytes, fungal endophytes are studied more till date. Fungal endophytes are of increasing interest due to growing list of benefits that they can confer on their hosts, including both biotic

Table 3.1 PGP and biocontrol properties of bacterial endophytes

Endophytes	Host plant		PGP/biocontrol	References
	Common name	Scientific name		
Bacillus megaterium LNL6, *Methylobacterium oryzae* CBMB 205	Rice	*Oryza sativa*	IAA, ACC deaminase, N fixation	Subramanian et al. (2014)
Gluconobacter diazotrophicus	Sugarcane	*Saccharum officinarum*	Systemic defense	Idogawa et al. (2014)
Burkholderia, *Azospirillum*, *Ideonella*, *Pseudacidovorax*, *Bradyrhizobium*	Potatoes	*Solanum tuberosum* L.	N fixation, phytohormone production, biocontrol of *Fusarium*, *Koribacter*, *Pectobacterium*	Pageni et al. (2014)
Paenibacillus, *Bacillus*, *Microbacterium*, *Klebsiella*	Rice cultivars	*O. sativa*	IAA, P solubilization, siderophore	Ji et al. (2014)
Burkholderia, *Klebsiella*, *Novosphingobium*, *Sphingomonas*	Rice	*O. sativa*	IAA, P solubilization, siderophore	Rangjaroen et al. (2014)
Bacillus subtilis var. *amyloliquefaciens* 72β24	Rice	*O. sativa*	Biocontrol of *Rhizoctonia solani*	Nagendran et al. (2014)
Bacillus sp., *Enterobacter* sp.	Corn	*Zea mays*	N fixation, IAA, siderophore	Szilagyi-Zecchin et al. (2014)
Pantoea dispersa	Cassava	*Manihot esculenta Crantz*	P solubilization	Chen et al. (2014)
Bacillus pumilus	Thulasi	*Ocimum sanctum*	P solubilization, IAA, siderophore, HCN	Murugappan et al. (2013)
Acinetobacter johnsonii strain 3–1	*Beet*	*Beta vulgaris*	IAA, P solubilization	Yingwu et al. (2011)
Martelella mediterranea, *Hoeflea alexandrii*	Japanese rose and annual sea blite	*Rosa rugosa*, *Suaeda maritime*	P solubilization, IAA, nitrate reduction, biocontrol of *Phytophthora capsici*, *Pythium ultimum*	Bibi et al. (2012)
Bacillus thuringiensis GDB-1	*Scots pine*	*Pinus sylvestris*	ACC, IAA, P solubilization, siderophore	Babu et al. (2013)
Bacillus, *Paenibacillus*, *Klebsiella*, *Acinetobacter*	Wheat	*Triticum* spp.	IAA, P solubilization, siderophore, biocontrol of *Gaeumannomyces graminis*	Duran et al. (2014)
Enterobacter sp. strain FD17	Maize	*Z. mays*	IAA, ACC,P solubilization, siderophore	Naveed et al. (2014)
Pseudomonas fluorescens PICF7	*Olive*	*Olea europaea*	Biocontrol of *Verticillium* wilt	Cabans et al. (2014)
B. subtilis NA-108, *B. subtilis* NA-120, *Enterobacter* sp. EMB-79	Strawberry	*Fragaria ananassa*	IAA, siderophore, N fixation	de Melo Pereira et al. (2012)
Bacillus spp.	Rose gum	*E. urophylla x E. grandis*	IAA, P solubilization, N fixation	Paz et al. (2012)

(continued)

Table 3.1 (continued)

Endophytes	Host plant		PGP/biocontrol	References
	Common name	Scientific name		
Stenotrophomonas maltophilia, *Pseudomonas putida*, *S. maltophilia*, *Achromobacter xylosoxidans*, *Achromobacter* sp.	Amaranth, tomato, calabaza	*Amaranthus hybridus*, *Solanum lycopersicum*, *Cucurbita maxima*	IAA, P solubilization, ammonia	Ngoma et al. (2013)
Escherichia fergusonii, *Acinetobacter calcoaceticus*, *Salmonella enterica*	Coffee	*Coffea arabica*, *C. robusta*	Phosphatase, siderophore, IAA	Silva et al. (2012)
Methylobacterium spp., *Micrococcus luteus*, *Lysinibacillus fusiformis*, *Stenotrophomonas maltophilia*	Citrus, Ginseng	*Citrus* sp., *Ginseng* sp.	Siderophore, IAA, P solubilization, N fixation	Vendan et al. (2010)

and abiotic. They have the ability to provide resistance against herbivores (Brem and Leuchtmann 2001), pathogens (Gond et al. 2010), temperature and salinity (Redman et al. 2002) and also stresses and heavy metals (Li et al. 2012). Fungal endophytes unlike mycorrhizal fungi colonize plant root and grow into rhizosphere. Plant tissue is the residence of the fungal endophytes which may grow in all or any part of the plants. There are numerous reports documenting the presence of fungal endophytes in distinct phyla. Petrini et al. (1992) reported that more than one type of fungal endophytes is found in single plant. Kharwar et al. (2008) evidence 13 isolates in leaf, stem, and root tissues of *Catharanthus roseus*. Fungal endophytes are predominantly found to be present in tropical, subtropical, and terrestrial ecosystems. Kharwar et al. (2011) also reported the isolation of total 149 fungal endophytic isolates belonging to 17 fungal genera in leaf, stem, and petiole. Among all tissues studied, leaves showed about 72 % endomycobiota compared to stem and petiole which are 68 % and 25.54 %, respectively. The predominant genera include *Cryptosporiopsis lunata* (4.18 %), *F. roseum* (4.07 %), *A. niger* (5.93 %), *Stenella agalis* (5.20 %), *Fusarium oxysporum* (5.18 %), and *Aspergillus alternata* (6.30 %).

3.5.1 Classification

A detailed study in the classification of the fungal endophytes has been reviewed by Rodriguez et al. (2009). Endophytes are broadly classified into two groups, viz., clavicipitaceous endophytes (class I) and nonclavicipitaceous endophytes (class II), based on evolution, taxonomy, ecology, and nature of the host. Depending upon the host range, the way they colonize, the pattern of transmission, tissue specificities, and symbiotically conferred benefits, they are of two more classes (III, IV).

3.5.1.1 Class I (Clavicipitaceous) Endophytes

These endophytes are defensive mutualism of host grasses. They include free-living and symbiotic species associated with insects and fungi (*Cordyceps* sp.) or grasses, rushes, and sedges (*Balansia* sp., *Epichloe* sp., and *Claviceps* sp.) (Bacon and White 2000). This class of endophytes is believed to begun from insect-parasitic ancestors and diversified through an interkingdom. The evolution of endophyte is thought to have begun with free-living insect parasite and then progressed to epibiotic plant gaining access to plant nutrients (Spatafora et al. 2007; Torres et al. 2007). These endophytes descend-

ing from insects do not possess enzymes or toxins for killing or degrading plant tissues but produce toxins that affect insects and other animals. The life history states that *Epichloe* spp. are endophytes present in grass which is present in intercellular spaces of leaf sheaths, rhizomes, and surface of leaf blades (Moy et al. 2000; Tadych et al. 2007). During flowering stage, fungus grows over to form a stroma, where inflorescence primordium remains at arrested stage preventing seed development. Some species exhibit stromata allowing partial seed production and vertical transmission. Inoculation of *E. festucae* in turf grasses showed significant resistance over uninoculated turf to two major leaf spot pathogens: dollar spot disease caused by *Sclerotinia homeocarpa* (Clarke et al. 2006) and red thread disease caused by *Laetisaria fuciformis* (Bonos et al. 2005).

3.5.1.2 Class II (Nonclavicipitaceous) Endophytes

They are a single group with diverse fungi and can be provisionally classified into at least three functional groups on life history, ecological intern, and traits. It comprises of diversified species, which are a member of Dikarya, most belonging to *Ascomycota* and minority of *Basidiomycota*. These fungi colonize plants via infection structures such as sporulation or by direct penetration of plant tissue via hyphae growth through plant tissue which is dominantly intracellular with little or no impact on host cells. These fungi rapidly emerge and sporulate during host senescence (Weber et al. 2004). Many endophytes protect host to some extent against fungal pathogens. Endophytic isolates of *F. oxysporum* and *Cryptosporiopsis* sp. conferred disease resistance against virulent pathogens in barley (*Hordeum vulgare*) and larch (*Larix decidua*), and resistance was correlated to an increase concentration of phenolic metabolites (Schulz et al. 1999). The uniqueness lies in the ability of the individual isolates to asymptomatically colonize and confer habitat-adapted fitness benefits on genetically distant host species representing monocots and eudicots (Rodriguez et al. 2009).

3.5.1.3 Class III Endophytes

These include the hyperdiverse endophytic fungi associated within leaves of tropical trees as well as ground tissues of nonvascular plants, seedless vascular plants, conifers, woody, and herbaceous angiosperm. Fungi with similar life histories of class III endophytes also occur with asymptomatic lichens and in that case are known as endolichenic fungi (Arnold 2008). Members of *Basidiomycota* belonging to *Agaricomycotina*, *Pucciniomycotina*, and *Ustilaginomycotina* also are class III endophytes. Reproduction is by spore formation which is released passively. Spores might be sexual or asexual.

3.5.1.4 Class IV Endophytes

The dark pigmented endophytes called as "mycelium radicis atrovirens" or dark septate endophytes are grouped as class IV endophytes. They are ascomycetous fungi that are either conidial or sterile and that form melanized structures such as inter- and intracellular hyphae and microsclerotia in the roots. These groups are less specific toward the host and have been reported about 600 plants including plants that are non-mycorrhizal, from Arctic, Antarctic, alpine, subalpine, tropic zones, temperate zones, coastal plains, and lowlands (Jumpponen 2001).

3.5.2 Role in PGP and Biocontrol

Fungal endophytes are valued more for its PGP traits and biocontrol potency (Azevedo and Araújo 2007; Suryanarayanan et al. 2012). Several investigations have performed to improve the plant growth and protect the plant. The endophytic fungi are beneficial to the host plants by inducing higher nutrient uptake (Lekberg and Koide 2005). Endophytic fungi are present right from the seed germination. At this stage, they degrade the cellulose of the cuticle and make carbon available for the plant germination and establishment. They colonize in the root of the host and result in promotion of growth and higher yield. They produce plant growth regulators, thereby promoting seed germination in crops (Bhagobaty and Joshi 2009).

Fungi are potent in producing wide variety of growth hormones, viz., gibberellins, auxins, and abscisic acid (You et al. 2012). Many endophytes have reported in vitro production of IAA and its effect on PGP (Govindarajan et al. 2008). IAA production further enhances plant growth under salinity, drought, and temperature stress (Redman et al. 2011). The sand flora of Korean coastal region showed a majority of 80.7 % growth promotion of Waito-C rice, thus indicating the induction of PGP hormones by fungal endophytes (Khan et al. 2012). A review by Mei and Flinn (2010) has listed US patents showing the significance of fungal and bacterial endophytes for plant growth promotion and stress tolerance.

Fungal endophytes have higher resistance toward insect herbivores, nematodes, and plant pathogens which is an important factor favoring crop protection. The defense against insects is enhanced by secreting growth-regulating compounds or metabolites. These in turn influence plant development and help in crop protection (Marina et al. 2011). The endophytes against crop diseases by fungus were reported by Webber (1981) for the first time where *Phomopsis oblonga* protects from *Physocnemum brevilineum*, a pest of elm trees. Plant hormones that act as defense signaling molecule include salicylic acid, jasmonic acid, etc. (Shinozaki and Yamaguchi-Shinozaki 2007). Endophytic genera of *Neotyphodium* and *Fusarium* suppress *Triticum* diseases and nematodes, respectively (Tunali et al. 2000). Several studies demonstrated that endophytic fungi can resist the plants against *Phytophthora palmivora*, *Moniliophthora roreri*, and *M. perniciosa* (Mejia et al. 2008) in which one of the endophytes *Gliocladium catenulatum* can reduce up to 70 % incidence of witches' broom disease (Rubini et al. 2005). *Piriformospora indica* induces systemic resistance in *Arabidopsis* against powdery mildew pathogen *Golovinomyces orontii* by activating the jasmonate signaling pathways (Stein et al. 2008). More examples of endophytic fungi controlling plant diseases caused by pathogenic fungi, nematodes, and bacteria are reviewed by Azevedo and Araújo (2007).

Inoculation with *P. indica* isolated from *Prosopis juliflora* and *Ziziphus nummularia* increased the plant growth in diverse host plants (Varma et al. 1999). Improved plant nutrition and increased tolerance to abiotic and biotic stress elucidate the plant growth stimulation mediated by endophytes. *Epichloe festucae* is a fungal endophyte that increases uptake of phosphorus on inoculation with *Festuca rubra*, by solubilizing rock phosphate from soil (Zabalgogeazcoa et al. 2006).

Many endophytes like *F. fujikuroi*, *Sphaceloma manihoticola*, *Phaeosphaeria* sp., *Neurospora crassa*, *Cladosporium* sp., *Penicillium* sp., *Gliomastix murorum*, *Arthrinium phaeospermum*, and *Aspergillus fumigatus* have been reported as growth promoters. Under extreme environmental conditions, these phytohormones producing endophytic fungi affect the production of several secondary metabolites like flavonoids to help the plant to tolerate/avoid stress (Schulz 2002; Waller et al. 2005; Khan et al. 2011). Representative fungal endophytes with PGP and biocontrol traits were tabulated (Table 3.2). Today's interest is toward the endophytic fungi which have residence in root tissues and secrete plant growth-regulating compounds to increase the crop yield and quality. On controlling the plant diseases and increasing the yield, the ideal strategy of sustainable agriculture can be reached. Though the molecular mechanism of the endophytic fungi in PGP and defense is not clearly known, several studies confirm that they play a key role in the crop protection and yield enhancement. The culturable and unculturable techniques are involved to explore still on the endophytes. Fungal endophytes have attracted the researchers and hence they are researched globally to combat crisis and demands in agriculture (Rai et al. 2014).

3.6 Endophytic Actinomycetes

Actinomycetes are Gram-positive filamentous bacteria belonging to the phylum *Actinobacteria* with 6 classes, 5 subclasses, 25 orders, 14 suborders, 52 families, and 232 genera. It is one of the

Table 3.2 PGP and biocontrol properties of fungal endophytes

Endophytes	Host plant		PGP/biocontrol	References
	Common name	Scientific name		
P. indica	Barley	*Hordeum vulgare* L.	Ethylene/phytohormone production	Schafer et al. (2009)
P. indica	Arabidopsis	*Arabidopsis thaliana*	Cytokinins, abscisic acid, gibberellins	Vadassery et al. (2009)
Cladosporium sp.	Cucumber	*Cucumis sativus*	Gibberellins	Hamayun et al. (2010)
Scolecobasidium humicolas	Tomato	*Solanum lycopersicum*	N fixation	Mahmoud and Narisawa (2013)
Penicillium sp., *Phoma glomerata*	Cucumber	*Cucumis sativus*	IAA, gibberellins, jasmonic acid	Waqas et al. (2012)
Pestalotiopsis sp.	Tomato	*Solanum lycopersicum*	IAA	Hoffman et al. (2013)
Aspergillus flavipes CanS-34A, *Chaetomium globosum* CanS-73, *Clonostachys rosea* CanS-43, *Leptosphaeria biglobosa* CanS-51	Oilseed rape	*Brassica napus*	Biocontrol of *Sclerotinia sclerotiorum*, *Botrytis cinerea*	Zhang et al. (2014)
Paraconiothyrium sp.	Taxus	*Taxus baccata*	Salicylic acid, benzoic acid	Soliman and Raizada (2013)
Penicillium verruculosum	Cinquefoils	*Potentilla fulgens*	IAA	Bhagobaty and Joshi (2009)
Curvularia, *Fusarium*, *Pestalotiopsis*, *Tolypocladium*	Cacao	*Theobroma cacao*	Biocontrol of *Phytophthora palmivora*	Hanada et al. (2010)
Penicillium sp.	Wheat	*Triticum* spp.	P solubilization	Wakelin et al. (2004)
Fusarium oxysporum	Banana	*Musa paradisiaca*	ISR against *Radopholus similis*	Vu et al. (2006)
Penicillium copticola	Cannabis	*Cannabis sativa* L.	Biocontrol of *Botrytis cinerea*, *Trichothecium roseum*	Kusari et al. (2013)
Aureobasidium pullulans, *Paraconiothyrium sporulosum*	Frailejón	*Espeletia grandiflora* and *Espeletia corymbosa*	Biocontrol of *Rhizoctonia solani*	Miles et al. (2012)
Paecilomyces formosus	Cucumber	*Cucumis sativus*	Gibberellin	Khan et al. (2012)
Trichoderma gamsii	*Lentil*	*Lens esculenta*	P solubilization, chitinase, ammonia, salicylic acid	Rinu et al. (2014)

largest taxonomic groups among the 18 known lineages within the bacterial domain (Stackebrandt and Schumann 2000). They are found in the internal tissue of the plant without harming the plant either as damage or in morphological change (Kunoh 2002; Hasegawa et al. 2006). Plant ecosystem is diversified and it is a rich reservoir of novel taxa actinomycetes (Inbar et al. 2005; Zin et al. 2007; Qin et al. 2009). They have wide range of host and found to be residing in many plants, viz., barley, rye, oats, and soybean (Sardi et al. 1992), rice (Tian et al. 2004), banana (Cao et al. 2005), cowpea (Dimkpa et al. 2008), medicinal plants (Qin et al. 2009), blue

lupin (Trujillo et al. 2010), tomato (de Oliveira et al. 2010), chickpea (Misk and Franco 2011), neem tree (Verma et al. 2011), and wheat (Sadeghi et al. 2012).

Among actinomycetes identified as endophytes, *Streptomyces* sp. is the predominant, and *Microbispora*, *Micromonospora*, *Nocardioides*, *Nocardia*, and *Streptosporangium* are the common genera. According to the study performed in roots and leaves of maize plants (*Zea mays* L.), *Microbispora* sp. was found to be the most common *Actinobacteria* (De Araujo et al. 2000), although *Streptomyces* and *Streptosporangium* spp. were also present. But a number of 619 actinomycetes were isolated from different cultivars of tomato, and all of them were *Streptomyces* spp. (Tan et al. 2006). Similarly Taechowisan et al. (2003) isolated 330 strains belonging to four different genera (*Streptomyces*, *Microbispora*, *Nocardia*, and *Micromonospora*) in 330 medicinal plants. Lee et al. (2008) reported 81 endophytic *Actinobacteria* including eight genera from Chinese cabbage roots, and *Microbispora* spp. were the most common isolates, followed by *Streptomyces* sp. and *Micromonospora* sp. Colonization takes place at higher rate in roots of the host. To date, more than 40 new taxa have been found by polyphasic taxonomic approaches, including four new genera, *Plantactinospora*, *Actinophytocola*, *Phytohabitans*, and *Jishengella*. The greatest diversity of endophytes occurs in the tropical and temperature regions. Janso and Carter (2010) reported a total of 123 endophytic actinomycetes isolated from plants collected from several locations in Mborokua Island, Papua New Guinea, and Solomon Islands. Filamentous *Actinobacteria* was found to be present in surface-sterilized roots of wheat plants (Coombs and Franco 2003). Misk and Franco (2011) observed a physiologically different endophytic group in legumes such as lentil, chickpea, pea, etc. Strobel and Daisy (2003) have reported that a great diversity of endophytic *Actinobacteria* is found in tropical and temperate regions. Taechowisan et al. (2003) isolated about 330 strains from 36 medicinal plants in Thailand which showed that the genera *Streptomyces*, *Microbispora*, *Micromonospora*, and *Nocardia* are predominant. *Actinobacteria* has attracted researchers in recent years where 50 new taxa have been identified from various plants in terrestrial environment. The identification and characterization is done by polyphasic approach which includes morphological, chemotaxonomical, and molecular techniques (Brusetti et al. 2008; Yuan et al. 2008). The next-generation sequencing, a high-throughput study, is another upcoming technique which is used in diversity and taxonomy studies (Mardis 2008, Lauber et al. 2010, Robinson et al. 2010).

3.6.1 Role in PGP and Biocontrol

Recently, actinomycetes have attracted the researchers' interest because of its potent biocontrol nature and significant role in plant promotion. However, the *Streptomyces* strain had the smallest population size (10^2–10^5 cfu/g) in a wheat rhizosphere; they relatively lived for a longer duration (1 year) than other organisms under the conditions tested (Yuan and Crawford 1995). Several studies have proved that endophytic actinomycetes can control many fungal pathogens and plant diseases (Quecine et al. 2008). This antagonistic ability is due to the production of bioactive compounds, cell wall-degrading enzymes, and competent in nutrition (El-Tarabily and Sivasithamparam 2006). They can also trigger ISR. The endophytic strain *S. galbus* R-5 released cellulose and pectinase and produced actinomycin X_2 and fungichromin to induce resistance in the rhododendron seedlings and triggered plant jasmonate-associated defense responses (Shimizu et al. 2005). Conn et al. (2008) observed that *Streptomyces* sp. EN27 and *Micromonospora* sp. strain EN43 led to increased resistance in *A. thaliana* leaves against pathogens such as *Erwinia carotovora* and *F. oxysporum* and triggered the expression of defense genes related to salicylic acid- or jasmonic acid-/ethylene-dependent signaling pathways in the absence of a pathogen. *Streptomyces* isolated from banana plant was found to have antibiosis property and was also capable in siderophore production (Cao et al. 2004). Similarly,

Micromonospora and *Streptomyces* from mangrove plants in China were potent to inhibit protein synthesis with antibiosis property (Hong et al. 2009). They promote plant growth by inducing the production of phytohormone production of siderophores to scavenge ferric iron from the environment, solubilization of inorganic phosphate, nitrogen fixation, and suppression of stress ethylene in plant by the production of ACC deaminase (Dimkpa et al. 2008; Kannan and Sureendar 2008; Trujillo et al. 2010; de Oliveira et al. 2010; Verma et al. 2011; Sadeghi et al. 2012). A wide range of pathogens can be controlled by actinomycetes including *Rhizoctonia solani*, *Verticillium dahliae*, *Plectosporium tabacinum*, *F. oxysporum*, *Pythium aphanidermatum*, and *Colletotrichum orbiculare* (Krechel et al. 2002; Shimizu et al. 2009). Several endophytic *Actinobacteria* isolated from winter rye produced IAA (Merzaeva and Shirokikh 2010). *Frankia* strains are symbionts in certain nonleguminous plants and can induce N_2-fixing root nodules (Benson and Silvester 1993). Tomato plants from Algerian Sahara were found to have many *Streptomyces* genera which were screened for the ability of IAA production and also potent in controlling *R. solani* (Goudjal et al. 2013, 2014). Endophytic actinomycetes isolated from various plants with PGP and biocontrol properties were summarized in Table 3.3.

Recently, our research group at ICRISAT has isolated from various rhizospheric soil and collected about 1500 microbes (bacteria and actinomycetes) in which many have documented agriculturally favorable traits. Actinomycetes such as *Streptomyces* spp., *S. griseorubens*, *S. caviscabies*, and *S. globisporus* subsp. *caucasicus* isolates have potency in in vitro PGP traits with upregulation of PGP genes such as IAA and siderophore-producing genes (Gopalakrishnan et al. 2012, 2013, 2014a). Apart from the PGP traits, they also have the capacity to act as biocontrol agents. The PGP actinomycetes were found to have inhibitory activity against *Fusarium oxysporum* f. sp. *ciceri* (FOC) and *Sclerotium rolfsii* Sacc., which causes *Fusarium* wilt and collar rot in chickpea, respectively (Gopalakrishnan et al. 2011a), and also against *Macrophomina phaseolina*, which causes charcoal rot in sorghum (Gopalakrishnan et al. 2011b). PGP bacteria such as *B. megaterium*, *B. subtilis*, *Serratia marcescens*, and *Pseudomonas geniculata* (Gopalakrishan et al. 2014b), a fungus *Metarhizium anisopliae*, and actinomycetes such as *S. cavourensis* sup sp. *cavourensis*, *S. cyaneofuscatus*, *S.bacillaris*, *S. antibioticus*, *S. albolongus*, *S. hydrogenans*, and *S. carpaticus* were found to have broad-spectrum insecticide against lepidopteran pests such as *Helicoverpa armigera*, *Spodoptera litura*, and *Chilo partellus* (Gopalakrishnan et al. 2011c; Vijayabharathi et al. 2014). Recently, five strains of *Streptomyces* sp. isolated from chickpea have been found to inhibit charcoal rot of sorghum and induce PGP of sorghum and rice. They have been found to have IAA and siderophore-producing genes (Gopalakrishnan et al. 2015). All these bacteria and actinomycetes with PGP and biocontrol ability need to be further evaluated for its endophytic ability by addressing the query of survival inside the endodermal layer. Plant growth-promoting properties of endophytic *Actinobacteria* and the recent increased understanding of some of the mechanisms suggest that this promising source merits further investigations for potential application in agriculture.

3.7 Future Prospects

The endophytic population is the gut population of the plants. They might be of bacteria, fungi, or actinomycetes. Majority of these are not identified yet. Endophytes make a renaissance in using microbes for biological control of plant pathogens for a sustainable agriculture where the emphasis mainly is on hazards associated with chemical pesticides and transgenic plants. They colonize inside and outside the host tissues and make a long-term friendship, actually a lifelong relation without making any harm to the host (Rodriguez et al. 2009). Though several decades of research has underwent in the field of symbiosis and their associations, there is a gap to know about the things needed for association and the way they maintain the association. The future

Table 3.3 PGP and biocontrol properties of actinomycete endophytes

Endophytes	Host plant		PGP/biocontrol	References
	Common name	Scientific name		
Streptomyces albosporus R13	Rice	*O. sativa*	Siderophore	Gangwar et al. (2012)
S. griseus	Wheat	*Triticum* spp.	IAA	Hamdali et al. (2008)
S. olivochromogenes, *Microbispora rosea* subsp. *rosea*	Chinese cabbage	*Brassica rapa*	Biocontrol of *Plasmodiophora brassicae*	Lee et al. (2008)
Streptomyces MBR-5, AOK-30	Alpenrose	*Rhododendron ferrugineum*	*Phytophthora cinnamomi*, *Rhizoctonia* sp.	Hasegawa et al. (2006)
Streptomyces sp. EN27 and EN28, *Micromonospora* sp. EN43, *Nocardioides albus* EN46	Arabidopsis	*Arabidopsis thaliana*	Systemic acquired resistance	Conn et al. (2008)
Streptomyces sp. MBCu-56	Cucumber	*Cucumis sativus*	*Colletotrichum orbiculare*	Shimizu et al. (2009)
Micromonospora sp., *Streptomyces* sp., *Actinoplanes* sp.	Lucerne	*Medicago sativa*	N fixation	Solans et al. (2009)
Streptomyces sp.	Neem	*Azadirachta indica*	IAA, siderophore, biocontrol of *Alternaria alternata*	Verma et al. (2011)
Streptomyces sp., *Nonomuraea* sp., *Actinomadura* sp., *Nocardia* sp.	Eaglewood	*Aquilaria malaccensis*	IAA, ammonia	Nimnoi et al. (2010)
S. griseorubiginosus	Banana	*Musa paradisiaca*	Biocontrol of *F. oxysporum* f. sp. *cubense*	Cao et al. (2005)
Streptomyces sp. PT2	Spiderflower	*Cleome arabica*	Biocontrol of *Rhizoctonia solani*	Goudjal et al. (2013)
Streptomyces sp.	Wheat	*Triticum* spp.	P solubilization, IAA, phytase, chitinase, siderophore	Jog et al. (2014)
Streptomyces sp. En-1	Chinese yew	*Taxus chinensis*	IAA	Lin and Xu (2013)
Streptomyces sp., *Nocardia* sp., *Nocardiopsis* sp., *Spirillospora* sp., *Microbispora* sp., *Micromonospora* sp.	Mandarin	*Citrus reticulata*	IAA	Shutsrirung et al. (2013)
Streptomyces sp. BSA25, *Streptomyces* sp. WRA1	Wheat, Faba bean	*Triticum* spp., *Vicia faba*	Siderophore, biocontrol of *Phytophthora medicaginis*	Misk and Franco (2011)
Streptomyces sp.	Maize	*Z. mays*	Biocontrol of *Pythium aphanidermatum*	Costa et al. (2013)

studies are queries lying in line to be solved. These include genomics of endophytes, signaling and dwelling in the same host, nutrient availability and sharing, etc. The diversity of the endophytes is very vast (Klitgord and Segre 2010), and with this nature assessing the common attribute in each and every endophyte is not possible. This complex environment in turn limits the uses of the endophytes. Next is that the use of the endophytes in vitro and in vivo has some limitations. Many metabolites are produced by these endophytes which sometimes are novel compounds also (Yu et al. 2010). These compounds are not the same when produced in vitro condition. High-throughput studies are carried to conduct screening strategies for increased production. In such cases with cultural modifications, the genetic and molecular level modifications are performed. The challenge here is picking out the specific genes that make such modification. Using the endophytes *in planta* is another big challenge where it should address the mechanism of action for protection and PGP which has not developed with higher success rate till date. Overall, isolating the unculturables and identifying them has brought molecular approaches and next-generation sequencing into the field (Draper et al. 2011). Thus, it is expected that many more endophytes will be identified, analyzed, and utilized. The future challenges are dependent on identifying, delineating, dissecting, and defining the mechanisms of the relation they have. A basement-level success in this research which is reached and further answers the above challenges might ensure the present and future successful technological applications of microbial endophytes mainly in growth promotion and in control of plant diseases.

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Omics-Driven Approaches in Plant–Microbe Interaction

4

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Abstract

Plant's life cycle involves interaction with various microorganisms in their environment. Studies are being focused to uncover the molecular components involved in plant–microbe interaction to understand the mechanism of pathogen infection or symbiosis. Recently, research studies in this area focus mainly on environmental issues to provide sustainable agriculture and to increase productivity. In this context, researchers use various high-throughput 'omics' approaches which include genomics, to study the structural and functional aspects of genes and also compare the degree of gene expression in contrasting genotypes, transcriptomics that quantifies mRNA transcripts, proteomics that analyse the protein composition and metabolomics which identifies and quantifies cellular metabolites. In this chapter, we discuss the advancement of 'omics' platforms, in exploring the complex metabolic networks and regulatory mechanisms during plant–microbe relationship. This has significantly improved our understanding about carbohydrate metabolism between legume plants and rhizobacteria. 'Omics' platforms are being used largely in understanding and selecting efficient endophytic or beneficial strains with various improved traits like nutrient uptake, imparting abiotic and biotic stress tolerance during their interaction with host. Comparative studies by large-scale genome analysis of host and pathogen have helped in identification of various effector genes and the nature of pathogenicity induced by pathogen and also the difference in defence mechanisms amongst hosts. Climatic changes that affect the agriculture production and the ever increasing population worldwide are the two challenging factors that need to be balanced cur-

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rently. In order to attain sustainable agriculture production and productivity, 'omics' is a promising tool to understand the plant–microbe interaction that aid in sustainable agriculture.

Keywords
Omics • Bacteria • Endophyte • Plant–microbe interaction • NGS technology

4.1 Introduction

A better understanding of what makes a plant–microbe interaction detrimental or beneficial to plants would provide an important insight into the efficient handling of microbes for agriculture production. This may offer unprecedented opportunities to increase crop productivity. The performance of next-generation sequencing (NGS) technologies continues to improve, whilst costs continue to fall, which enables researchers to conduct whole transcriptome sequencing (RNA-seq) studies of interactions between plants and microbes in many systems (Wang et al. 2009).

4.2 Plants and Pathogens

Plants have evolved themselves in many different shapes and colours and make up majority of the earth's living environment as trees, shrubs, climbers, grasses, creepers and so on. Directly or indirectly, they contribute to make up all the food on which humans and all animals depend, and also whether cultivated or wild, they grow and produce well as long as they are provided with sufficient nutrients, light and temperature within a certain normal range. A normal and healthy plant carries out its physiological functions to the best of its genetic potential where the meristematic cells of a healthy plant divide and differentiate as needed, and different types of specialised cells absorb water and nutrients from the soil, translocate these to all plant parts to carry out the photosynthesis and translocate, metabolise or store the photosynthetic products leading to production of seed or other reproductive organs for survival and multiplication. Like humans, plants also get affected by diseases caused by various microbes and in that state show various types of symptoms in some parts of the plants or whole plants and sometimes ultimately leading to the death of the plants.

Plants are affected by several environmental conditions including biotic and abiotic stresses which undoubtedly play a major role in limiting plant productivity. For example, all crops can be significantly affected by diseases with the potential to reduce both yield and quality, if not kill the crop. Plants in their natural habitats are surrounded by a large number of microorganisms – the disease-causing agents such as viruses, bacteria, fungi, protozoa, nematodes and insects – and unfavourable environmental conditions such as lack or excess of nutrients, moisture and light and the presence of toxic chemicals in air or soil.

Pathogenic microorganisms (pathogens) are those agents that can cause diseases in plants by disturbing the metabolism of plant cells through enzymes, toxins, growth regulators and other substances that they secrete and also by absorbing foodstuffs from the host cells for their own use. Pathogens make their survival by multiplying in the internal tissues like phloem and xylem of plants and blocking the passage of nutrients and water through the tissues. Abiotic factors outside a certain range of tolerance lead to disease in plants. In addition to these, a wide range of microorganisms are beneficial to the plant which include nitrogen-fixing bacteria, endo- and ectomycorrhizal fungi and plant growth-promoting bacteria and fungi. These biocontrol microorganisms may adversely affect the population density, dynamics and metabolic activities of soilborne pathogens via mainly three types of interactions, which are competition, antagonism and hyperparasitism.

In general, plant disease can be summed up as 'Any harmful deviation or alteration from the normal functioning of physiological responses of plant cells and tissues to a pathogenic organism or environmental factor that result in adverse changes in the form, function or integrity of the plant and may lead to partial impairment or death of plant parts or of the entire plant' (Agrios 2005).

4.3 Concept of Disease in Plants

The concept of plant diseases is important because of the loss they cause. When a plant is attacked by a pathogenic organism or an adverse environmental factor, the cells or tissues fail to carry out the physiological functions or alter the activities of the cells that are essential for the plants. At first, the infection is localised to only a few cells and is invisible, and then the reaction spreads widely by which the affected plant parts develop visible changes. These visible changes are nothing but the ultimate symptoms of the disease.

4.4 Types of Plant Diseases

The emergence and existence of plant diseases greatly varies from season to season and also depending on the presence of the pathogen, the environmental conditions and the crops and varieties grown. Some plant varieties are prone to outbreaks of diseases, whilst others are more tolerant to them. Each crop can be affected by many plant diseases and is categorised based on host plant, plant parts affected, symptoms, the cause and its occurrence.

4.5 Plant–Pathogen Interaction

Plants in natural environments establish multiple interactions with many different microorganisms throughout their lifetime. Significance of host–pathogen interactions provides information that can help scientists and researchers understand the disease pathogenesis, the biology of one or many pathogens, as well as the biology of the host. Every organism on earth associates with their neighbours in order to sustain life. This association is facilitated by the chemical substances released and exchanged between the host and the symbionts. In these interactions, plant roots exude chemicals to effectively communicate with others in the rhizosphere. The concept of interaction between plant and pathogen is depicted in Fig. 4.1.

Microbes that live in the rhizosphere are of particular importance because this is where most interactions between plants and microbes occur. Various plant–microbe interactions can be broadly categorised as beneficial, detrimental or neutral. Most microbe effects on plants appear to be neutral, but these microbes may utilise plant-derived organic compounds as substrates for energy production and thus may still play key roles in nutrient cycling and modifying plant environments. Beneficial microbes make their presence everywhere right in the soil to the roots, plant surfaces and also sometimes within the plant tissues, where it's very difficult to differentiate between the beneficial and detrimental microbes. In spite of this, beneficial microbes have a significant role in improved nutrient acquisition, production of growth regulators and biosynthesis of pathogen-inhibiting compounds and thereby improve plants health. The most noticeable because of their detrimental effects are plant pathogenic fungi, oomycetes, bacteria and viruses that can cause diseases on plants. The potential outcome of these plant–microbe interactions is further influenced by abiotic stress factors such as drought, temperature, salinity, soil acidity and water logging.

4.6 How Pathogens Attack Plants

After the emergence of infection, the pathogen will continue its growth and produce spores which will find an exit through the host surface and spread to repeat the same process. Many pathogens spread tremendous distances at a remarkable speed, and then there are pathogens

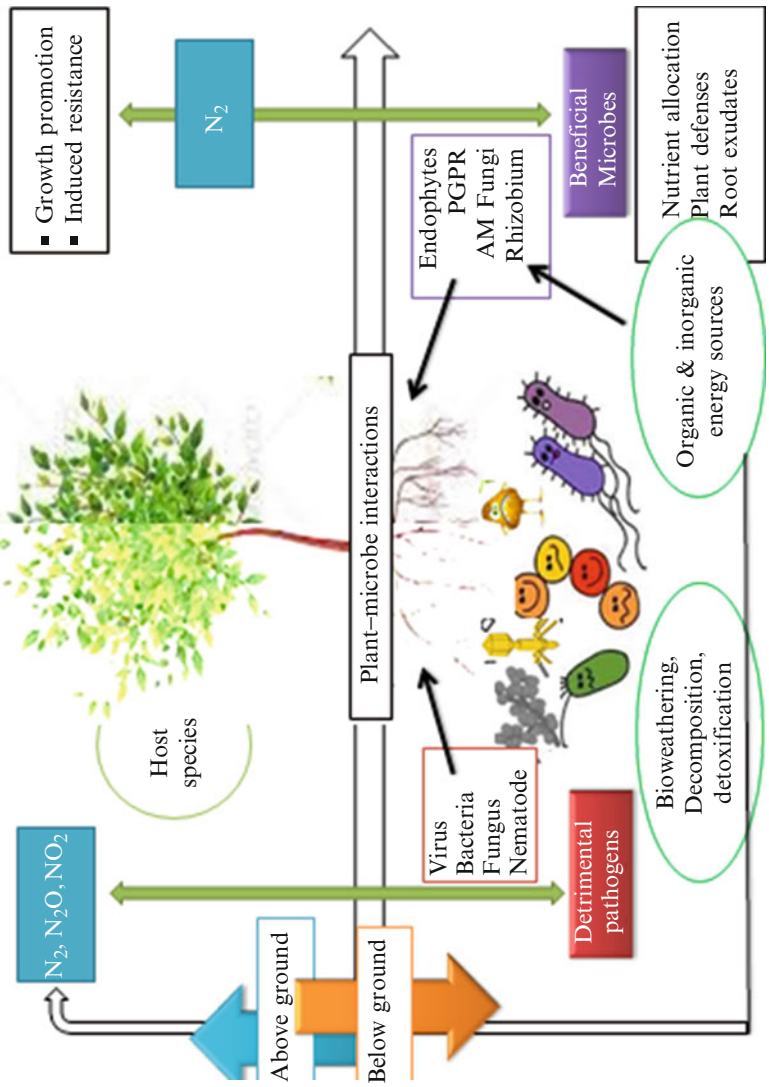

Fig. 4.1 *Plant–microbe interactions.* This model explains the complex network of interactions occurring between the plant roots and microorganisms at the root–soil interface. The beneficial microbes promote plant growth and/or suppress plant diseases via a variety of mechanisms, which include improved nutrient acquisition, production of growth regulators and biosynthesis of pathogen-inhibiting compounds

which depend for their dispersal of spores and propagules on rain or water, whilst others need a vector, like man, insects and nematodes.

4.7 How Plants Defend Themselves against Pathogen

4.7.1 Plant Response to Pathogen Attack

Plants have developed both highly specialised defence responses to prevent and limit disease spread. Many disease responses are activated locally at the site of infection and can spread systemically when a plant is under pathogen attack. Plants respond to pathogen attack by employing a highly coordinated series of molecular, cellular and tissue-based defence mechanisms, and if these mechanisms are activated too little or too late or in the wrong place, then they will fail to restrict the entry of the pathogen, ultimately making the plant susceptible. Pathogens respond by escaping or suppressing plant defence responses or by rendering these responses impotent, for example, by detoxifying the plant antibiotics.

4.7.2 Plant Resistance to Pathogens

Resistance is the ability of a plant variety to restrict the growth and development of a specified pathogen or the damage they cause when compared to susceptible plant varieties under similar environmental conditions and pathogen pressure. In order to protect themselves from damage, plants have developed a wide variety of constitutive and inducible defences. Both of these defence mechanisms ultimately contribute towards protecting the plants from the attack of pathogens either by developing preformed barriers like thickening of cell walls and coating with waxy epidermal cuticles (constitutive defence) or either with detection and set off defence mechanism (inducible defence) by release of toxic chemicals, pathogen-degrading enzymes or leading to cell suicidal.

Based on plants' response to pathogen, the resistance mechanism can be broadly classified as:

Nonspecific resistance – complex defence mechanism involving multiple genes to all the races of particular pathogen

Specific resistance – defence mechanism based on the presence of particular pathogen race with involvement of single gene or small no of related genes and further subdivided into three main categories.

(a) Race-specific resistance

The term race specific itself indicates resistance to particular race of pathogens and not to others, and it is inherited. Basically, this involves the interaction of specific genes of host and pathogen. Race-specific resistance relies on the variations at the gene level which lead to the production of altered proteins and thus result in interaction with specific pathogens only.

(b) Cultivar-specific resistance

In case of cultivar-specific resistance, specific resistance is seen in case of specific host plant and not in specific pathogen. In this resistance mechanism, all races of pathogen are taken into account. Here, the genetic variation is seen only in specific plant species or genes leading to altered proteins and finally altering the outcome of interaction in certain plant species only.

(c) Race–cultivar-specific (gene-for-gene) resistance

Gene-for-gene complementarity occurs most frequently in plant–pathogen interactions which involves both obligate and biotrophic pathogens which are highly specialised and have a narrow host range (Ellingboe 1976; Heath 1981; Keen 1982). If the involvement is from both the pathogen and the host to contribute towards plant disease resistance which is very specific, then the interaction is termed as race–cultivar-specific resistance because it involves the role of both avirulence genes (avr) in the pathogen and resistance gene (R) in the host. In this case, interaction between the receptors of host and elicitors of pathogen

is followed by signal transduction and activation of genes involved in defence mechanism (Dixon and Lamb 1990; Keen and Dawson 1992; Scheel and Parker 1990). The mechanism of recognition is not known yet. Also, the work on the fungal pathogen *C. fulvum*, the causal agent of tomato leaf mould, has provided new insight into the mechanism of induction of hypersensitivity response by fungal avirulence gene products (Van den Ackerveken et al. 1993). A recent study addresses the issue through the use of a model system to understand plant–microbe interactions that exploit *Pseudomonas aeruginosa* strain PA01 and two varieties of sugar beet (*Beta vulgaris* L.), variety (var.) Celt and var. Roberta (Mark et al. 2005).

As a result, a combination of major and minor genes for resistance against a pathogen is the most desirable make-up for any plant variety.

4.8 Control of Plant Diseases

In modern agriculture, both ecological and molecular approaches are being integrated to achieve higher crop yields whilst minimising negative impacts on the environment. The study of the symptoms, causes and mechanisms of development of plant diseases has an extremely useful purpose; it allows for the development of methods to combat plant diseases. Thereby, it increases the yield and improves the quality of plant products available for use.

The various control methods can be classified as regulatory, cultural, biological, physical and chemical depending on the nature of the agents employed. The cultural control methods mostly used aim at helping plants avoid contact with a pathogen, creating environmental conditions unfavourable to the pathogen or avoiding favourable ones and eradicating or reducing the amount of a pathogen. Most biological and some cultural control methods aim at improving the resistance of the host or favouring microorganisms antagonistic to the pathogen, whilst the physical and chemical methods aim at protecting the plants from pathogen inoculum that has arrived/is likely to arrive/curing an infection that is already in progress (Agrios 2005).

4.9 'Omics' Approach in Plant–Microbe Interaction

Integration of molecular profiling technologies in plant developmental biology and plant–microbe interaction has just begun where these parallel profiling technologies probe many genes, transcripts, proteins or metabolites at once and contribute to plant biology. 'Omics'-based approaches have allowed addressing the complex global biological systems that underlie various plant functions. These technological advances have also accelerated the development of genome-scale resources in applied and emerging model plant species and have promoted translational research by integrating knowledge across plant–microbe species. The computer-based annotation and comparative genomic analyses of DNA sequences have provided biologists with information regarding gene function, genome structures, biological pathways, metabolic and regulatory networks and evolution of microbial genomes, which has greatly enhanced our understanding of microbial metabolism (Fig. 4.2).

Genomics, in the form of genome sequences from various organisms, has increased our understanding of gene content, gene function and evolution. diArk (http://www.diark.org), a central hub for all sequenced eukaryotes, reports about 2600 eukaryotes with 6000 genome and transcriptome assemblies (Kollmar et al. 2014).

A transcriptome is a collection of all messenger RNA molecules in a cell. The information gained from transcriptomics can provide a platform for the researchers to gain a better understanding of how genes and pathways are involved in biological processes. Transcriptomics basically focus on DNA sequencing using NGS approaches and also quantitatively measuring the expression of mRNA, and their variations occurred under various stress conditions. As this is the latest and most widely used technology, it has been used to explore the genome-wide tran-

scriptional activities in both plants and microbial communities. The use of this technology has also been employed to study the plant–microbe interactions in addition to microarray.

The deep-sequencing technologies (NGS – next-generation sequencing) deliver large amount of data at faster rate and are inexpensive. Additionally, the progress in bioinformatics approaches, both as hardware and software for the analysis of data, permits us to enhance our knowledge on ever improving management and mining of such large datasets. RNA-seq, a recently developed approach for transcriptome profiling by means of deep-sequencing technologies, provides a precise measurement of the level of gene transcripts and their isoforms than other methods. A major advantage of next-generation sequencing over the traditional sequencing method is that it dramatically increased the degree of parallelism which can be represented by the number of reads in a single sequencing run and the number of sequenced bases per day.

Proteomics is the study of proteins including protein abundances, their modifications and binding nature with interacting partners. It has revolutionised agricultural and clinical research fields. Various techniques that are being applied to promote our understanding of proteins are gel-based techniques like two-dimensional gel electrophoresis (2DE) and fluorescent two-dimensional 'difference gel electrophoresis' (2DDIGE) and gel-free techniques like isotope-coded affinity tags (ICAT), isobaric tagged for relative and absolute quantitation (iTRAQ), multidimensional protein identification technology (MudPIT) and the widely used primary tool mass spectrophotometry (MS) and MALDI-TOF. High sensitivity is the advantage of MS. Genomics provides only the protein sequence and transcript level but fails to reveal the post-translational modifications. An

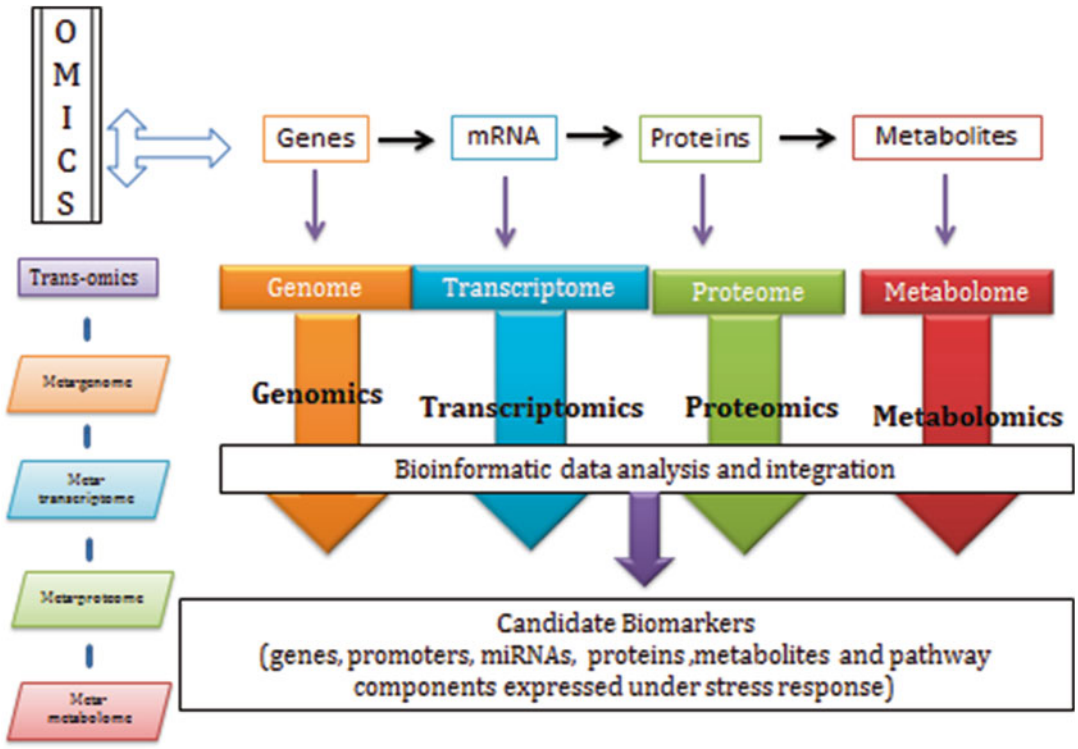

Fig. 4.2 *Omics approach in plant–microbe interactions.* This model explains the use of an integrated approach to understand the degree and complexity of plant–microbe interactions through the application of modern 'omics' technologies. These approaches would produce more information about microbial responses to plant signals and contribution of specific gene products to the establishment of integration with the host

mRNA produced may be translated inefficiently or degraded rapidly, resulting in a change in amount of protein synthesised. Hence, transcript profile may not give the complete picture on cellular regulations. This has triggered broad protein-focused research to assess the plant–microbe-associated proteins to apply in the crop improvement programmes. Metabolomics generates in-depth information on metabolites, which are the end products of cellular metabolism often combining with other 'omics' (Saito and Matsuda 2010). Different techniques being applied to study metabolic profile are nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC) and the combination of chromatography with MS that helps to detect more number of complex compounds. Widely used methods are combinations of gas chromatography (GC)–MS and liquid chromatography (LC)–MS.

A functional analysis pipeline would be useful in identifying not only the functions of individual genes and RNA molecules but also proteins and metabolites during plant–microbe interactions. Datasets from diverse studies like genomics, transcriptomics, proteomics and metabolomics need to be combined using bioinformatics and statistical tools that will help to identify and integrate key biological processes as well as make predictions through modelling.

4.10 Plant–Microbe Beneficial Interaction

Plants are continuously being challenged by the world around them as they are involved in a complex network of interactions with microorganisms where some of those are beneficial whilst others are detrimental. There are several types of plant–microbe interactions: competition, commensalism, mutualism and parasitism. Beneficial interactions are caused by symbiotic or non-symbiotic bacteria and by a highly specialised type of fungi, the mycorrhizae. A symbiotic lifestyle where the two or more different species live together is a widely existing process in nature. This interaction between plant and different microorganisms like bacteria and fungi, which has known as typically beneficial for both, appeals attention from researchers because of its potential application in agriculture. The interaction between plant and various organisms like plant growth-promoting rhizobacteria (PGPR), *Pseudomonas*, bacilli, *Trichoderma*, diazotrophs, arbuscular mycorrhizal fungi (AMF), phosphate-solubilising fungi and bacteria and cellulose-degrading bacteria and fungi are broadly studied. During the interaction, the nature of the benefit varies where focusing the benefit to plants, the microbes promote plant growth by increasing nitrogen fixation in legumes; improving the supply of nutrients like phosphorus, sulphur, iron and copper, plant hormone production controlling fungal and bacterial diseases; and helping in bioremediation of contaminated soils.

New biotechnological methods for crop protection are based on the use of beneficial microorganisms applied as biofertilisers and/or biocontrol agents; this approach represents an important tool for plant disease control and could lead to a substantial reduction of chemical fertiliser use, which is an important source of environmental pollution. Microbial inoculants, some of which have a historical record for safe use, are being widely applied in modern agriculture as biofertilisers and biocontrol agents. Linking plant phenotype to gene and protein expression and also to metabolite synthesis and accumulation is one of the main challenges for improving agricultural production worldwide. Here, the recent contribution of 'omics'-based studies on plant–microbe relationship is presented.

4.10.1 Nitrogen Fixation

Nitrogen fixation is the natural form of fertilisation, and research on this topic provides successful path to a sustainable agriculture. A few recent studies that are focused on this subject to further understand the nitrogen-fixing mechanism are highlighted below. Miche et al. (2006) by transcriptome analysis have investigated the obligate nitrogen-fixing endophyte *Azoarcus* sp. strain

BH72, which expresses nitrogenase (*nif*) genes inside rice roots.

Proteomics as well as metabolomics is an ideal platform to examine the symbiotic interaction between root nodules and nitrogen-fixing bacteria, which has been analysed vastly, and it provides broad spectrum of proteins/metabolites secreted by both the partners. In the first phase of plant–microbe interaction (symbiotic relation), both the partners secrete signals into soil, various secondary metabolites like flavonoids, phenolic acids, isoprenoids and alkaloids which are perceived by the roots and microbial receptors inducing subsequent physiological as well as morphological changes. The studies of these signal molecules and the changes aid in important role in agricultural applications. The best studied symbiotic relationship is nitrogen fixation which involves efficient signalling of both the partners, and flavonoids are the primary coordination signal and the well-studied metabolites that are induced by host plants which act as chemoattractants (Peters and Vermas 1990). They activate expression of rhizobial nod genes. Many studies are focused on the role of flavonoids in nodulation process (Schmidt et al. 1994; Peck et al. 2006; Zhang et al. 2009).

The mass spectrometry-based proteomic profile of *G. diazotrophicus*–sugarcane interaction was studied by Lery et al. (2011). The SP70-1143 genotype of sugarcane which contributes high nitrogen fixation showed overexpression of signal cascade proteins. Also, they identified presence of glutamate ammonia lyase in SP70-1143 plants grown with *G. diazotrophicus* indicating the efficiency of nitrogen metabolism, and nine bacterial proteins which are induced by plant signals were also identified in the roots. Van Noorden et al. (2007) have identified 131 proteins in *M. truncatula* in nodule formation during interaction with *Sinorhizobium meliloti*. They also reported that auxin treatment induced many redox-related proteins, isoflavone reductase, a late-embryogenesis-like, etc., during nodule formation. A review on proteomic approaches written by Salavati et al. (2013) gives insights on plant–bacteria symbiosis during root nodule formation.

Recently, distribution of metabolites in root nodules and roots of *Medicago truncatula* during nitrogen fixation by association with *Sinorhizobium meliloti* has been studied by employing MALDI/mass spectrometric imaging (MSI). The difference in metabolite profile between roots and nodules and also between nitrogen-fixing and non-fixing nodules was studied. Various amino acids, organic acids, sugars and flavonoids were detected (Ye et al. 2013). Furthermore, during metabolite profiling of extracts of *Medicago truncatula*, it was identified that within the first hour of in planta nod factor treatment, suppression of one metabolite resembling oxylipin was observed. Both oxylipin metabolite and jasmonic acid inhibited the nod factor signalling (Zhang et al. 2012). 2610 metabolites in nitrogen-fixing bacterium (*Bradyrhizobium japonicum*) inoculated root hairs of soya bean were identified by using GC–MS/UPLC. 166 metabolites were significantly regulated, and trehalose was found to be induced strongly (Brechenmacher et al. 2010).

During symbiosis, alteration of proteins is studied in both the partners, which depicts the clear picture of its mechanism. *Rhizobium tropici* strain PRF 81 owes to significant nitrogen-fixing efficiency and is being used as commercial inoculants for application to common bean. As it also exhibits thermotolerance, Gomes et al. (2012) have attempted to examine the proteins responsible to heat tolerance, and they identified upregulation of molecular chaperones and many oxidative stress-responsive proteins. Another strain CPAC 7 belonging to new species *Bradyrhizobium diazoefficiens* is well known for its efficiency in nitrogen fixation and is being used in soya bean commercial inoculants. Its protein profile under free-living conditions carried by Gomes et al. (2014) showed the expression of proteins, namely, inositol monophosphatase, *NifH* and chaperones and other unknown proteins that play a role in symbiosis with soya bean. Recent study on proteomes of *Bradyrhizobium* sp. ORS278 symbiosis with *Aeschynomene indica* revealed the requirement of *fixA* locus in symbiotic efficiency (Delmotte et al. 2014). Different environmental stress leads to the loss of

nitrogen-fixing efficiency; hence, studies described above for screening strains capable of efficient nitrogen fixation under various stress conditions help in evolving a sustainable agriculture system. Particular rhizobial strains nodulate only specific legumes but not with others. What is the reason that all plants cannot form a symbiosis with rhizobia? In spite of vast research on rhizobial symbiosis, there is no clear information to answer this question.

4.10.2 Growth Promotion

Pseudomonas fluorescens helps to promote growth in rice. By MS analysis, *Pseudomonas fluorescens* strain KH-1 was found to induce proteins, namely, thioredoxin h, p23 co-chaperone, glutathione S-transferase protein, ribulose-bisphosphate carboxylase, etc. (Kandasamy et al. 2009). This increase in photosynthetic proteins upon inoculation with *Sinorhizobium meliloti* contributes to growth promotion in rice, which was identified by gel-based proteomic approach (Chi et al. 2010). Transcriptional profiling study on arbuscular mycorrhiza and *Petunia hybrida* interaction revealed a new role for phosphate P(i) in repressing essential symbiotic genes in the host. This finding has important implications in managing the levels of P(i) under field conditions to maximise plant growth and yield by taking advantage of both P supply and the beneficial effect of the symbiosis.

Secondary metabolites help in the growth and development of plants which are also involved in tolerance to environmental stresses. Changes in the secondary metabolites and enhanced growth of host plants in interaction with different beneficial microbes have been studied recently. Metabolite analysis of mycorrhizal and nonmycorrhizal roots of *Medicago truncatula* revealed the increase in the amount of particular amino acids (Glu, Asp, Asn), fatty acids (palmitic and oleic acids), isoflavonoids and accumulation of apocarotenoids, and cell wall-bound tyrosol exclusively in *AM* roots was found. This study shows the difference in a secondary metabolism in normal development and in symbiosis-dependent changes in *M. truncatula* (Schliemann et al. 2008). Increase in concentration of bioactive primary and secondary metabolites like flavonoids, phenols and total tannins in cebil (*Anadenanthera colubrina*) seedlings was also enhanced by mycorrhizal inoculation (Pedone-Bonfim et al. 2013).

Secondary metabolite analysis in roots of *Lotus japonicus* in a symbiotic interaction with *Mesorhizobium loti* resulted in the changes in 14 phenolic acids compared to non-inoculated plants (Rispail et al. 2010). Change in root phenolics was also studied in actinobacterium *Frankia* interaction with *Myricaceae* plant species by HPLC analysis (Popovici et al. 2011). Hence, these studies designate the changes and adaptation or regulation in the secondary metabolism of host plants based on the specific strains. Also, secretion and changes in some fatty acids and flavonoids in microbial community were observed.

4.10.3 Bioremediation

Bioremediation by microbes is another hot topic in agriculture, which is the process to mitigate adverse environmental conditions. Heavy metal contamination is the major problem which has an adverse effect on human health and agriculture. Plant growth-promoting bacteria (PGPB) and AM are found to be involved in phytoremediation of heavy metal-contaminated soil. By MS analysis, Cheng et al. (2009) have investigated the response of bacterium *Pseudomonas putida* UW4 to nickel stress and identified the involvement of several mechanisms including anti-oxidative stress, general stress adaptation and heavy metal efflux proteins. Arsenic-treated arbuscular mycorrhiza (AM) fungi (*Glomus mosseae* or *Gigaspora margarita*) on colonisation with *Pteris vittata* induced the expression of 130 leaf proteins. In this study, the main role of glycolytic enzymes and arsenic transporter PgPOR29 in arsenic metabolism has been identified (Bona et al. 2010).

2-DE/MALDI-TOF-based comparative proteomic analysis revealed that the alleviation of Cd toxicity by *Medicago truncatula* shoots on

mycorrhizal exposure was assisted by the increase in photosynthetic proteins and chaperones (Aloui et al. 2011). Significant reduction in metal pollution (zinc and copper concentrations) in mycorrhizal-inoculated poplar plants describing the phytoremediation character of mycorrhiza was reported by Lingua et al. (2012). By 2DE and MS, they showed that after 16 months growth on metal-polluted soil, mycorrhizal plants resulted in the upregulation of the RuBisCO large subunit, Hsp70, a small Hsp and downregulation of 43 spots majorly related to carbohydrate metabolism and oxidative stress. A study conducted by Farinati et al. (2011) showed a reduction in Zn and Cd concentration and modulation of the shoot proteome of *Arabidopsis halleri* plants grown in metals with bacterial strains (isolated from contaminated *A. Halleri* rhizospheric soil). This concludes that screening strains for multiple roles help in the application of biocontrol agents with multiple benefits.

4.10.4 Biocontrol

Biological strategies are being integrated widely to control disease in agriculture. Here, the microbial community, bacteria and fungi colonise the roots of the plants but do not harm the host, and its interaction leads to the activation of defence mechanisms. This strategy is being utilised commercially in concern with the environmental pollution as biopesticides or biofertilisers.

There are several other reports on transcriptional profiling of plants or beneficial microbes such as *Pseudomonas* spp. under laboratory conditions. For instance, substantial differences in transcript levels were found in *Arabidopsis* roots during *Pseudomonas fluorescence* WCS417r-mediated ISR, as well as in shoots inoculated with the leaf pathogen *Pseudomonas syringae* pv. tomato DC3000. Mark et al. (2005) examined the influence on the *Pseudomonas aeruginosa* PA01 transcriptome of exudates from two varieties of sugar beet that select for genetically distinct *Pseudomonas* populations in the rhizosphere. Homologues of the genes identified in the genomes of both beneficial and pathogenic root-associated bacteria suggest that this strategy may help to elucidate molecular interactions that are important for biocontrol, plant growth promotion and plant pathogenesis. Recent transcriptome analyses of interactions such as *Trichoderma* (beneficial fungi)–*Arabidopsis* (host)–*Pseudomonas syringae* (pathogen) and *Piriformospora indica* (beneficial fungi)–barley (host)–*Blumeria graminis* f. sp. *hordei* (pathogen) have supported the hypothesis that these beneficial fungi have little effect on host gene expression profiles in the absence of pathogens. A study has been conducted (Franks et al. 2006) on molecular approaches for the isolation and characterisation of bacterial endophytes and plant-associated bacteria and communities. Microbial communities inhabiting stems, roots and tubers of various varieties of plants were analysed by 16SrRNA gene-based techniques such as terminal restriction fragment length polymorphism analysis, denaturing gradient gel electrophoresis as well as 16S rRNA gene cloning and sequencing.

In general, studies on biocontrol of plant diseases are being flooded, but application of proteomics to explore the mechanism of microbial control or to study the changes in plant proteome is still lacking. Some *Trichoderma* strains are found to have direct impact on plants by inhibiting plant pathogens through their antagonistic and mycoparasitic effects (Viterbo and Horwitz 2010). Proteomic analysis of *Trichoderma harzianum* T39 (T39) interaction with grapevine downy mildew using iTRAQ carried by Palmieri et al. (2012) showed that R-induced resistance involves the changes in proteins associated with stress and redox and also the accumulation of ROS and formation of callose at infection sites, suggesting an active defence response against the disease. Endochitinase, pathogenesis-related protein PRMS (pathogenesis-related maize seed), GTP-binding protein, isoflavone reductase and other proteins related to respiration were found to be induced by biocontrol agent *Trichoderma harzianum Rifai* in maize seedlings infected by *Pythium ultimum* which causes damping off (Chen et al. 2005). 2DE protein profiling and MS analysis of the roots of cucumber plants on interaction with *Trichoderma asperellum* strain T34

revealed expression of 28 proteins after colonisation. Proteins involved in ROS scavenging, photorespiration, stress response, etc. were differentially expressed, where this study helps in understanding the nature of *Trichoderma*-treated plants resist the pathogen attacks (Segarra et al. 2007).

Through proteomics and phenotypic analysis, Klaponski et al. (2014) have identified that the *Pseudomonas chlororaphis* strain PA23 needs a LysR-type transcriptional regulator (LTTR), designated as ptrA (*Pseudomonas* transcriptional regulator) for its biocontrol action, where the mutant was not capable of inhibiting fungal growth. In beans, proteome study during interaction with any of the two pathogens (*Botrytis cinerea*, *Rhizoctonia solani*) and/or *T. atroviride*, many disease-related factors and pathogenesis-related proteins were identified (Marra et al. 2006). The biocontrol agent *Pseudomonas chlororaphis* strain PA23 is able to suppress the fungal pathogen *Sclerotinia sclerotiorum* (Savchuk and Fernando 2004), by producing antibiotics phenazine and pyrrolnitrin with other components. Few other studies have also given the protein profile of host plant on interaction with beneficial bacteria (Keyung-Jo et al. 2007; Kierul et al. 2015). To investigate the biocontrol mechanism or repressive action of *Bacillus* strains EU07 and FZB24 on *Fusarium oxysporum*, proteomic analysis was done by Baysal et al. (2013) and identified the presence of lytic enzymes, 1,4-beta-glucanase, proteases and cellulases that digest the fungal cell wall. Further, it was found that proteins that function in protein degradation, protein folding, recognition and signal transduction network play a significant role in the inhibition of *Fusarium oxysporum*.

Metabolomics approaches to investigate the biocontrol action of microbes which protect plants from serious diseases. Gene expression and metabolite analysis revealed induction of systemic resistance to powdery mildew caused by *Blumeria graminis* f. sp. *hordei* (mycorrhiza) in *Piriformospora indica* barley. Along with the increase in pathogenesis-related genes, metabolic shift from carbohydrate metabolism to increase in sucrose biosynthesis was analysed in resistant plants which were colonised by *mycorrhiza* (Molitor et al. 2011). Biocontrol activity of *Trichoderma* that isolates against *Fusarium* wilt on melon plants was analysed. By employing HPLC–MS analysis, the disease inhibition action was found to be related to changes in the level of the hormones ethylene, abscisic acid and cytokinin transzeatin riboside (Martinez-Medina et al. 2014).

Spectroscopic and 2D NMR analysis has allowed to identify the structure of new metabolite isoharzianic acid (iso-HA), from the culture filtrate of the *T. harzianum*. Mycelial growth of *Sclerotinia sclerotiorum* and *Rhizoctonia solani* was inhibited by the in vitro application of iso-HA and also enhanced the germination of tomato seeds and improved disease resistance (Vinale et al. 2014). Many peptaibols which are antibiotic peptides have been discovered in *Trichoderma*. Peptaibols have attracted great attention as they act as potential inhibitors of pathogen growth. This quality of *Trichoderma* is being used to utilise it as a biocontrol agent. Peptaibol profile of the *Trichoderma asperellum* TR356 strain, which is an efficient biocontrol agent of *Sclerotinia sclerotiorum* and structural elucidation of few asperelines and trichotoxins, was carried out recently (Brito et al. 2014). GC–MS analysis revealed alterations in 11 poplar plant metabolites on interaction with endophytic *Paenibacillus* strain. Downregulation of amino acids and sugars and an increase in urea and asparagine accumulation indicating efficient nitrogen fixation in the mutualistic relationship were observed (Scherling et al. 2009). Root profile analysis of corn inoculated by the *rhizobacterium*, *Azospirillum brasilense*, was studied by infecting with *D. speciosa* larvae. The alteration of host selection by larvae towards non-inoculated versus inoculated due to the higher emission of (E)-β-caryophyllene (sesquiterpene) in rhizobacterium-inoculated corn was identified. The study suggested the use of *A. brasilense* in integrative pest management programme of corn protection (Santos et al. 2014). The results of recent studies promise that identification and analysis of new metabolites help to select the new beneficial strains and thus effectively help in inhibition of new pathogens.

4.10.5 Abiotic Stress Tolerance

Efforts are being made currently to screen microbial community for their efficiency in helping plants in various abiotic stress tolerance like heat, drought, salinity, etc. simultaneously in their application in biocontrol. The study conducted by Cho et al. (2013) by microarray analysis showed that colonisation of *Pseudomonas chlororaphis* O6 induced the expression of drought-responsive genes along with stimulation of systemic defence response genes in *Arabidopsis* resulting in drought-tolerant phenotypes. Similarly, the *Gluconacetobacter diazotrophicus* PAL5-inoculated sugarcane plants showed better tolerance for drought stress than the non-inoculated plants. By RNA-seq analysis, the activation of transcription factors that involve in ABA-dependent signalling was found in shoots suggesting that these factors may act as key elements in drought tolerance mechanism (Vargas et al. 2014). Also *rhizobacterium*, *P. fluorescens* MSP-393, was identified to involve in salt-stress tolerance. Increased salt stress alters the expression of glutamic acid, acyl carrier protein, ABC transporter, tryptophan synthase, 60 kDa chaperonin, etc. This was analysed by 2DE and MALDI-TOF. They are also a good biocontrol agent against bacterial blight disease of rice (Diby Paul et al. 2006). Enhanced secondary metabolites leading to alleviation of low temperature stress and increased growth were observed in *arbuscular mycorrhiza* (AM)-treated cucumber seedlings than non-AM plants (Chen et al. 2013).

4.11 Plant–Pathogen Interaction

Plants have evolved with a sophisticated defence response against microbes. When a microbe challenges a plant, coevolutionary multilayer defence strategies get triggered in the plants as well as in the microbes. The strategy used by the plants basically depends on the resources needed to mount the defence. Basically induced response requires lesser resources than the constitutive response, but often the environmental conditions prompt plants to take up the constitutive response. In general, the ultimate outcome of plant–microbe interactions is governed by the host and microbe genotypes and the environmental conditions.

The pathogenic or detrimental interaction of microbes with plants has viruses, bacteria and fungi and leads to infectious diseases affecting only the plant kingdom. An advantage of studying these interactions helps us to understand natural phenomena that affect our daily lives and could lead to applications resulting in sustainable resources, less impact on the environment, clean-up of pollution and influence on atmospheric gases on a global scale.

Plant defence mechanisms are characterised by a combination of constitutive and inducible responses. Both the defence responses are exhibited in a different mode of action where the constitutive responses generally consist of barriers and biochemical defences, whilst the inducible responses are either localised or systemic in nature but proceed with a systematic mode of action right from pathogen recognition till the expression of defence genes. The general barriers or pre-existing biochemical defences involve the recognition of the pathogen by the host plant, signal transduction and expression of several genes. In a localised response, plant tissues react against pathogens by a type of programmed cell death, whilst in systemic defence, a signal spreads from the site of interaction, and the signal is mediated by several molecules which function as messengers in plants, for example, salicylic and jasmonic acid or even volatiles such as nitric oxide and ethylene (Baker et al. 1997). The messenger molecules are considered to be very important in bringing about the activation of pathogenesis (PR)-related gene expression, and the products of these genes are the enzymes which are basically involved in the secondary metabolism and production of phenolic compounds or phytoalexins, for example, peroxidases, lipo-oxygenases, superoxide dismutases and phenylalanine ammonia lyase (PAL).

Transcriptomics, the advanced and frequently used omics platform, has contributed a major role in understanding the concept of plant fungal plant diseases. This platform provides us with an

enhanced expression profiling data for various stresses under in vitro conditions. In some studies, this approach has been used to study pathogenicity, defence genes and protein in various crops (Mehta et al. 2008). Recent studies that have employed 'omics' approaches to study plant–pathogen interaction are listed in Table 4.1.

Transcriptome analysis of both interacting partners, rice and blast fungus, in the infected plant tissue was studied by Kawahara et al. (2012). Two hundred and forty transcripts of fungus encoding secreted proteins like cutinases, glycosyl hydrolases and LysM domain-containing proteins that may act as effector genes in causing initial infection processes were identified. In rice, phytoalexin biosynthetic genes and pathogenesis-related proteins were upregulated. Similarly, transcriptome characterisation of pea–*Sclerotinia sclerotiorum* interaction revealed 142 ESTs encoding secretory peptides and 93 ESTs to be involved in virulence of *S. sclerotiorum* and 277 pea ESTs that play a role against biotic and abiotic stresses (Zhuang et al. 2012).

During lettuce–*B. cinerea* interaction, upregulation of the large number of phenylpropanoid and terpenoid biosynthesis pathway genes and downregulation of photosynthetic genes were observed in lettuce, at 48 h post inoculation (De Cremer et al. 2013). Genes involved in energy metabolism and in redox mechanism, particularly transcripts encoding glutathione S-transferase (GST), were accumulated significantly in *Arabidopsis* during its interaction with *Botryosphaeria dothidea* pathogen that causes blister canker (Liao et al. 2014). Illumina sequencing platform was used for de novo assembly of the *Pyrenochaeta lycopersici* genome. Functional characterisation by integrating RNA-seq data was carried to analyse effectors and virulence mechanisms of the pathogen (Aragona et al. 2014). In another study, a high-quality genome assembly of *R. solani* AG8 causative agent of the bare patch of wheat, barley and legume species had a set of 13,964 genes supported by RNA-seq, where the whole genomes of AG8, the rice pathogen AG1-IA and the potato pathogen AG3 were observed to be systemic and colinear. A comparative study for pathogenicity genes amongst the pathogens (AG8, AG1-1A and AG3) was done to focus genes and functions which were unique to *R. solani* anastomosis group (Hane et al. 2014). List of genomes of plant pathogens that are sequenced by NGS is given in Table 4.2.

For several years, proteomics approaches are being utilised to study plant–pathogen interaction and played a significant role in identifying proteins and their changes upon infection by pathogens. A few recent studies are highlighted in this chapter. *Rph15* gene that confers resistance to over 350 isolates of fungal pathogen *Puccinia hordei* which causes leaf rust foliar disease in barley is of significant interest in resistance breeding. Protein profile of resistant and susceptible near-isogenic lines was studied using LC/MS/MS analysis to investigate the *Rph15*-based defence response by Bernardo et al. (2012). Here, many pathogen-responsive proteins were identified in *Rph15* resistant line at 4dpi; proteins that are involved in carbohydrate metabolism, photosynthesis, protein degradation and defence were also identified. Proteome study by 2D gel analysis of the *Flavescence doree* (grapevine disease caused by phytoplasma)-affected grapevine identified 48 proteins that were differentially expressed. Proteins like glutathione S-transferase and isocitrate dehydrogenase that play an antioxidant role were increased in infected plants (Margaria et al. 2013). Many studies are there on the proteomic approaches to unravel the plant response to fungal pathogens (Bregar et al. 2012; El Hadrami et al. 2012; Vincent et al. 2012; Yang et al. 2013).

Proteomic profile of *Vigna mungo* on the interaction with mung bean yellow mosaic virus showed 109 differentially expressed proteins. It was found that photosystem II electron transports were the major targets during pathogenesis and the incidence of downregulation of photosynthetic proteins in susceptible genotypes (Kundu et al. 2013). Tomato infection by *Pseudomonas syringae* pv. tomato DC3000 (*Pst*) that causes bacterial speck disease was studied by iTRAQ proteomic approaches and identified 2,369 proteins in tomato leaves, where 477 were *Pst* responsive. PR1, glutamate dehydrogenase,

Table 4.1 Few recent studies that applied 'omics' approaches to study plant–pathogen interaction

Host	Pathogen	Reference
Transcriptomics		
Oryza sativa	*Magnaporthe oryzae*	Kawahara et al. (2012)
		Mosquera et al. (2009)
Pisum sativum	*Sclerotinia sclerotiorum*	Zhuang et al. (2012)
Lactuca sativa	*Botrytis cinerea*	De Cremer et al. (2013)
Brassica napus	*Leptosphaeria maculans* 'brassicae'	Lowe et al. (2014)
Arabidopsis thaliana	*Hyaloperonospora arabidopsidis*	Asai et al. (2014)
Populus tomentosa	*Botryosphaeria dothidea*	Liao et al. (2014)
Solanum lycopersicum	*Phytophthora capsici*	Jupe et al. (2013)
Solanum tuberosum	*Phytophthora infestans*	Gyetvai et al. (2012)
		Gao et al. (2013)
Musa acuminata	*Mycosphaerella musicola*	Passos et al. (2013)
Triticum	*Blumeria graminis* f. sp. *tritici*	Xin et al. (2012)
Proteomics		
Hordeum vulgare	*Puccinia hordei*	Bernardo et al. (2012)
Vitis vinifera	*Candidatus Phytoplasma vitis*	Margaria et al. (2013)
Vigna mungo	Mung bean yellow mosaic virus	Kundu et al. (2013)
Solanum lycopersicum	*Pseudomonas syringae* pv. tomato	Parker et al. (2013)
Musa acuminata	*Fusarium oxysporum* f. sp. *cubense*	Li et al. (2013)
Actinidia chinensis	*Pseudomonas syringae* pv. *actinidiae*	Petriccione et al. (2013)
Metabolomics		
Hordeum vulgare	*Fusarium graminearum*	Kumaraswamy et al. (2011)
Oryza sativa	*Magnaporthe grisea*	Jones et al. (2011)
Asparagus officinalis	*Fusarium proliferatum*	Waskiewicz et al. (2013)
Vitis vinifera	*Botrytis cinerea*	Hong et al. (2012)
Solanum tuberosum	*Phytophthora infestans*	Yogendra et al. (2014)
Nicotiana tabacum	*Phytophthora nicotianae*	Ibanez et al. (2010)
Glycine max	*Rhizoctonia solani*	Aliferis et al. (2014)
Nicotiana benthamiana	*Pseudomonas syringae* pv. *tabaci*	Lee et al. (2013)
Arabidopsis thaliana	*Pseudomonas syringae* pv. tomato	Allwood et al. (2010)
Glycine max	*Fusarium tucumaniae*	Scandiani et al. (2015)
Triticum	*Fusarium graminearum*	Pasquet et al. (2014)

redox proteins like thioredoxin, glutathione S-transferase and superoxide dismutase were the major proteins upregulated (Parker et al. 2013). There are many review articles on proteomic applications to study plant–pathogen interaction (Abdin et al. 2013; Afroz et al. 2013; Delaunois et al. 2014; Zimaro et al. 2011). Thirty-eight differentially expressed proteins were identified in banana on *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (*Foc*4) infection, and PR proteins, antifungal protein synthesis and cell wall strengthening-related proteins were mainly involved in defence in resistant genotype (Li et al. 2013). It was analysed that proteins that involve in ROS scavenging, PCD and photosynthesis are found to be greatly affected. Hence, broad-spectrum screening and analysis of these proteins in different genotypes and strains help to select the resistant plants as well as beneficial strains to be utilised in future agriculture.

Discovering the key metabolites that are induced in both plants as well as pathogens during their interaction is the focus of recent metabolic studies. Using metabolomics tool, resistance-related metabolites were identified in resistance barley genotypes against *Fusarium*

Table 4.2 List of plant pathogens whose genomes are sequenced using next-generation sequencing technology

Sl. no	Pathogen	Genome size (Mb)	Reference
1	*Phytophthora ramorum*	65	Tyler et al. (2006)
2	*Phytophthora sojae*	95	Tyler et al. (2006)
3	*Marssonina brunnea*	52	Zhu et al. (2012)
4	*Magnaporthe oryzae*	40.9791	Kim et al. (2014)
5	*F.oxysporum (Foc1)*	47.838	Guo et al. (2014a)
6	*F.oxysporum (Foc4)*	53.111	Guo et al. (2014b)

head blight caused by *Fusarium graminearum*. The identified metabolites which can be used as potential biomarkers are p-Coumaric acid, phenylalanine, jasmonate, linoleic acid and deoxynivalenol-3-O-glucoside (D3G) (Kumaraswamy et al. 2011). The metabolic alteration of rice upon infection with compatible and incompatible *Magnaporthe grisea* strains that cause rice blast was studied using NMR and GC/LC–MS. Along with metabolites, namely, malate, glutamine, cinnamate and proline, alanine was found to be increased to a greater extent during compatible interaction than the resistant lines (Jones et al. 2011). Through HPLC analysis, production of mycotoxins by *Fusarium oxysporum* or *F. proliferatum* on infecting *Asparagus officinalis* L was identified, along with an increased level of SA and free radicals in the host (Waskiewicz et al. 2013).

Metabolite alterations associated with *Botrytis cinerea* infection in grape were studied by NMR spectroscopy. The presence of flavonoid and phenolic compounds and sucrose including succinate, gluconic acid and glycerol which were significantly produced only in infected berries was suggested to be associated with the grape defence system (Hong et al. 2012). Furthermore, in potato, following infection by *Phytophthora infestans*, flavonoids, phenylpropanoids and alkaloids was largely induced in resistant than susceptible genotype (Yogendra et al. 2014). Changes in alkaloids, phenols, oxylipins and carbohydrates as *Nicotiana tabacum* defence response against *Phytophthora nicotianae* were studied by Ibanez et al. (2010). *Rhizoctonia solani* infection of soya bean resulted in changes in components that help in antioxidant mechanism like flavonoids, phytoalexins, coumarins and few hormones likely to counter play the pathogen invasion (Aliferis et al. 2014). Employing UPLC-qTOF-MS, 49 extracellular metabolites were identified from *Pseudomonas syringae* pv. *tabaci* (Pstab) extracts, which suppressed the defence response like stomatal closure and HR cell death induced by non-host bacterial pathogen *Pst* T1 in *N. benthamiana* (Lee et al. 2013).

The reports on plant–pathogen interaction show that the significant changes appear in metabolites that help in antioxidant mechanism flavonoids and phytoalexins along with phenylpropanoid metabolism. Further investigation of individual metabolites in specific host–pathogen interaction helps to develop more sustainable biochemical markers. Comparative studies by large-scale genome analysis of the host and the pathogen utilising 'omics' approaches have been done. This helps to understand the difference in various host defence response and pathogenicity nature in pathogen by revealing the difference in effector secretion or other virulent genes/proteins secreted during interaction.

But the evolutionary arms race, where the coevolution of plant pathogens with the evolution of plant defence responses resulted in the modification of pathogen virulence strategies and plant defence mechanism, occurred by genetic drift. Other mechanisms that lead to the evolution of new pathogen lineages or species are mutations, sexual recombination, lateral gene transfer, whole genome exchange and chromosomal instability (Anderson et al. 2010). Chitin is the good example of evolutionary arms race (Malinovsky et al. 2014). Ma et al. (2010) by comparative genomics approach demonstrated that transfer of lineage-specific (LS) chromosome 14 between strains of *F. oxysporum* converts a non-pathogenic strain into a pathogen. Reports on outbreaks of new diseases compel immediate action of researchers to understand the plant–pathogen

interactions to identify new strategies to avoid the disease. Hence, the advanced genomic tools could be the best option to study the intricate plant–pathogen interaction.

4.12 Metagenomics

Metagenomics which allows microbial community profiling based on DNA directly extracted from an environmental sample has led to the discovery of new species, genomes, genes and new molecules with potential applications in agriculture and other fields.

Metatranscriptomics is an emerging field that focuses on characterising patterns of gene expression displayed by microbial communities by sequencing of expressed genes. This approach leads to the discovery of potentially interesting (yet unknown) plant–microbe relationships. Study of samples collected from different environment helps to reveal the diversity of microbes and the nature of plant–microbe interactions to isolate plant growth-promoting microbes and biocontrol agents (Spaepen et al. 2009). Recent advances in metagenomics and metatranscriptomics of microbial communities could be applied to rhizosphere microorganisms and in combination with plant transcriptomics provide further insights into multiple interactions between them.

Twenty *Halomonas* strains showing resistance to different abiotic stresses including efficient nitrogen fixation and phosphate solubilisation were identified (Mapelli et al. 2013). 16S rRNA pyrosequencing data analysis allowed to characterise and identify the efficient productivity-related rhizobacteria in wheat (Anderson and Habiger 2012). Hence, collecting and screening microbial community for their beneficial roles including tolerance to various stresses help to exploit those strains in biofertiliser formulates. Metagenomic analysis of microbial community of the *L. japonicus* rhizosphere with respect to phytic acid utilisation, which is the prominent form of organic phosphate in many soils, was reported by Unno and Shinano (2013). The study identified bacterial classes *Betaproteobacteria*, *Bacteroidetes*, *Methanobacteria*, etc. that helps in plant growth-promoting and phytic acid utilisation. From a barley rhizosphere soil, functional metagenomic analysis was carried to characterise phosphate solubilisation trait, where a number of genes related to phosphorus uptake and solubilisation were identified (Chhabra et al. 2013). Metagenome analysis of endophyte bacteria present inside the roots of rice helps to predict the metabolic processes necessary for the bacterial lifestyle. They include plant cell wall-degrading enzymes, detoxification of ROS, iron storage, protein-secreting systems and mainly nitrogen-fixing proteins (Sessitsch et al. 2012).

Multispecies transcriptomics may lead to the discovery of key plant and microbial genes that characterise the interaction and further help in evolving new strategies for disease resistance. As the data output is from vast different microbial species, bioinformatics is in demand to make it more meaningful; therefore, computational methods are being expanded in metagenomics field. These tools have the capacity to revolutionise research on plant–microbe interactions, as they facilitate investigation of dynamic microbial transcriptomes in response to plants. Such studies focusing on functional characteristics linked to plant growth promotion like nitrogen fixation, phosphate utilisation, antibiotic production and hormonal production help in providing sustainable crop production.

4.13 The Integrated Genomics

As discussed earlier, transcriptomics provide only information on expressed gene levels, which does not give details about the post-translational modifications but is provided by proteomic approach. However, metabolites are the end products of cellular processes that are provided by metabolomics platform. Hence, studies are being carried out with a combination of 'omics' approaches which helps to build bridges between all aspects of cellular changes. This helps to understand the exact picture of the complex dynamics of cellular systems in both the partners during plant–microbe interactions. The few stud-

ies that deal with integrated 'omics' approaches are discussed here. *Bradyrhizobium japonicum* bacteroid metabolism in root nodules of soya bean was studied by compiling the datasets of proteomics and transcriptomics (Delmotte et al. 2010). Based on the dataset, a significant number of proteins corresponding to different types of bacterial metabolism were discovered that were not previously considered to be present during symbiosis. Combined transcriptomic and proteomic analysis of potato during compatible and incompatible interaction with *Phytophthora infestans* was investigated (Ali et al. 2014). Alterations in abundance of over 17000 transcripts and 1000 expressed proteins were identified. Kunitz-like protease inhibitor, RCR3-like protein and transcription factors were found to be induced only during incompatible interaction. The corresponding change at the transcript level was coincided with the change in half of the differentially abundant proteins.

The mechanism of resistance against *Fusarium graminearum* wheat (near-isogenic line (NIL)) containing *Fusarium* head blight resistance locus, *Fhb1*, was examined by metabolo-proteomic approach by Gunnaiah et al. (2012). Metabolites of phenylpropanoid pathway like flavonoids and hydroxycinnamic acids were induced or highly induced in resistant NIL than susceptible line, after pathogen infection. The presence of these metabolites was confirmed by fragmentation pattern using LC-LTQ-Orbitrap and demonstrated that wheat resistance is derived from cell wall thickening by deposition of phenylpropanoid metabolites. Different responses by genetically close resistant and susceptible tomato lines against tomato yellow leaf curl virus (TYLCV) infection were identified by comparing protein and metabolite profile. Antioxidant, pathogenesis-related and wound-induced proteins were significant in susceptible, whereas homeostasis was maintained by protein and chemical chaperones. Further, carbon and nitrogen metabolism was less affected in resistant than susceptible plants (Moshe et al. 2012).

By employing and comparing multidisciplinary 'omics' approaches, the abundance, presence or absence of a particular biological component can be assessed exactly. This chapter on current 'omics'-based studies enhanced our understanding on the plant–microbe interaction. Hence, the strategies being evolved based on these integrated approaches would lead to long-term applications resulting in more sustainable crop improvement.

4.14 Conclusion

'Omics' approaches have expanded our knowledge in understanding plant–microbe interaction especially in the fields of nitrogen fixation and biocontrol. Focused research studies to reveal the mechanism and effects of plant–pathogen interaction and to enhance the host defence response are done in various crop systems. A common change observed under biotic or abiotic stress imposed on the host is an alteration of the redox or ROS-scavenging molecular components. Earlier studies were concentrated to unravel nitrogen fixation mechanism and the role of flavonoids. In order to identify the most efficient strains, efforts are being made to examine the potential determinants active during the process. Recently, 'omics' has helped to characterise known components, namely, *fixA*, *nifH*, inositol monophosphatase and nod factors. 'Omics' has also helped to identify other unknown proteins involved in nitrogen fixation which were induced under genistein (isoflavone known to regulate nodulation, Gomes et al. 2014). During metal toxicity, growth promotion in the host by an increase in photosynthetic proteins, chaperones and transport proteins and during drought and temperature stress, various chaperones, namely, DnaK and GroEL and heat shock protein induction were identified (Gomes et al. 2012, 2014).

During plant–pathogen interaction, majorly ROS-scavenging and PR component synthesis are enhanced. Currently, by employing 'omics' platform, the biological components profile of both the plant and its interacting microorganisms can be analysed. Such studies should be formulated, because gene expression in symbiotic (beneficial) or parasitic relationships between plants and microbes is tightly linked. This helps to iden-

tify resistance genes and their corresponding avr proteins during interaction. Analysis of symbiotic genes or the end products of toxins produced by pathogen helps to evolve the strategies to enhance the plant productivity. As already discussed, the virulence product deoxynivalenol produced by a pathogen (*Fusarium graminearum*) and its detoxification product DON-3-O-glucoside (D3G) including other phenolic acids were identified as potential biomarkers against *Fusarium* head blight in wheat. Also, *LysR*-type transcription regulator responsible for the biocontrol action of *Pseudomonas chlororaphis* was identified Klaponski et al. (2014).

'Omics' helps to study and analyse the complex cellular mechanism during plant–microbe interaction. Genomics has led to compare different host, microbe or host–microbe interaction simultaneously in simplified ways. Evolution of new strains also can be studied. Hence, by applying the concepts of integrated genomics and comparative genomics understanding of plant–microbe interaction has made significant achievements and further that may help in developing strategies for sustainable agriculture.

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Strategies for Taxonomical Characterisation of Agriculturally Important Microorganisms

5

Om Prakash, Rohit Sharma, Prashant Singh, and Amit Yadav

Abstract

Agriculturally important microorganisms mainly comprise bacteria, fungi, cyanobacteria, phytoplasmas and other groups like viruses. Most of the bio-inoculant technology for plant growth promotion (PGP) and biological control of plant diseases is based on bacteria, fungi and cyanobacteria. Plant Pathogenic organisms like Phytoplasmas cause serious diseases in economically important plants. Rapid and authentic identification of agriculturally important microorganisms is imperative before their use in bio-inoculant formulation as well as for diagnosis of pathogens to prevent the crops from damage. Many agricultural microbiologists and plant pathologists are still using traditional approaches of identification of an organism resulting into poor resolution of its taxonomic status. Polyphasic approach of microbial classification using the phenetic, chemotaxonomic and genotypic methods in combinations is the recent approach in microbial taxonomy. In current chapter, we discussed the recent advances in the taxonomy of bacteria (including cyanobacteria and phytoplasma) and fungi. We appeal agricultural microbiologists and plant pathologist for the use of polyphasic approach for better delineation of organism in focus in addition to traditional approaches.

Keywords

Taxonomy • Polyphasic approach • Bio-inoculant • Diagnosis • Identification

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5.1 Introduction

The microbial world of agricultural soil comprises bacteria, fungi, actinobacteria (previously known as actinomycetes), cyanobacteria (blue-green algae), archaea (archaebacteria), viruses and microalgae. Microbes are the key component of the soil microflora, and activities of the soil microorganisms directly or indirectly affect the nutritional status of the soil and plant health, growth and productivity. They work as bio-fertilisers, biopesticides and biocontrol agents (Compant et al. 2005; Aseri et al. 2008; Gulati et al. 2009; Khan et al. 2013) and modulate plant immunity and health by different activities like phosphate solubilisation, nitrogen fixation, plant growth promotion, production of plant growth-stimulating hormones and other metabolites and disease suppression (Bertin et al. 2003; Bakker et al. 2007; Prakash et al. 2015). In addition, soil microflora is also responsible for the degradation of organic compounds, soil formation, humification, composting and biogeochemical cycling of materials (Cunningham and Kuiack 1992; Gulati et al. 2009; Lugtenberg and Kamilova 2009; Morgan and Connolly 2013). They make soil fertile and assist in movement and uptake of nutrients in the soil (Bhatia 2008; Hayat et al. 2010). Microbial colonisation on different parts of the plants enables them to cope with the condition of various abiotic and biotic stresses like high salinity, pH and drought and protects them from the invasion of pathogenic microorganisms (Prakash et al. 2015). The area of plant microbes' interaction is thus a fascinating field of research amongst the agricultural microbiologists and biotechnologists and attract the attention of new generation scientists and reserachers (Wu et al. 2009; Khan et al. 2013; Seth and Taga 2014)

In addition to promoting the plant growth and yield, microorganisms also play an important role in allied sectors of agriculture, aquaculture, veterinaries and dairy. They are the key players of agricultural waste management and generation of bioenergy and biogas. Microbes are also used in wastewater treatment, degradation of agricultural chemicals likes fertilisers, pesticides and herbicides and clean-up of environmental pollutants (Hayat et al. 2010; Prakash et al. 2015). Thus, when looking at the potential of agriculturally important microorganisms in agriculture and its allied sectors, it is considered that they are the hope of future food safety and security for the growing world population and backbone of sustainable agriculture, clean energy and second green revolution.

Despite the immense importance of agriculturally important microorganisms in food security and human health, the species and strain level taxonomic characterisation is still in infancy and needs more attention before their active use as bio-inoculants. In the current chapter, we would mainly focus on the taxonomic status of different groups of agriculturally important microorganisms along with suggesting the recent methods used for their characterisation.

5.2 Strategies for Characterisation of Bacteria

Bacteria constitute one of the very valuable groups of agriculturally important microorganisms. Most of the organisms used in PGPR, biofertiliser and biopesticide formulations belong to this class. In addition, they also work as causative agents of disease of livestock, fisheries and aquaculture. Therefore, there is a practical need for taxonomic characterisation of these organisms for authentication as well as for future applications. Progress and resolution of any field of science depend on technological advancements. In comparison to the past, the current bacterial taxonomy is more refined and provides better resolution at species and strain levels. Conventional bacterial taxonomy was mainly focused on morphological and growth characteristics of the organisms, and the levels of resolution were not very good. The recent concept of bacterial classification is based on the polyphasic approach (Sharma et al. 2015; Prakash et al. 2007; Tindall et al. 2010). In the polyphasic approach, researchers use phenotypic, phylogenetic, chemotaxonomic and genotypic approach for classification of organisms of interest. Typing based on phenotypic data includes shape, size, pigmentation and arrangement of flagella on bacterial cells (morphological features),

whilst physiological features include requirement/tolerance for salinity, pH, temperature and utilisation of source of carbon and energy. It also includes oxygen relationship of the organisms like microaerophilic, aerobic, facultative anaerobes and anaerobe. Chemotaxonomic data includes similarity and dissimilarity in structural or chemical constituents of the bacterial cells like peptidoglycan, lipids, quinone, fatty acids, proteins, sugars and polyamines (Prakash et al. 2007; Tindall et al. 2010).

Small subunit ribosomal RNA (16S rRNA) gene sequence-based typing and study of phylogenetic relatedness are the most popular and simple aspect of current bacterial characterisation. Although 16S rRNA-based approach does not provide species level resolution in most of the cases, bacterial taxonomists usually use it as first step for typing purposes (Tindall et al. 2010). According to the current practice, a cut-off of <95 % and <97 % 16S rRNA gene sequence similarity based on near about complete length (>1400 bp) with closely related and validly published type species is the criteria for the creation of new genus and species, respectively. If sequence similarity exceeds more than 97 %, in that situation decision of novelty of the species should be confirmed using DNA-DNA hybridisation (DDH) experiment. In addition, according to Stackebrandt and Ebers (2006), the cut-off of 97 % for DDH should be extended to 98.7 %, in case of good quality >1400 bp sequence used for similarity search. Species is the basic unit of bacteria classification. It is an assemblage of more than one bacterial strain showing most of the common features and isolated from similar or different kinds of habitats. Description of novel species in an existing genus is based on DDH data. If a new bacterium shows less than 70 % DDH value with closely related and validly published species with greater than 97 % sequence similarity in that situation, it would be a novel species of an existing genus (Wayne et al. 1987; RosselloMora 2006).

DDH value is still considered as the gold standard for bacterial species delineation, but it is difficult and time-consuming, and the results vary from laboratory to laboratory. Revolution in sequencing technologies and availability of next-generation sequencing platforms made whole genome sequencing cheap and less time-consuming. Now comparison of whole genome sequence of closely related organisms and calculation of average nucleotide identity (ANI) value of conserved genes are an emerging alternative of DDH data in bacteria taxonomy (Goris et al. 2007; Henz et al. 2005; Kurtz et al. 2004; Ramasamy et al. 2014; Jongsik and Rainey 2014). According to the comparative study of ANI value and DDH value of related organisms, it was concluded that a 95–96 % ANI value corresponds to 70 % DDH value which is a threshold for bacterial species delineation.

5.3 Strategies for Characterisation of Agriculturally Important Fungi

Fungi are the causative agents of many diseases of agricultural crops and vegetables and are also used as bio-fertilisers and biopesticides to promote and protect the plants, respectively. Characterisation and identification of fungi have not been easy since the history of fungal taxonomy and systematics. Unlike other microorganisms, fungi have a complex life with two morphotypes, teleomorphs (sexual stage) and anamorphs (asexual stage) (Fig. 5.1). Some strains of the fungi form either one or both the stages in their life cycle, and based on the above observations, taxonomists developed rules of fungal classification. Hence, four major phyla were known, *Basidiomycetes*, *Ascomycetes*, *Zygomycetes* and *Oomycetes*. Those fungal strains which did not form any sexual stages or until their sexual stage were discovered were classified under a separate phylum *Deuteromycetes*. However, with the development of molecular techniques, it became easier to assign an anamorphic stage fungus to its teleomorphic stage using internal transcribed spacer (ITS) region sequencing. Thus, with time, the class *Deuteromycetes* gradually became obsolete. Moreover, many fungi previously belonging to kingdom fungi have been positioned in either other kingdoms or other phyla. For example, *Oomycetes* are now placed in kingdom

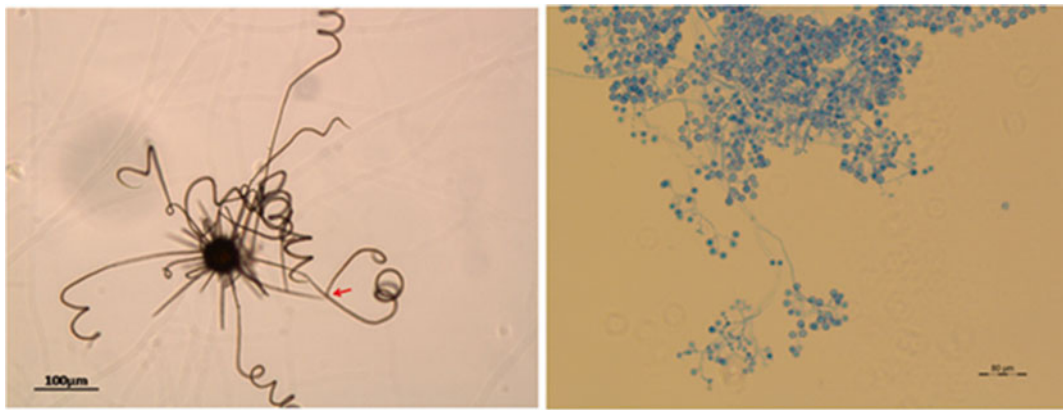

Fig. 5.1 One fungus two names. *Chaetomium* sp. is a pleomorphic fungus which reproduces sexually (teleomorphic stage) by forming ascospores within ascomata (L) and one of the asexual form *Botryotrichum* sp. (anamorphic stage) reproducing by forming conidia (R) (Image courtesy of Rohit Sharma)

Fig. 5.2 A diagrammatic representation of change in classification of fungi following phylogenetic analyses using molecular techniques. It includes some representative plant pathogens, common saprophytes and mycorrhizal species

Chromista (Fig. 5.2). True fungi contain the following phyla, *Basidiomycota*, *Ascomycota*, *Glomeromycota*, *Blastocladiomycota*, *Kickxellomycota*, *Entomophthoromycota*, *Mucoromycota*, *Neocallimastigomycota*, *Chytridiomycota* and *Microsporidia*.

Incidence of fungal plant diseases is increasing around the world. It has been found that climate change, susceptible varieties and virulent fungal pathogens are playing an important role in the spread of the fungal diseases. There are three main points related to the study of plant pathology: (1) symptom-based detection of fungal pathogen, (2) detection of non-symptomatic pathogens and latent or quiescent symptom-causing fungi and (3) authentication or identification of fungal pathogens using appropriate tools. For the management of any disease, authentic

identification or characterisation is the first priority. Only a handful of fungal strains are virulent and cause diseases, but still we do not have complete data (morphological, biochemical and molecular) for their authentic diagnosis during disease outbreak. Similarly, before the use of fungal strains as biocontrol agent or in bio-inoculant formulation, their authentic identification is a must.

Systematics is the study of biological diversity which includes taxonomy, nomenclature and phylogeny. These three principal divisions of systematics guide the description, nomenclature and classification of a fungus. Scientific name does not only tell the name of a fungus but also its biology, behaviour and all peripherals about the organism (habitat, interaction with other organisms). For example, *Phytophthora* suggests a potential pathogen and *Chaetomium* represents a non-pathogenic cellulolytic fungus (Rossman and Palm 2006). Any change in the systematics of a fungus will affect its identity thus affecting the identity of a plant pathogen or a bio-fertiliser agent (Fig. 5.3). Therefore, an authentic identification of fungi is important for control of diseases, understanding the biology of pathogen, mechanism of spread of disease, knowing the correct identity of pathogens when multiple organisms give same symptoms, quantifying the pathogen for estimation of disease loss, assessing the variation in strains, identifying new pathogens and selection of the better biocontrol agents. Pathogens can be present in various habitats like in plant leaves, seeds or soils and plant debris and can move by air and water from infested area to un-infested area. A thorough knowledge of fungal life cycle, habitat, information about host plant(s) and pathogenesis of diseases is necessary for the control of fungal pathogen.

Several methods have been proposed for the characterisation of fungi including the morphology, physiology, immunological features, cellular chemical composition and molecular methods (Fig. 5.4). These are related to the species concept and/or criteria of a fungus (Sharma et al. 2015). Agriculture mycology has travelled a long distance from the time of symptomatic studies to characterising by molecular and biochemical markers. In this segment of the chapter, we discuss usefulness and limitations of these methods and discuss some specific techniques used in characterisation of agriculturally important fungi. We also discuss the impact of recent changes in fungal taxonomy on agriculturally important microbes.

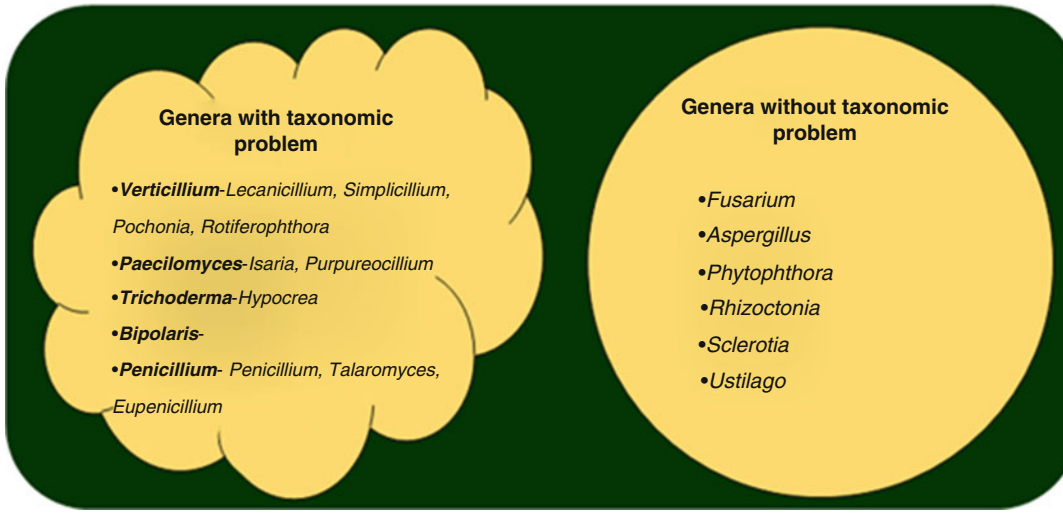

Fig. 5.3 Genera with taxonomic problem in agriculturally important fungi

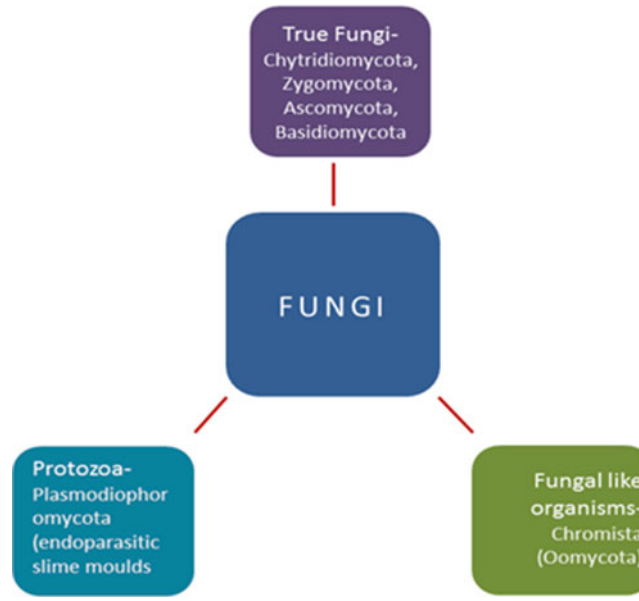

Fig. 5.4 Strategies for characterisation of agriculturally important microbes

5.3.1 Morphological and Physiological Characterisation

Characterisation of fungi based on morphology is the preliminary step of fungal taxonomy, and it starts on the basis of the symptoms it produces. For example, the white root rot of pulses is only caused by species of *Sclerotinia* (*S. minor*, *S. sclerotiorum* and *S. trifoliorum*), the black root rot is caused by *Fusarium solani* and wet root rot is caused by *Rhizoctonia solani.* The second step for characterisation is the culturing of the fungal pathogen in desired medium for the study of morphological features like colony characteristics and spore morphology.

Unlike other groups, smut (*Ustilaginomycotina* and *Pucciniomycotina*, *Microbotryales*) and rust fungi (*Pucciniomycotina*, *Pucciniales*) are one of the most economically important group of plant pathogens (Vánky 2011; Shivas et al. 2014), and their identification is mostly based on morphology and knowledge of the host species. Morphological identification of smut fungi is reliant on differences between sori and teliospores (Vánky 2013). In case of rust fungi, morphological characters of the teliospore and urediniospore stages, such as size, apex shape and wall thickness, ornamentation, germ-pore position and numbers are useful for species identification. Due to the absence of literature, experience and knowledge of the stages in life cycles, it may be difficult to identify the rust fungi for which molecular methods are required. Advancement in technology and microscopy such as Nomarski differential inference contrast (DIC) and scanning electron microscope (SEM) has made morphological characterisation easy and more authentic. Sometimes physiological characters like tolerance and optima of temperature, salinity and pH also help in resolving the species at the strain level, and classification of *Aspergillus*, *Penicillium* and *Fusarium* using physiological traits is a good example of the role of morphological features in classification (Frisvad and Samson 2004; Li et al. 2012).

5.3.2 Molecular Characterisation

In cases of sterile mycelia and inconclusive morphology, molecular identification based on sequence data from the large subunit (LSU) region or internal transcribed spacer (ITS) region

of nuclear ribosomal DNA may identify species or genera of smut and rust fungi (Schoch et al. 2012). The molecular methods used in characterising agriculturally important microbes have come a long way from RAPD, RFLP to sequencing of specific regions. Still, the gel-based techniques are used for strain level differentiation. Internal transcribed spacer (ITS) which is about 500–600 bp long (ITS 1/5.8S/ITS 2) is now considered as the barcode region of fungi. It has been widely used to differentiate species and strains in several agriculturally important fungi like *Colletotrichum*, *Fusarium*, *Pythium*, *Alternaria*, *Cercospora*, *Puccinia*, *Rhizoctonia* and *Verticillium*. It has also helped in the identification of several cryptic species in these genera. In *Trichoderma* alone, 30 cryptic species were identified based on the sequencing of ITS region. In *Peronospora*, corrected taxonomy was provided by Göker et al. (2009) using ITS sequencing. Moreover, it is also helpful to identify mushroom strains cultivated commercially like *Agaricus bisporus* (button mushroom), *Pleurotus ostreatus*, *P. sajor-caju* (oyster mushroom), *Volvariella volvacea* (paddy straw mushroom) and medicinally important fungi *Ganoderma lucidum* (reishi mushroom). Since it has the robust database in NCBI, UNITE and EzTaxon, the ITS region is considered a reasonably good marker for fungal strain identification. Still, authentic and complete databases are lacking for the same which has been highlighted in articles by other workers also (Sharma 2012; Nilsson et al. 2014). Moreover, incomplete and erroneous database of reference sequences also poses problems in right identification (Kang et al. 2010). For yeasts, large subunit (LSU) which is approximately 1400 bp long is considered as the primary region for sequence comparison. However, D1 and D2 regions of LSU which are considered as hyper-variable regions are usually used. Several other regions have also been used for complete characterisation and better resolution at the species level. These regions include nuclear and mitochondrial rDNA regions [18S ribosomal RNA small subunit (18S-SSU), 28S ribosomal RNA large subunit (28S-LSU), internal transcribed spacer (ITS), intergenic spacer (IGS1) region, mtSSU, and mtLSU], as well as protein-coding genes, such as RNA polymerase II (*rpb1* and *rpb2*), β-tubulin (*β-tub*), calmodulin (*cal*), γ-actin (*act*), ATP synthase (*atp6*), translational elongation factor 1α (*ef-1α*), etc. Multilocus sequence typing (MLST) is a handy tool to delineate various fungi like *Alternaria*, *Botryosphaeria* and *Chaetomium* (Brun et al. 2013; Slippers et al. 2013; Sharma et al. 2013). The MLST sequencing has helped to separate several cryptic species in *Fusarium*, *Trichoderma*, *Aspergillus*, *Penicillium*, etc. Sharma et al. (2015) have discussed in detail the various species concepts used in characterisation of fungal species. However, the database for obligate fungi is limited. According to Shivas et al. (2014), only 3 % of rust fungi (310 LSU sequences and 210 ITS sequences) and 21 % of smut fungi (346 ITS sequences) have reference sequences in GenBank. The sequences are used to construct phylogenetic tree based on different algorithms (maximum parsimony, maximum likelihood or neighbour joining) to calculate the evolutionary distance between different fungal species. Based on the distance, a fungal strain is assigned a particular species in the genus.

Apart from the above criteria, some other techniques like DNA microarray, fluorescent in situ hybridisation (FISH), real-time PCR (RT-PCR) and microsatellite markers are also used for fungal characterisation. DNA microarray, also known as DNA array or reverse dot blot hybridisation (RDBH) technique, uses a pre-labelled DNA probe of specific region (ITS, 18S, protein-coding gene, etc.) which is hybridised with immobilised oligonucleotides on a solid support. It has been developed for the detection of plant pathogens in a wide range of environmental samples, such as greenhouse crops, potatoes, ginseng and fruits (Tsui et al. 2011). Microarrays are also effective diagnostic tools for the detection of phytopathogenic fungi and fungus-like organisms like *Phytophthora*, etc. (Chen et al. 2009). DNA microarray was able to detect species of *Phyllosticta*, *Alternaria*, *Pestalotia* and *Pilidium* from a single frozen cranberry fruit sample (Robideau et al. 2008). Fluorescent in situ hybridisation (FISH) has been used for simultaneous detection of many fungi

including endophytes and mycorrhizal fungi (arbuscular and ectomycorrhizal) within the roots of different plants (Vági et al. 2014). It is a good method of in situ detection of fungi colonising and infecting a crop plant. The technique detects RNA or DNA in the organelle or cytoplasm, thus detecting metabolically active fungi in the sample. It involves labelling of a fluorescent marker/label to a nucleic acid sequence to form a probe. This probe is then hybridised with the DNA or RNA of biological material to identify the fungal organism. It uses mostly ribosomal and mitochondrial genes. It was first applied to *Aureobasidium pullulans* identified from phylloplane of apples. Although it is a good, specific, rapid technique, Tsui et al. (2011) have pointed some drawbacks of the same like nonspecific fluorescence, autofluorescence emitted by some organisms, etc. Conventional PCR-based diagnosis has been used for the identification of phytopathogenic fungi as in *Fusarium oxysporum* f. sp. *cubense* (Dita et al. 2010). In the past decade, some reviews have appeared on real-time PCR focussing on detection of soilborne fungal pathogen (Taylor et al. 2001). Even though a molecular technique resolves the fungus to species and/or strain level, sometimes biochemical characterisation is required in some complex genera.

5.3.3 Biochemical Characterisation

Although in biochemical characterisation only profiles of secondary metabolites and matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) profiles of ribosomal proteins are considered as taxonomic characters, several other techniques are popularly used in the diagnosis of fungal strains like isozyme analysis, electrophoretic mobility of proteins and Raman spectroscopy. Characterisation of secondary metabolites is an important tool in fungal taxonomy. In the past decade, most of the species of *Alternaria*, *Aspergillus*, *Fusarium*, *Hypoxylon*, *Penicillium*, *Stachybotrys* and *Xylaria* were characterised using secondary metabolite profiles of isolated strains (Frisvad et al. 2008). In fact, within the *Aspergillus* section *Nigri*, there are more than 19 species which are distinguished from each other by their secondary metabolite profile. Secondary metabolites of *Daldinia* and *Hypoxylon* have been studied in detail (Stadler et al. 2014). The volatile metabolites can be characterised using gas chromatography-mass spectroscopy (GC-MS), and non-volatile compounds can be characterised using thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC). Apart from these, isozyme analysis is also done by many plant pathologists to separate fungal strains, and it is a simple, efficient and less expensive technique for evaluating taxonomy, genetics and virulence of plant pathogens especially fungi. Moreover, the characteristics determined by this technique are generally accepted to be of independent genetic origin (Kohn 1992).

Fatty acid profile, cell wall composition, type of ubiquinone and API system are also being used for the identification of different human pathogenic fungi and are equally applicable for the taxonomic characterisation of agriculturally important fungi. However, creation of authentic database for comparison is essential before using these methods. Other methods are also employed for diagnosis not specifically for characterisation of fungi like electrophoretic mobility of proteins, enzyme-linked immunosorbent assay (ELISA) and Raman spectroscopy. These methods are mainly used for human pathogenic fungi but seldom used with plant pathogens or bio-fertilisers. These are low-cost (running), rapid techniques and should also be employed to agriculturally important fungi. A good database can be made, with phytopathogens added in their library. Reports have showed that ELISA is being used in nurseries and tissue culture labs for monitoring the early infection of *Sclerotinia sclerotium* and *Venturia inaequalis*, even before the fungi produce symptoms. Kennedy et al. (2000) showed rapid detection and quantification of ascospores of *Mycosphaerella brassicicola* and conidia of *Botrytis cinerea* by using ELISA. However, the other techniques are less time-consuming than ELISA, and we should promote their use in diagnosis of plant pathogens.

5.3.4 New Strategies and Platforms

The development of next-generation technologies and improvement of the bioinformatics tools enhance our ability to sequence and analyse metasequence data in short period of time and in less cost. In the future, genome sequencing will be key to fungal characterisation and taxonomic designation. So far more than 40 fungal genomes have been sequenced (16 out of them are phytopathogens, viz., *Aspergillus nidulans*, *Ustilago maydis*, *Phytophthora infestans*, *Fusarium oxysporum*), and 300 genome projects are under way. The other criterion relatively recently being used for identification of fungi is profiling of ribosomal proteins using MALDI-TOF. Major studies have been undertaken with human pathogenic fungi, and therefore the database for such strains is more robust. Recently, people have also studied agriculturally important fungi, and a few proteomics studies on fungal spores have been published (Wu et al. 2009; Barreiro et al. 2012; Gonźalez-Fernández 2010). The characterisation of *Penicillium* spores by MALDI-TOF MS with different matrices has been demonstrated for the classification of fungal spores (Welham et al. 2000). Sulc et al. (2009) have reported protein profiling of intact *Aspergillus* spp. spores, including some plant pathogenic species, by MALDI-TOF MS. Till now, several phytopathogenic fungi have been studied using MALDI-TOF, viz., *Phytophthora palmivora*, *P. infestans*, *Ustilago maydis*, *Pyrenophora tritici-repentis*, *Rhizoctonia solani* and *Fusarium* sp. Gonźalez-Ferńandez et al. (2010) provided a tabular list of phytopathogenic fungi with detailed proteomics studies. Chalupová et al. (2012, 2013) and Brun et al. (2013) performed development and evaluation of a methodology for IC/IS MALDI-TOF MS of fungal and fungal-like pathogens representing obligate biotrophic parasites of crop plants like *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium* and *Trichoderma* and also identified them. In MALDI-TOF, either intact spore or cell is chosen for extraction of surface proteins. In the current situation, a combination of traditional and modern methods should be used for better characterisation and better understanding of a fungus and its biology. More focus should be given to genomics in any group of organisms, but we think in case of phytopathogens, we should more concentrate on proteomics to understand about the proteins involved in the plant-pathogen interactions.

Study of taxonomic details is one of the common problems amongst the agricultural microbiologists, and agriculturally important fungi have also faced series of confusions about their clear taxonomic status. Fungal taxonomy has undergone some major changes in recent times which have been communicated from time to time by taxonomists in scientific journals publishing research articles on taxonomy and systematics. Figure 5.5 shows changes in taxonomy of some agriculturally important microbes. Changes in fungal taxonomy have made a great impact on agriculturally important fungi, be it plant pathogens or bio-fertiliser agents (Fig. 5.5). Typically, many plant pathologist and bio-fertiliser researchers have not kept the pace by which taxonomic methods used in systematic studies are changing. Many plant pathologists still do not adopt modern methods of identification and characterisation of plant pathogens which are fast and more authentic, viz., sequencing and MALDI-TOF. Moreover, many methods like ELISA, DNA microarray, serological methods, Raman Spectroscopy, etc. are considered more of a diagnostic technique rather than using for complete characterising of a fungus. These methods can show strain level variation but due to database dependency have their own limitations. Therefore, availability of a good database of pathogenic fungi for typing or identification of strains by plant pathologist working on a particular fungus is essential.

5.4 Strategies for the Characterisation of Cyanobacteria

The use of cyanobacteria (formerly blue-green algae) is being promoted nowadays as a beneficial agricultural practice. These cyanobacteria are phototrophic in nature, produce auxins and

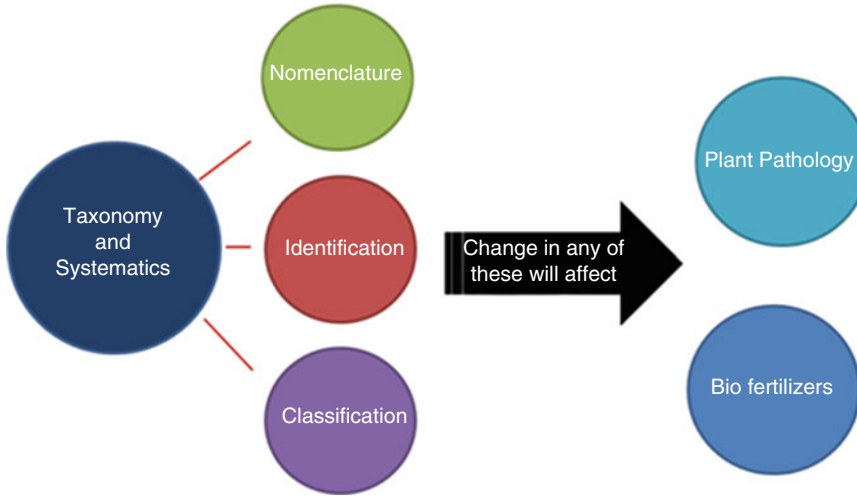

Fig. 5.5 Changes in fungal taxonomy have made a great impact on agriculturally important fungi, be it plant pathogens or bio-fertiliser agents

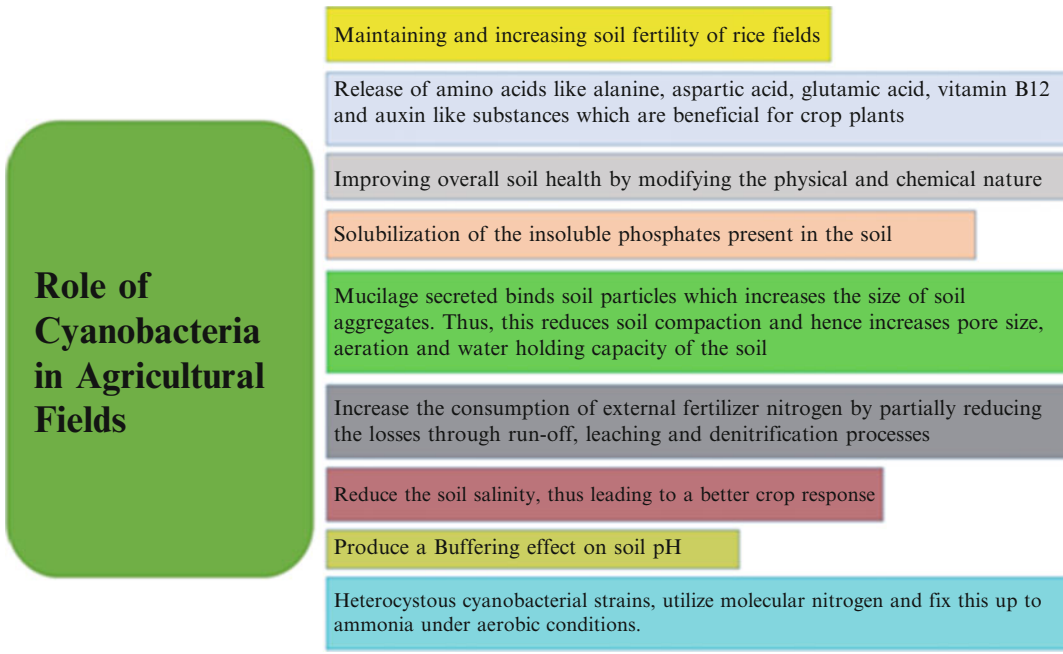

Fig. 5.6 Beneficial roles of cyanobacteria in agriculture

gibberellins and, most importantly, fix 20–30 kg nitrogen/ha in submerged rice fields. Because of their abundance in paddy fields, they are frequently referred as 'paddy organisms'. Cyanobacteria impart a lot of significant advantages when being used as bio-fertilisers in agricultural fields (Fig. 5.6).

Amongst the noteworthy technical constraints for the use of cyanobacteria as bio-fertilisers, the problem of proper strain identification is one of the premier ones, and it has long persisted as one of bottlenecks in bio-fertiliser technology. The taxonomic assignments of many members of the cyanobacteria are still highly debated (Litvaitis

2002). The lack of consensus, regarding the treatment of cyanobacteria according to the botanical or the bacterial system, has plagued cyanobacterial taxonomy till date.

The huge variety in the phenotypes of cyanobacteria is accompanied by very huge morphological plasticity also that changes in response accordingly to different environmental or culture conditions. This can result in misidentifications when being judged only on the morphological scale alone (Lyra et al. 2001). Thus, the proper inclusion of other nonplastic characteristics, such as genetic information, is an imperative complement for the accurate identification and classification of cyanobacteria.

Taking into consideration the limitations of the above morphological characters with environmental extremes and culture conditions, new approaches have now come into shape for deciding the phylogenetic affinities and classificatory schemes of heterocystous cyanobacteria. A number of innovative and important techniques for delineating the taxonomic affiliations have been developed in recent times especially PCR-based molecular techniques such as DNA fingerprinting methods like denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), single-strand conformational polymorphism (SSCP), ribosomal intergenic spacer analysis/automated ribosomal intergenic spacer analysis (RISA/ARISA), terminal restriction fragment length polymorphism (T-RFLP), along with some non-PCR-based approaches like DNA-DNA hybridisation, guanine-cytosine ratio, photopigment compositions, etc. (Kumari et al. 2009).

5.5 Molecular Markers in Assessment of Cyanobacterial Taxonomy

As has been mentioned, a lot of monumental work is going on in the past decade for addressing the problems in the cyanobacterial phylogeny and taxonomy. In this chapter, we would dwell with a selected few markers that have recently been used and, more importantly, reported by few groups.

The 16S rRNA sequence, in spite of having hyper-variable and extremely informative regions along with much conserved regions, is often not divergent enough to give good resolutions in closely related species of the same genus (Normand et al. 1996). It is also too well conserved in the hyper-variable region for studying species identity (Fox et al. 1992) or intraspecies variations (Ward et al. 1992), thus shedding serious doubts on the use of this parameter as a molecular marker.

Phylogenetic studies of *nif*D and partial *nif*H sequences have, in general, supported the occurrence of vertical inheritance in diazotrophs (Zehr et al. 1997; Henson et al. 2004). Nevertheless, several studies have also shown proof of evidence of instances of a possible lateral gene transfer in the *nif*D (Henson et al. 2004), *nif*H (Cantera et al. 2004) and *nif*K genes (Hirsch et al. 1995). Ambiguities between the *nif* and 16S rRNA gene phylogenies are in fact an attribution of an assortment of possibilities that may be a result of lateral gene transfer events, varied rates of evolution between the genes, selective adaptations and divergent/convergent radiations, uncertain taxonomic classifications, computational artefacts during the phylogeny constructions or combinations of many or all of these factors.

The *psb*A gene is an important functional gene that has hugely been neglected for phylogenetic assessments of cyanobacteria. It has been known to code for the D1 protein of the photosystem II reaction centre with an amplicon of size approximately 990 bp (Junier et al. 2007). There is a strong dearth of reports on cyanobacterial diversity using the *psb*A gene with one of the only reports being of Singh et al. (2014).

The *rbcl* gene, just like the *psb*A gene, has been studied very less till now. Apart from being a single-copy gene, approximately 1430 bp in length, it is also known to be free from length mutations except at the far 3′ end and, thus, has a fairly conservative rate of evolution (Gugger et al. 2002). Reports about the phylogenetic assessment of the *rbcl* gene are very less (Gugger et al. 2002; Morden and Golden 1991) and do not encompass many of the cyanobacterial genera.

5.6 A Word of Caution for the Use of Cyanobacteria as Bio-fertiliser

It seems appropriate to assume that, still, the current level of understanding especially for the heterocystous cyanobacterial phylogeny and systematics is weak and pretty unsteady. Using different kinds of markers and various other approaches also, it is still unclear that what exactly could be the reliable scheme of taxonomy in case of cyanobacteria. The issues especially those between species of the same genera are indeed very tough to resolve and need a proper polyphasic treatment, which itself is tough to decide in case of cyanobacteria. Comprehensive and large-scale work with a broad range of molecular parameters is still to be done for assessing the molecular phylogeny and evolution of heterocystous cyanobacteria. Thus, using a multilocus approach that addresses the study of genetic diversity of cyanobacteria using more than one molecular marker and thereafter treats all the DNA information at the same stage for phylogeny reconstruction must be done. For the use of cyanobacteria as bio-fertilisers and bio-inoculants, it is thus essential to first select the properly identified strain through a multilocus polyphasic method and then apply it into the fields. Failure to adopt any of the above-mentioned standard practices may lead to erroneous identification, improper field results and most importantly an unnecessary waste of both money and time of the farmer involved.

5.7 Strategies for the Characterisation of Phytoplasmas

Phytoplasmas, formerly termed as mycoplasma-like organisms (MLOs), are a large group of obligate, endophytic, cell wall-less bacterial parasites classified within the class Mollicutes (Wei et al. 2007). Phytoplasmas are known to infect more than 1000 plants species including many economically important plants and crop species (Hogenhout et al. 2008). The typical symptoms shown by phytoplasma-infected plants include whitening, yellowing or reddening of the leaves indicating chlorosis, shortening of the internodes leading to stunted growth, smaller leaves and excessive proliferation of shoots resulting in a 'broom' phenotype, loss of apical dominance and phylloidy (Lee et al. 2000). Reviews published from time to time have given good insights of phytoplasma studies including its taxonomy, aetiology, transmission and interaction with insect and plant hosts (Lee et al. 2000; Weintraub and Beanland 2006; Hogenhout et al. 2008; Sugio et al. 2011). Many economically important crops, including food, vegetable, fruit crops, ornamental plants, timber and shade trees, are infested with phytoplasma. The impact of phytoplasma diseases and their distribution in different geographical areas depend on the host range of the phytoplasma as well as the polyphagous feeding behaviour of the insect vector (Weintraub and Beanland 2006; Foissac and Wilson 2010).

Phytoplasmas were thought to be of viral origin since it could not be cultured in artificial media and could pass through a bacteria-proof filter. These causal agents of many yellows, dwarf and witches' broom diseases and similar diseases were referred as mycoplasma-like organisms (MLOs), till 1994 (Doi et al. 1967; McCoy et al. 1989). In 1994, the name 'phytoplasma' was adopted by the Phytoplasma Working Team at the 10th International Congress of the International Organization for Mycoplasmology, replacing the term MLO (ICSB-Mollicutes 1993 and 1997).

The Phytoplasma Working Team of the International Committee on Systematic Bacteriology (ICSB) subcommittee for the taxonomy of Mollicutes (ICSB-Mollicutes), the International Research Programme on Comparative Mycoplasmology (IRPCM), adopted a taxonomic rule and proposed to erect a genus-level provisional taxon '*Candidatus* (*Ca.*) Phytoplasma' (IRPCM 2004) based on near-full-length sequence of phytoplasma 16S rRNA. As per IRPCM rules, a novel '*Ca.* Phytoplasma' species description should refer to a single, unique 16S rRNA gene sequence of greater than 1200 bp and share less than 97.5 % sequence

similarity to that of any previously described '*Ca*. Phytoplasma' species unless the phytoplasma under consideration clearly represents an ecologically separated population (IRPCM 2004). The 16S rRNA gene of a novel '*Ca*. Phytoplasma' species should possess at least one unique sequence region in addition to the signature sequence that is characteristic of phytoplasmas: 5′-CAAGAYBATKATGTKTAGCYGGDCT-3′, representative of annealing site of universal primer R16F2n (IRPCM 2004).

The 16S rRNA genes have served as the primary character for phytoplasma molecular taxonomy and classification as they contain ample information for differentiation of a wide array of phytoplasma strains. The genus-level taxon '*Candidatus* Phytoplasma' and existing phytoplasma classification schemes were established based on 16S rRNA gene sequences. Currently, there are two widely accepted phytoplasma classification schemes: one is based on phylogenetic analysis of 16S rRNA gene sequences (Kirkpatrick and Fraser 1989; Namba et al. 1993; Schneider et al. 1993; Gundersen and Lee 1996; Smart et al. 1996) and another is based on restriction fragment length polymorphism (RFLP) analysis of a 1245 bp PCR-amplified 16S rRNA gene fragment (Wei et al. 2008; Zhao et al. 2009). Whilst both schemes can reliably classify diverse phytoplasmas into groups, the latter offers a faster mechanism, by distinguishing subtle RFLP pattern differences, to identify and differentiate distinct subgroup lineages amongst phytoplasmas within individual groups.

To automate the RFLP analysis, Zhao et al. (2009) designed an interactive online tool, *iPhyClassifier*, to expand the efficacy and capacity of the current 16S rRNA gene sequence-based phytoplasma classification system. The *iPhyClassifier* performs sequence similarity analysis, simulates laboratory restriction enzyme digestions and subsequent gel electrophoresis and generates virtual restriction fragment length polymorphism (RFLP) profiles. Based on calculated RFLP pattern similarity coefficients and overall sequence similarity scores, *iPhyClassifier* makes suggestions on tentative phytoplasma 16Sr group/subgroup classification status and '*Candidatus* Phytoplasma' species assignment. However, the *iPhyClassifier* requires a full- or near-full-length (~1245 bp), good quality 16S rRNA query sequence, generally amplified using phytoplasma 16S universal primers R16F2n and R16R2 (Gundersen and Lee 1996; Zhao et al. 2009). Till now, the PCR-RFLP-based classification scheme has delineated 31 phytoplasma groups and more than 100 subgroups.

The phytoplasma 16S–23S rRNA intergenic spacer (IGS) region which is about 232 bp (varies in different species) contains a portion that codes for the highly conserved $tRNA^{Ile}$. However, the flanking sequences that extend from the $tRNA^{Ile}$ to 16S rRNA and to 23S rRNA are variable amongst various phytoplasmas. IGS region can serve as a useful tool for differentiation of phytoplasma groups and subgroups. Overall, the IGS region is comparable to the 16S rRNA gene sequence in its capacity for use in delineating distinct phytoplasma lineages (Smart et al. 1996). Combined analysis of the entire 16S rRNA gene plus IGS region sequence proved to be useful in several cases for differentiating distinct type of strains within a given 16S rRNA subgroup (Marcone et al. 2000; Andersen et al. 2006).

The *tuf* gene, encoding the elongation factor, EF-Tu, is another highly conserved gene that has been frequently used to distinguish and classify phytoplasmas. It was found that *tuf* gene, like 16S rRNA gene, emerged as a potential marker for classification of phytoplasma (Makarova et al. 2012). The nucleotide sequence similarities amongst the aster yellows (AY), peach X-disease and stolbur (STOL) phytoplasma groups ranged from 87.8 to 97.0 %. Phytoplasma groups and subgroups were also differentiated based on RFLP analyses. The resolving efficacy for separation of distinct lineages amongst phytoplasmas was found to be lower than that of the 16S rRNA gene (Marcone et al. 2000). Further, DNA sequences of *sec*A and *sec*Y (Lee et al. 2005) and 23S rRNA gene (Hodgetts et al. 2008) were employed for classification of phytoplasmas. The sequence similarity for 480 bp amplicon of *sec*A ranged from 69.7 to 84.4 % for phytoplasma strains representing 12 16S rRNA groups. Several molecular markers, other than the 16S rRNA

gene identified, have thus shown much-improved resolving power in delineation of these ecological strains.

5.8 Conclusion

Now, it is evident that the agriculturally important microorganisms are the hope of sustainable agriculture and backbone of agricultural-based economy. Bacteria, fungi and cyanobacteria are most extensively studied agriculturally important microorganisms in terms of plant growth promotion and causative agents of animal and plant diseases. Other microbes like phytoplasmas and viruses cause diseases in valuable plants and crops and are responsible for the economic loss. Current practice of bio-inoculant formulations is mainly based on bacteria, fungi and cyanobacteria. In order to produce the right bio-inoculants and for quick identification of plant pathogens, species and strain level identification of microorganisms is mandatory.

It is evident from the above discussion that the taxonomical characterisation of microorganisms using single aspects of identification is not complete and it provides misleading information about the taxonomic status of the organisms. Several examples of reclassification of previously classified microbes are available in the literature which indicates the lacuna of traditional characterisation. Polyphasic approach of microbial classification is the current practice in microbial taxonomy. Therefore, authentic characterisation of microorganisms using polyphasic approach is a must before using them as bio-inoculants or developing of the kits for quick identification of pathogen. Unfortunately, most of agricultural microbiologists and plant pathologists still use either traditional morphological and physiological approach or just simply doing small subunit rRNA gene sequencing for identification and do not bother about complete characterisation of the organisms due to time-consuming nature of the polyphasic study. Extensive work has been done in the area of bacterial taxonomy and species, and strain level typing is possible in short time and less labour. In comparison to bacteria, little work has been done in fungal and cyanobacterial taxonomy. Plant pathologists and mycologists generally use traditional approach of classification. Therefore, the species and strain level resolution in fungi and cyanobacteria is not very clear and needs more work using modern tools and polyphasic approaches of microbial classification.

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Microbial Inoculants: Identification, Characterization, and Applications in the Field

6

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Abstract

Microorganisms play a very important role in recycling of nutrients and organic compounds. They are also involved in improving structure and fertility of soils and managing plant health and ecosystem functioning. Microbes show interactions with plants, animals, and soils, working sometimes as pathogens while sometimes for mutual benefits.

Currently very advanced biochemical, microbial, and molecular tools and techniques have been developed which provide accurate, rapid methods for determining microbial diversity in any ecosystem. About 99 % of the microbes in the environment are non-culturable; therefore, much more efforts are required to make them culturable and then identifiable.

Microorganisms are potentially useful for accelerating plant growth and increasing crop yields. It has been observed that significant numbers of microbial species, usually associated with the plant rhizosphere, are able to exert a beneficial effect upon the growth of plant.

They possess inevitable role in nutrient supply (N_2 fixation, P solubilization, IAA production, etc.) or biocontrol mechanism. In field, the beneficial effects of microbial inoculants has been proved by various researchers. In this chapter, various approaches employed in identification and characterization of culturable microbes, their various plant growth-promoting features, and role as bioinoculants have been given.

Keywords

Microbes • PGPR • ARDRA • RISA • Molecular methods • BIOLOG • FAME • N_2 fixation • P solubilization • HCN production

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6.1 Introduction

Microorganisms are cosmopolitan and generally single-cell organisms representing the oldest form of life which are present everywhere in the environment. Microbes play an important role in the various processes of multicellular organisms such as plants and animals. Microorganisms are important component of agriculture, food, and other technology including engineering and medical sciences (Davinson 1988; Whitman et al. 1998; Glick 1995; Sloan et al. 2006; Ghazanfar et al. 2010). Till now, very little is known about various aspects related to microbes since around 99 % are microbes are unculturable. Microorganisms play a key role in the recycling of various nutrients and organic compounds, plant health and nutrition, ecosystem functioning, structure and fertility of soil, etc. However, despite of the advancement of various technology, our knowledge about these tiny and most powerful organisms are still very less.

In addition, free-living microbes are also attached with certain plants and animals, sometimes as pathogens while sometimes for mutual benefits. Study about these interactions is very useful for particular ecological niche. Additionally, gene transfer between microbes is another important characteristic which provides clues about their interaction with environment (Lorenz and Wackernagel 1994). Due to their gene transfer ability, microorganisms are not only present in diverse habitats but there is also diversification in their evolution. During this review, we are going to provide various roles and identification methods of microbes and their applications in the field.

6.2 Microbes as Plant Growth Promoters

Microbes play a direct role in plant growth promotions. Under natural environmental conditions, plants' root interacts with a large number of microorganisms, which play a major role in plant growth and multiplications (Lynch 1990; Glick 1995). Significant numbers of bacteria associated with the rhizosphere provide beneficial effect to plant either in its growth or disease control. These are termed as plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978). Therefore, they are either used as biofertilizers or as controlling agents for agriculture improvement for several years (Kloepper and Schroth 1978; Suslov 1982; Davinson 1988; Kloepper 1994; Glick 1995). Several beneficial strains from various genera, including *Acinetobacter*, *Azospirillum*, *Burkholderia*, *Bacillus*, *Enterobacter*, *Erwinia*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Alcaligenes*, *Arthrobacter*, *Flavobacterium*, etc., have been identified and characterized for their plant growth promotion activity (Shahi et al. 2011).

Plant-associated bacteria can be categorized into beneficial, neutral, and deleterious groups on the basis of their effects on plant (Dobbelaere et al. 2003). Plant growth-promoting bacteria (PGPB) exert their positive effect on plant growth either by direct or indirect mechanism (Glick 1995).

Bacteria play a direct role in growth promotion via N_2 fixation (Christiansen-Weneger 1992); phosphate solubilization from inorganic phosphate to organic phosphate, which makes phosphorous available to the plants (Krasilnikov 1961; Gaur and Ostwal 1972; Shahi et al. 2011); biosynthesis of phytohormones (Xie et al. 1996); decrease in membrane potential of the roots (Bashan and Levanony 1991); synthesis of enzymes like ACC deaminase which modulate the level of plant hormones (Glick et al. 1998); etc.

In addition to their direct role, bacteria also play an indirect role in plant growth promotion by "preventing them from harmful effect of pathogenic microorganisms," usually due to the synthesis of antibiotics or siderophores (Leong 1986; Sivan and Chet 1992).

Microbial inoculants have been utilized to enhance the plant yields in many countries, and their commercial products are available in the market. For example, several biofertilizers are commercially available for different crops, generally using strains of *Azotobacter*, *Azospirillum*, *Rhizobium*, *and Burkholderia*.

Several researchers have described about the potential use of plant-associated bacteria as plant growth promoters and their role in managing soil and plant health (Rovira 1965; Glick 1995; Hallman et al. 1997; Sturz et al. 2000; Welbaum et al. 2004; Shahi et al. 2011).

Plant growth-promoting bacteria (PGPB) are related to different plant species and present in various environments. The most commonly studied plant growth-promoting bacteria are PGPR (plant growth-promoting rhizobacteria) which colonize the surface of the root and the closely adhering soil interface, termed as rhizosphere (Bashan and Holguin 1998; Kloepper and Schroth 1978; Kloepper et al. 1999). In brief, bacteria which colonize plant roots and promote plant growth are termed as PGPR. Also, PGPR showed endophytic associations with plants in which PGPR enter into the root and form colonization (Kloepper et al. 1999; Gray and Smith 2005). The level of endophytic colonization reflects the ability of bacteria to adapt in specific ecological niches (Gray and Smith 2005). In endophytic association, an intimate associations between host plants and bacteria can be formed without harming the plant (Hallman et al. 1997; Kloepper et al. 1999; Whipps 2001; Lodewyckx et al. 2002; Compant et al. 2005).

In spite of different ecological niches, endophytic bacteria as well as free-living rhizobacteria use approximately similar mechanisms to promote plant growth and control phytopathogens (Glick 1995; Sturz et al. 2000; Bloemberg and Lugtenberg 2001; Lodewyckx et al. 2002; Dobbelaere et al. 2003). In addition to its growth promotion activity, PGPB are involved in biocontrol in the host plants to a broad range of pathogens (biotic stress) and abiotic stresses (Glick 1995; Haas et al. 2000; Bloemberg and Lugtenberg 2001; Ryu et al. 2004).

6.3 Isolation and Identification Methods

For microbial isolation, the first requirement is that it can be cultured in the laboratory. Isolation process requires knowledge of optimal temperature, optimal oxygen requirements, and optimal nutritional needs. There are two main ways to isolate organisms: (1) streaking for isolation on an agar plate and (2) pour plate method. Streaking on an agar plate involves the successive dilution so that recognizable individual colonies appear. This is a rapid qualitative isolation method which is commonly employed in the isolation of discrete colonies. During streaking, the four ways or quadrant streak is mostly done. In the pour plate method, samples should dilute sufficiently before its pouring on the plate. The isolated cells give rise to individual colonies. This method yields individual colonies on the surface of the agar plate.

6.4 Microbial Identification

Under laboratory condition, microbes are cultured on widely used common or specific media. These conventional methods followed by physiological and biochemical tests are involved in the characterization of microbes (Amann et al. 1995). Various techniques which involve different energy sources for culture, identification of metabolites, etc., are employed for the taxonomic classification and identification which led to the recognition of mostly uncharacterized microbial life (Abbaszadegan 2004). Since 99 % of the microorganisms in the environment are nonculturable, much efforts will be required to culture the microbial communities (Hanada 2003; Kamagata and Tamaki 2005; Rappe and Giovannoni 2003; Sekiguchi 2006). Conventional methods for microbial community analysis concentrate on cultivation and isolation of isolates, separated from several niches. Only restricted approaches and culture media are available for this purpose. For the identification of cultures, selective media are in use, but it should be verified by the conventional biochemical (BIOLOG) and molecular methods (Garbeva et al. 2004; Ghazanfar et al. 2010; Hugenholtz 2002; Kirka et al. 2004).

Knowing that morphology and colonial diversity are widely used parameter for the distinction of bacteria, they cannot give precise

identification of microbial communities (Ghazanfar et al. 2010). Various methods for the identification of microbes, such as DNA-based molecular methods including polyphasic approaches, are employed by some workers (Griffiths et al. 2000). Now polyphasic techniques which mainly rely on 16S rRNA coupled with other methods give much insight on the prokaryotic diversity (Griffiths et al. 2000). Currently for prokaryotic diversity analysis, multiple statistical methods are used including estimated species richness, diversity indices, and rarefaction curve analysis (Hughes et al. 2001). The following various methods are involved in the identification of culturable microbes (Table 6.1):

1. Plate count method
2. FAME (Fatty Acid Methyl Esters) analysis
3. Physiological profiling/carbon substrate utilization
4. Random amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF)
5. Amplified ribosomal DNA restriction analysis (ARDRA)
6. Multilocus sequence typing (MLST)
7. Fluorescence in situ hybridization (FISH)
8. Ribosomal intergenic spacer analysis (RISA)

6.4.1 Plate Count Method

This is one of the initial methods used for the identification and discrimination of microbes, which is based on colony spreading on which microbes and spore-germinating fungi are allowed for their growth (Dix and Webster 1995). This method provides the valuable information about the different component of the microbial colonies. Apart from the above mentioned features, this method also have some limitations, like maintaining various growth parameters (e.g., pH, temperature, light), source of microbes isolation (like soil, water, etc.), and a large number of bacteria and fungi are still unculturable (Tabacchioni et al. 2000).

6.4.2 Fatty Acid Methyl Esters (FAME) Analysis

This recent technique is employed in the identification and characterization of various microbes. The FAME analysis employs fatty acids grouping for species-level microbial identification and also describes community composition. Major taxonomic groups in any microbial community possess signature fatty acids, which can differentiate them from other microbes, and hence any deviation in the fatty acid profile would reflect change in the microbial community of any specific ecosystem. It is frequently employed for the study of the microbial community composition and population dynamics analysis, particulary when microbes are subjected to chemical contaminants (Siciliano and Germida 1998; Kelly et al. 1999) and for identification of new microbial species in an ecosystem.

6.4.3 Physiological Profiling (BIOLOG)

BIOLOG or physiological profiling is based on profiling of variable sole source carbon utilization. It was developed by Garland and Mills (1991). It includes 96-well microtitre plate, where different plates are available for gram-positive and gram-negative bacteria. Each plate possesses diverse carbon sources in 95 well, whereas last well is without substrate and serves as control. This method was originally developed for clinical bacteria, but now a days, it is widely used for soil microbial community identification. As certain fungal communities are not able to grow on gram-positive and gram-negative plates, separate plates are also available for fungal identification (Derry et al. 1999).

6.4.4 RAPD (Random Amplified Polymorphic DNA) and DAF (DNA Amplification Fingerprinting)

RAPD (random amplified polymorphic DNA) and DAF (DNA amplification fingerprinting) are

Table 6.1 Approaches for identification of microbes able to grow in laboratory conditions

S. no.	Methods	Features	References
1	Plate count method	Based on colony spreading	Dix and Webster (1995) and Tabacchioni et al. (2000)
2	FAME	Based on fatty acids grouping	Siciliano and Germida (1998) and Kelly et al. (1999)
3	Physiological profiling/carbon substrate utilization	Based on different sole source carbon utilization profiling	Garland and Mills (1991) and Derry et al. (1999)
4	Random amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF)	Based on amplification of DNA with a short-length primer which anneals randomly at multiple sites on the genomic DNA under low annealing temperature	Franklin et al. (1999)
5	Amplified ribosomal DNA restriction analysis (ARDRA)	Based on alteration in the nucleotide sequence present in PCR product of 16S rRNA genes	Cook and Meyers (2003), Laurent et al. (1999), Steingrube et al. (1997), and Wilson et al. (1998)
6	Multilocus sequence typing (MLST)	Based on the use of DNA sequences from multiple regions in the genome	Maiden et al. (1998) and Xu (2006)
7	Fluorescence in situ hybridization (FISH)	Based on oligonucleotide probes containing a fluorescent dye at the 5′ end which bound to cellular rRNA	Amann et al. (1995) and Pernthaler et al. (2002)
8	Ribosomal intergenic spacer analysis (RISA)	Based on PCR amplification of a portion of the intergenic spacer region (ISR) present between the small (16S) and large (23S) ribosomal subunits	Fisher and Triplett (1999)

types of molecular markers in which amplification of single sample of DNA at many loci or gene part occurs in a single PCR reaction. Due to its simplicity, RAPD/DAF is widely applied in the fingerprinting of closely related microbial species (Franklin et al. 1999).

In this method, about ten nucleotide long primer is used to anneal randomly at many locations on the genomic DNA keeping annealing temperature at low (Franklin et al. 1999), which results in varying size PCR amplicons in a single reaction. Amplicons can be separated on agarose/PAGE. Results of RAPD/DAF depend on the quality and quantity of template DNA as well as primer used. RAPD/DAF is very sensitive to experimental conditions and thus optimization of primers and reaction conditions are required for precise determination of microbial communities. Amplicons generated is employed for the microbial identification.

6.4.5 Amplified Ribosomal DNA Restriction Analysis (ARDRA) and Restriction Fragment Length Polymorphism (RFLP)

ARDRA is based on 16S rRNA genes. This technique uses differences between the PCR amplicons of 16S rRNA genes, and it is useful for identification of microbes at species and genus level. After amplification of 16S rRNA gene from environmental DNA, PCR product is digested with restriction endonucleases (e.g., *Alu*I and *Rsa*I), and then gel electrophoresis is carried out for digested fragments. Along with sequence information, ARDRA is better identification tool of microbes in any environmental samples.

Similar to ARDRA, restriction fragment length polymorphism (RFLP) is employed for

the analysis of diversity among other genes. RFLP is very effective for the characterization of microbes at genetic as well as species level (Laurent et al. 1999; Steingrube et al. 1997; Wilson et al. 1998) and is helpful in the screening of various types of microbes and diversity of microbial communities (Alves et al. 2002; Laurent et al. 1999; Sjöling and Cowan 2003).

The above techniques are helpful for the comparison of microbial diversity or screening of microorganisms over time. They have some limitation that it is too hard to separate restriction fragment by agarose/PAGE (Rastogi and Sani 2011).

6.4.6 Multilocus Sequence Typing

Multilocus sequence typing (MLST) is low-cost and efficient DNA sequencing technologies employed in the characterization of microbial populations. MLST have accelerated microbial research to higher level. It uses DNA sequences from multiple regions of the genome for separating microbial isolates from mixed population. This approach was first employed in 1998 for typing human bacterial pathogens by Maiden et al. (1998); now it is also used for the characterization of various microbial, ecological, and evolutionary patterns. Additionally, MLST is also called as multiple gene genealogical analysis (MGGA) or comparative genealogical analysis (CGA) (Xu 2006). Data generated with MLST has various advantages because much more information can be generated due to sampling from multiple regions of the whole genome. When MLST compared with other methods like RAPD, RFLP, PCR fingerprinting, and AFLP (amplified fragment length polymorphisms) is employed in microbial population analysis, it is more beneficial because unambiguity of nucleotides in a DNA sequence provides much certainty for various analyses. Additionally, evolutionary history can be better represented well by MLST since it uses DNA segment information of nucleotide. MLST is employed in the analysis of ecological genetics of several microbial communities which provide scope for genetic and genotypic diversity analysis among the populations.

6.4.7 Fluorescence In Situ Hybridization (FISH)

This technique (FISH) is based on hybridization of entire cell with oligonucleotide probes (Amann et al. 1995). Here a probe of 18–30 nucleotides length is employed which contains fluorescent dye at the 5′ end. Fluorescent dye help in the checking of probe hybridization with cellular rRNA by epifluorescence microscopy. Here by utilizing intensity of fluorescent signals, growth rates can be determined and also estimation of metabolic condition of the cells can be done.

In case of mixed microbial population determination, FISH can be coupled with flow cytometry (Caracciolo et al. 2010). Various probes are available to target different bacterial groups.

Catalyzed reporter deposition (CARD) FISH technique is advance version of FISH which uses tyramide-labeled fluorochromes and accumulate numerous fluorescent probes at the target site leading to enhanced signal intensity and sensitivity (Pernthaler et al. 2002). Other modified technique is secondary-ion mass spectrometry (SIMS) (Li et al. 2008).

6.4.8 Ribosomal Intergenic Spacer Analysis (RISA)

RISA involes amplification of rRNA gene operon between the 16S and 23S ribosomal subunits, known as intergenic spacer region (ISR) (Fisher and Triplett 1999). This technique is involved in the community analysis from dissimilar environments. ISR is dissimilar in terms of length and nucleotide sequence throughout various microbial groups. In RISA each band suggests a particular bacterial population.

Additionally, in automated RISA (ARISA), fluorescence-labeled primer is used which is detected by a laser detector. It can examine multiple samples at the same time; the main restriction with this technique is that it gives an overestimate of microbial richness and diversity (Fisher and Triplett 1999). ARISA is also employed during the analysis of bacterial species from different geographical soils which results in

diverse profiles (Ranjard et al. 2001). Based on the above statements, it can be concluded that ARISA is a sensitive, rapid, and effective method for evaluating complex microbial community at different scales.

6.4.9 Use of Bioinformatics in Microbial Identification

During the past decades, in the area of biology and molecular biology, much improvement are made, which results in vast amount of datasets that require storage, analysis, and management. Advancements in the area of biology/molecular biology and database gave rise to the development of multidisciplinary subject, termed as bioinformatics. Bioinformatics helps in the identification process of microbes by developing online databases and various computational approaches (Carriço et al. 2013). Area of bioinformatics provides deeper insight into the lifecycle, evolution, and working phenomenon of microbes (Fang et al. 2010).

Bioinformatics is very useful for the microbial research because it helps researchers in collection, analysis, and interpretation of data related to microbial identification and characterization, microbial diversity, molecular taxonomy, and community analysis (Singh et al. 2012). Here computational methods (bioinformatics) are helpful in better understanding of microbial systems.

The above mentioned approaches are widely used during identification of microbes. However, due to advancements in technology, new techniques are evolving continuously and are giving a better understanding of diverse microbial world.

6.5 Characterization of Microbes for Agriculturally Important Traits

Microorganisms are potentially useful for accelerating plant growth and increasing crop yields (Burr et al. 1984). Significant number of microbial (bacterial) species, usually associated with the plant rhizosphere, are able to exert a beneficial effect upon the growth of plant. Today researchers are capable to use them successfully in field experiments. Increased growth and yields of potato, sugar beet, radish, and sweet potato have been reported (Farzana et al. 2009). At commercial level, applications of microbes are being tested and successful results are coming. But still much more microbial plant interactions are needed which result in higher success rate of field applications (Burr et al. 1984). Agriculturally important characteristics of different microbial communities are briefly described below:

6.6 Biological Nitrogen Fixation

Microbes play an important role in biological nitrogen fixation. A large number of bacteria from genera *Acinetobacter*, *Azospirillum*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Erwinia*, *Enterobacter*, *Flavobacterium*, *Pseudomonas*, *Rhizobium*, and *Serratia* are associated with the rhizosphere and are able to exert a beneficial effect on plant growth including nitrogen fixation (Egamberdiyeva 2005; Tilak et al. 2005; Shahi et al. 2011). It has been reported that biological nitrogen fixation provide 180×10^6 metric tons/year nitrogen globally, in which symbiotic microbial associations provide 80 % and the rest 20 % of nitrogen fixation done by free-living or associative microbial system (Graham 1998). Bacteria and Archaea perform this nitrogen fixation activity from the atmosphere to soil (Young 1992). Biological nitrogen fixation includes symbiotic nitrogen fixation and free-living (nonsymbiotic) nitrogen fixation as follows:

(a) Symbiotic nitrogen fixers

Symbiotic nitrogen-fixing microbes show symbiotic association with plant. Its two groups have been studied in details, which includes *Rhizobia* and *Frankia* where *Rhizobium* is obligate symbionts in leguminous plants while *Frankia* in non-leguminous trees. It has been reported that *Frankia* forms root nodules in around 280 species of woody plants belonging to 8 different families (Schwintzer and Tjepkema 1990).

(b)Nonsymbiotic nitrogen-fixing microbes

Nonsymbiotic microbes do not show any symbiotic association, and they can fix nitrogen free-living, associative, or endophytic state. Examples of these groups are cyanobacteria, *Azospirillum*, *Azotobacter*, *Acetobacter diazotrophicus*, *Azoarcus*, etc. This type of N_2 fixation has a great agronomical importance. Some beneficial nonsymbiotic nitrogen-fixing bacteria include *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Bacillus*, *Acetobacter*, *Azospirillum*, *Bacillus megaterium*, *Azotobacter* sp., *Achromobacter*, *Azoarcus* sp., *Arthrobacter*, *Azomonas*, *Beijerinckia*, *Clostridium*, *Corynebacterium*, *Derxia*, *Rhodospirillum*, *Xanthobacter*, and many more (Saxena and Tilak 1998; Vessey 2003; Shahi et al. 2011).

6.7 HCN Production

Microorganisms can also work as biocontrol agents when it colonized with plant root surfaces (Suslow and Schroth 1982). Cyanide is a highly toxic chemical which acts as a metabolic inhibitor and biocontrol agent and is produced by certain microbes (Heydari et al. 2008). Biosynthesis, excretion, and metabolism of cyanide are done by some bacteria, algae, and fungi. During HCN production by associated microbes, host plants are usually not affected by the negative impact of cyanide; hence, certain specific rhizobacteria can act as biological weed-control agents (Zeller et al. 2007).

HCN is a type of secondary metabolite which negatively affects metabolism and growth of root (Schippers et al. 1990) and has a potential role as a biological control agent for the weeds (Heydari et al. 2008). HCN production commonly occurred in *Pseudomonas*, *Bacillus*, etc., in the rhizospheric soil and plant root nodules where it works as a biocontrol agent (Ahmad et al. 2008).

6.8 Phosphate Solubilization

Microbes also play an important role in phosphate solubilization. They are key element in the natural phosphorus cycle which occurs by means of the cyclic oxidation and reduction of phosphorus compounds. P solubilizing bacteria increase P uptake by the plant which leads to increase in crop yield. Several bacterial strains have been reported (Shahi et al. 2011) in which *Bacillus*, *Pseudomonas*, *Rhizobium*, etc., are good phosphate solubilizers. Mechanism for mineral P solubilization is the production of organic acids, and phosphatases play a key role in the mineralization of organic phosphorous in the soil. Phosphate-solubilizing microbes (PSM) give an alternative route for sustainable agriculture to fulfill the P demands of plants. PSM include many bacteria and fungi. Efficient PSM belong to bacterial genera *Bacillus*, *Rhizobium*, *Pseudomonas*, etc., and fungal genera *Aspergillus*, *Penicillium*, etc. Rivas et al. (2006) reported *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* as good phosphate solubilizers.

6.9 Siderophore Production

Iron is an essential element for all organisms. During iron-limiting circumstances, microorganisms including bacteria synthesize low-molecular-weight compounds termed as siderophores to competitively acquire ferric ion (Whipps 2001; Miethke and Marahiel 2007). In greek, siderophores means "iron carrier." These iron-chelating compounds are secreted by microorganisms like bacteria, fungi, and others (Neilands 1995; Miethke and Marahiel 2007; Shahi et al. 2011).

Microorganisms liberate siderophores to scavenge iron and furthermore formation of its soluble Fe^{3+} complexes which can be utilized by active transport mechanisms. Siderophores are non-ribosomal peptides (Miethke and Marahiel 2007), while several biosynthesized independently (Challis 2005). Apart from this, in case of pathogenic bacteria, siderophores are also important due to their role in acquisition of iron (Miethke and Marahiel 2007).

6.10 Other Beneficial Activities by Microbes

Microbes also play a very important role in plant growth promotion through the synthesis of various plant hormones such as auxins (including IAA),

cytokinins, gibberellins, ethylene, abscisic acid, etc. Synthesis of indole-3-acetic acid (IAA), which is a type of auxin, has been reported in several bacterial genera, *Pseudomonas*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Acetobacter*, *Azospirillum*, *Bacillus megaterium*, *Azotobacter*, *Rhizobium*, and others (Shahi et al. 2011). Some plant growth-promoting rhizobacteria play a role in ACC (1-aminocyclopropane-1-carboxylate) deaminase activity. Since ACC is an immediate precursor of ethylene, by hydrolyzing, it promotes root growth by lowering ethylene levels in the root environment.

6.11 Applications of Microbial Inoculants in the Field

Various workers have proved the role of microbial inoculants in the fields (Çakmakçi et al. 2006; Egamberdiyeva 2007; Roesti et al. 2006). For example, Çakmakçi et al. (2006) studied growth promotion of plants by selected growth-promoting rhizobacteria including *Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Rhodobacter*. They conducted their experiment in greenhouse and field in two soil type in order to investigate seed inoculation of sugar beet. They observed that under greenhouse, inoculations with PGPR increased weight of sugar beet root by 2.8–46.7 % depending on the species. It was observed that plant growth promotion responses were variable and dependent on the soil organic matter content, growing stage, inoculant strain, harvest date, and growth parameter evaluated.

Effect of plant growth-promoting bacteria on growth and nutrient uptake of maize was studied by Egamberdiyeva (2007). During this study the influence of two different soil types on the stimulatory effect of PGPR for maize was observed. Results indicate that plant growth stimulates efficiency of bacterial inoculants affected by soil nutritional condition. The bacterial inoculation has a much better stimulatory result on plant growth in nutrient-deficient soil than in nutrient-rich soil. Roesti et al. (2006) studied about the bacterial community during a growing season in three wheat fields which differ primarily by fertilizer management and yield and also studied about the effects of PGPR/AMF bioinoculations on the wheat growth and bacterial community structure. They observed that wheat rhizobacterial community structure is highly dynamic and influenced by various factors like plant's age, fertilizer input, and type of bioinoculant. Also, they obtained a distance-related effect of the root on the bacterial community. During the study, they concluded that a combined bioinoculation can synergistically improve the nutritional quality of the grain without negatively affecting mycorrhizal growth.

Daza et al. (2000) studied about growth and survival of *Rhizobium leguminosarum* bv. *phaseoli*, *R. tropici*, *Bradyrhizobium japonicum*, and *Bacillus megaterium* in peat and perlite-based inoculants. Generally, it was observed that survival was similar for all strains in both carriers. Better survival was noticed when inoculants were kept at 4 °C compared to 28 °C. Stephens and Rask (2000) successfully described the formulation and commercial-grade production of *Rhizobium* or *Bradyrhizobium* legume inoculants.

6.12 Conclusion

Based on the conclusion of the above literature, it has been proved that microbes show beneficial interactions with soils, plants, and animals. Microbes play a significant role in agriculture by improving fertility of soils and helping them via biocontrol mechanism. Since very large proportions of microbes are non-culturable, much more efforts are required to make them culturable and identifiable. Large number of techniques and approaches are explained and focused for the identification of microbes. Conventional methods like plate count method, physiological profiling using BIOLOG, and FAME analysis are biochemical-based methods for the identification. Apart from this, several molecular methods like ARDRA, RAPD, FISH, and multilocus sequence typing are better elaborated and elucidated identification methods for microbial system.

Microbes play a direct role in nutrient supply (N_2 fixation, P solubilization, IAA production, etc.) or biocontrol mechanism. Based on above observation, we can conclude that microorganisms are potentially helpful in accelerating plant growth and increasing crop yields. Microbes play a direct role in nutrient supply or in biocontrol mechanism. In field the beneficial role of microbial inoculants has been proved by various researchers.

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Endophytic Microbes in Crops: Diversity and Beneficial Impact for Sustainable Agriculture

7

Archna Suman, Ajar Nath Yadav, and Priyanka Verma

Abstract

Endophytic microbes are ubiquitous in most plant species. Endophytic microbes enter plants mainly through wounds, naturally occurring as a result of plant growth or through root hairs and at epidermal conjunctions. Besides gaining entrance to plants through natural openings or wounds, endophytic microbes appear to actively penetrate plant tissues using hydrolytic enzymes like cellulase and pectinase. Diverse community structure of endophytes can be analyzed using culture-dependent and culture-independent method. Endophytic bacteria belong to different phyla such as Acidobacteria, Actinobacteria, Ascomycota, Bacteroidetes, Basidiomycota, Deinococcus-Thermus, and Firmicutes. Endophytic archaea (Euryarchaeota) were reported using only culture-independent method. Endophytic microbes were most predominant and studied and belonged to three major phyla Actinobacteria, Proteobacteria, and Firmicutes. Among reported genera *Achromobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Pantoea*, *Pseudomonas*, *Rhizobium*, and *Streptomyces* were dominant in most host plants. Along with common endophytic microbial genera, there were many niche-specific microbial genera that have been reported from different host plants. Application of associative microbes for sustainable agriculture holds immense potential. Endophytic microbes are known to enhance growth and yield of plants by fixing atmospheric nitrogen and solubilization of phosphorus, potassium, and zinc; production of phytohormones (cytokinins, auxins, and gibberellins), ammonia, hydrogen cyanide, and siderophores; and possession of antagonistic activity as well as reducing the level of stress ethylene in host plants. Endophytes seem to contribute to plant fitness and development, displaying beneficial traits that can be

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exploited in agricultural biotechnology. The interactions between endophytes and plants can promote plant health and play a significant role in low-input sustainable agriculture for both food and nonfood crops. This chapter summarizes part of the work being done on endophytic microbes, including their isolation, identification, diversity, distribution, and applications for sustainable agriculture.

Keywords
Endophytic microbes • Diversity • Plant growth promotion • Biocontrol • Sustainable agriculture

7.1 Introduction

The endophytic microbes are referred to those microorganisms, which colonize in the interior of the plant parts, *viz:* root, stem, or seeds without causing any harmful effect on host plant. The word *endophyte* means "in the plant" and is derived from the Greek words *endon* (within) and *phyton* (plant). The usage of this term is as broad as its literal definition and spectrum of potential hosts and inhabitants, e.g., bacteria (Kobayashi and Palumbo 2000), fungi (Stone et al. 2000), and insects in plants (Feller 1995). Endophytes have been defined by various authors in somewhat different ways (Rosenblueth and Martínez-Romero 2006; Mercado-Blanco and Lugtenberg 2014). Microbial endophytes can be isolated from surface-disinfected plant tissue or extracted from internal plant tissues (Hallmann et al. 1997). Endophytes inside a plant may either become localized at the point of entry or spread throughout the plant. These microorganisms can reside within cells (Jacobs et al. 1985), in the intercellular spaces or in the vascular system (Bell et al. 1995). Endophytic microbes enter in host plants mainly through wounds, naturally occurring as a result of plant growth or through root hairs and at epidermal conjunctions (Quadt-Hallmann et al. 1997). Other entry sites for endophytic microbes include flowers, stomata, and lenticels (Kluepfel 1993). Endophytic microbes have an ecological advantage over the epiphytic microbes in that they are protected from adverse external environmental conditions of temperature, salinity, drought, pH, osmotic potentials, and ultraviolet radiation.

Endophytic microbes can promote plant growth directly through N_2- fixation; phytohormones production (IAA and gibberellic acids) and solubilization of phosphorus, potassium, and zinc; and production of siderophore or indirectly through inducing resistance to pathogen by production of ammonia, hydrogen cyanide, siderophores, lytic enzymes, and antibiotics. Endophytic microbes may promote plant growth in terms of increased germination rates, biomass, leaf area, chlorophyll content, nitrogen content, protein content, roots and shoot length, yield, and tolerance to abiotic stresses like draught, temperature, flood, salinity, pH, etc. (Hallmann et al. 1997; Rosenblueth and Martínez-Romero 2006; Verma et al. 2013, 2014, 2015b).

Among the microbial groups, arbuscular mycorrhizal (AM) fungi are known to promote activities which can improve agricultural developments. Thus, these microorganisms appear as a research target with regard to sustainability purposes. Mycorrhizal fungi are a heterogeneous group of diverse fungal taxa, associated with the roots of over 90 % of all plant species. Endomycorrhizae have several functions, the major one being nutrient acquisition. Endomycorrhizae facilitate the exchange of nutrients between the host plant and the soil. Mycorrhizae help plants in the uptake of water, inorganic phosphorus, mineral or organic nitrogen, and amino acids. In exchange for the mycorrhizae providing all of these nutrients, the plant

in turn provides the mycorrhizae with carbon (Finlay 2008; Bonfante and Genre 2010).

Endophytic microbes live in plant tissues in the form of symbiotic association to slightly pathogenic without causing substantive harm to the host. Endophytic microbes have been isolated from a variety of plants including wheat (Coombs and Franco 2003; Jha and Kumar 2009; Verma et al. 2013, 2014, 2015b), rice (Naik et al. 2009; Piromyou et al. 2015), mustard (Sheng et al. 2008), chili (Kang et al. 2007; Yang et al. 2009), sugarcane (Suman et al. 2000, 2001; Mendes et al. 2007), maize (Araújo et al. 2000; Montanez et al. 2012; Thanh and Diep 2014), citrus (Andreote et al. 2008), potato (Manter et al. 2010; Rado et al. 2015); tomato (Hallmann et al. 1997), soybean (Hung and Annapurna 2004; Mingma et al. 2014), pea (Tariq et al. 2014), common bean (Suyal et al. 2015), sunflower (Forchetti et al. 2010; Ambrosini et al. 2012), cotton (Quadt-Hallmann et al. 1997), chickpea (Saini et al. 2015), pearl millet (Rokhbakhsh-Zamin et al. 2011), and strawberry (Hardoim et al. 2012). A large number of endophytic bacterial species belonging to different genera including *Achromobacter*, *Azoarcus*, *Burkholderia, Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, *Microbispora*, *Micromonospora*, *Nocardioides, Pantoea*, *Planomonospora*, *Pseudomonas*, *Serratia*, S*treptomyces, Thermomonospora*, etc. (Mcinroy and Kloepper 1995; Hallmann et al. 1997; Reinhold-Hurek and Hurek 1998; Suman et al. 2000, 2001; Rosenblueth and Martínez-Romero 2006; Ryan et al. 2008; Pageni et al. 2013; Verma et al. 2013, 2014, 2015b; Mercado-Blanco 2015) have been isolated from different host plants.

7.2 Isolation, Characterization, and Identification of Endophytic Microbes

Colonization of microbes in plant tissues is largely influenced by the environmental circumstances surrounding the host plants such as the type and pH of soil, the content in soil, rainfall, salinity of soil, and temperatures. Endophytic microbes may occur in low numbers and sometimes in localized positions within plants, so that it is almost impossible to find their specific affiliation to their host plant. For isolation of endophytes, attention needs to be paid to avoid contamination with undesirable epiphytic microbes. It is recommended to first sterilize the entire surface of the samples, followed by cutting their organs and tissues into pieces with a sterilized knife, if necessary. Sodium hypochlorite is the most commonly used disinfectant. Plant samples usually are sterilized by sequential immersion in 70 % ethanol for 1–3 min and 1–3 % sodium hypochlorite for 3–5 min, followed by repeated rinsing in sterile water to remove residual sodium hypochlorite. Hydrogen peroxide and mercuric chloride are also effective disinfectants (Guerny and Mantle 1993; Bandara et al. 2006; Verma et al. 2015b; Coombs and Franco 2003). The surface treatment with ethanol alone is not sufficiently effective to endophytic bacteria. Double or triple surface sterilization with a combination of ethanol and other disinfectants is also recommended to eliminate epiphytic microbes. All the samples (roots, flowers, shoots, and seeds) are then macerated independently with 10 mL sterile 0.85 % NaCl using a mortar and pestle and further homogenized by vortexing for 60 s at high speed. The solutions are then used for further isolation of microbes. In another method, segments of the sterilized samples are placed onto an appropriate agar medium, followed by incubation at an appropriate temperature (5–45 °C). There are another method for isolation, in which initially, the samples are ground with 5 mL of aqueous solution (0.9 % NaCl) using a sterile mortar and pestle. The tissue extract is subsequently incubated at 30 °C for 3 h to allow the complete release of endophytic microorganisms from the host tissue. The endophytic microbes were isolated through enrichment method, using the standard serial dilution plating technique (Costa et al. 2012) (Fig. 7.1). Different specific medium can be used for isolation of archaea, eubacteria, and fungi (Table 7.1). The different growth and specific mediums were used to isolate the maximum possible culturable morphotypes (Table 7.1). To isolate different

groups of microbes, all medium and condition can be used such as for halophilic (with 5–20 % NaCl concentration), drought tolerant (7–10 % polyethylene glycol), acidophilic (pH 3–5); alkaliphilic (pH 8–11), psychrophilic (incubation at >5 °C temperature), thermophilic (incubation at >45 °C temperature), etc (Yadav et al. 2015d). The plates were incubated for up to 15 days, and the colonies were selected according to their time of growth and morphology (color, size, shape). After 15 days of incubation, all of the colonies were counted and expressed as colony-forming unit (CFU) per gram of fresh tissue. Endophytic microbes can be screen for tolerance to temperature, salt (NaCl concentration), drought, and pH according to the method described earlier (Yadav et al. 2015d).

For identification of endophytic microbes, isolates should be grown in specific broth, until they reached an OD 600 nm >1.0. The cells are pellet from 5 mL culture, washing thrice with TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0), and the pellet are resuspended in 750 µL TE buffer. Genomic DNA can be isolated from the sus-

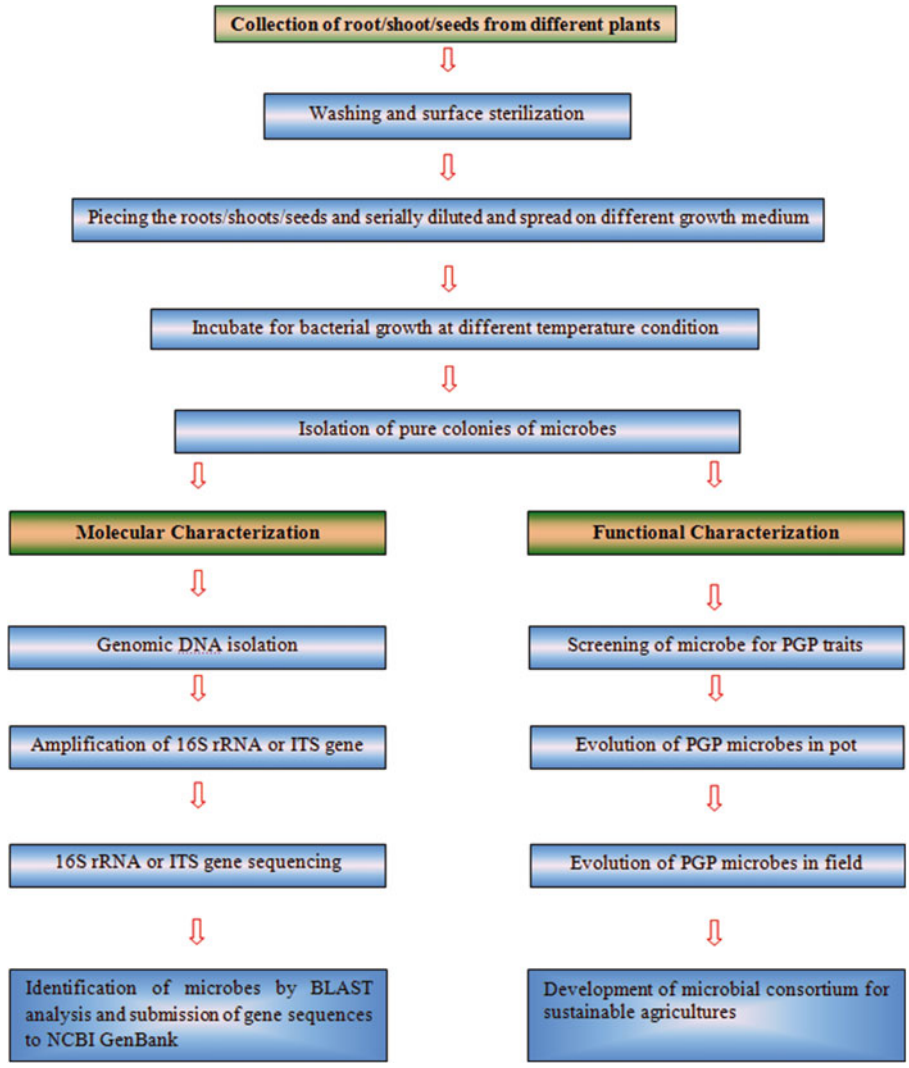

Fig. 7.1 A schematic representation of the isolation, characterization, identification, and potential application of endophytic microbes for sustainable agriculture

Table 7.1 The different media used in isolation of endophytic eubacteria, archaea, and fungi

Media and composition per liter
Eubacteria
1. Nutrient agar: 5 g peptone; 5 g NaCl; 3 g beef extract; 20 g agar
2. T_3 agar: 3 g tryptone; 2 g tryptose; 1.5 g yeast extract; 0.005 g $MnCl_2$; 0.05 g NaH_2PO_4; 20 g agar
3. Tryptic soy agar: 17 g tryptone; 3 g soya meal; 2.5 g $C_6H_{12}O_6$; 5 g NaCl; 2.5 g K_2HPO_4; 20 g agar
4. King's B agar: 20 g protease peptone; 1.5 g K_2HPO_4; 1.5 g $MgSO_4.7H_2O$; 10 mL glycerol; 20 g agar
5. Jensen's agar: 20 g sucrose;1 g K_2HPO_4; 0.5 g Mg_2SO_4; 0.5 g NaCl; 0.001 g Na_2MoO_4; 0.01 g $FeSO_4$; 2 g $CaCO_3$; 20 g agar
6. R_2A agar: 0.5 g proteose peptone; 0.5 g casamino acids; 0.5 g yeast extract; 0.5 g dextrose; 0.5 g soluble starch; 0.3 g K_2HPO_4; 0.05 g $MgSO_4.7H_2O$; 0.3 g sodium pyruvate; 20 g agar
7. Ammonium minerals salt: 0.70 g K_2HPO_4; 0.54 g KH_2PO_4; 1.00 g $MgSO_47H_2O$; 0.20 g $CaCl_2$. $2H_2O$; 4.00 mg $FeSO_4.7H_2O$; 0.50 g NH_4Cl; 100 µg $ZnSO_4$. $7H_2O$; 30 µg $MnCl_2.4H_2O$; 300 µg H_3BO_3; 10 µg $CuCl_2$. $2H_2O$; 200 µg $CoCl_2$. $6H_2O$; 20 µg $NiCl_2$.$6H_2O$; 60 µg $Na_2MoO_4.2H_2O$; 20 g agar
8. Luria-Bertani Media: 10 g casein acid hydrolysate; 5 g yeast extract; 10 g NaCl; 20 g agar
9. Modified Dobereiner medium: 10 g sucrose; 5 g malic acid; 0.2 g $K_2HPO_4.H_2O$; 0.4 g $KH_2PO_4.H_2O$; 0.1 g NaCl; 0.01 g $FeCl_3$; 0.002 g Na_2MoO_4; 0.2 g $MgSO_4.7H_2O$; 0.02 g $CaCl_2.H_2O$; 20 g agar
10. Yeast extract mannitol agar: 1 g yeast extract; 10 g mannitol 0.5 g $K_2HPO_4.H_2O$; 0.002 g $MgSO_4.7H_2O$ 0.1 g NaCl; 20 g agar
Archaea
1. Chemically defined medium: 5 g casamino acids; 5 g yeast extract; 1 g sodium glutamate; 3 g tri-sodium citrate; 20 g $MgSO_4$; 2 g KCl; 100 g NaCl; 36 mg $FeCl_2$; 0.36 mg $MgCl_2$; 20 g agar
2. Standard growth media: 7.5 g casamino acids; 4 g $MgSO_4$; 2 g KCl; 150 g NaCl; 3 g Tri-sodium citrate; 2.3 mg $FeCl_2$;7 mg $CaCl_2$; 0.044 mg $MnSO_4$; 0.05 mg $CuSO_4$; 20 g agar
3. Halophilic medium: 100 g NaCl; 2 g KCl; 1 g $MgSO_4 \cdot 7H_2O$; 0.36 g $CaCl_2 \cdot 2H_2O$; 0.23 g NaBr; 0.06 g $NaHCO_3$; 5 g protease peptone; 10 g yeast extract; 1 g glucose; trace $FeCl_3$; 20 g agar
Fungi
1. Potato dextrose agar: 4 g potato infusion (from 200 g potato); 20 g dextrose; 15 g agar; supplemented with 50 µg/mL chloramphenicol; 20 g agar
2. Rose Bengal agar : 5 g enzymatic digest of soybean; 10 g dextrose; 1 g g KH_2PO_4; 0.5 g $MgSO_47H_2O$; 0.05 g Rose Bengal; 15 g agar; supplemented with 50 µg/mL chloramphenicol; 20 g agar

pended pellet using Zymo Research Fungal/Bacterial DNA MicroPrep™ following the standard protocol prescribed by the manufacturer (Kumar et al. 2014a; Verma et al. 2015a). Different primers can be used for amplification of 16S rRNA gene for archaea and bacteria while 18S rRNA gene for fungi (Yadav 2015). The amplification can be carried out in a 100 µL volume, and amplification conditions can be used as described by Pandey et al. (2013). PCR-amplified 16S or 18S rRNA genes have to purified and sequenced. Sequencing employed a dideoxy cycle with fluorescent terminators and was run in a 3130xl Applied Biosystems ABI prism automated DNA sequencer (Applied Biosystems, Foster City, CA). The partial 16S or 18S rRNA gene sequences are compared with sequences available in the NCBI database. Isolates can be identified to species level on the basis of 16S rRNA gene sequence similarity of ≥97 % with the sequences in GenBank. Sequence alignment and comparison used the multiple sequence alignment tool CLUSTALW2 with default parameters. The phylogenetic tree can be constructed on aligned data sets using the neighbor-joining (NJ) method (Saitou and Nei 1987) and the program MEGA 4.0.2 (Tamura et al. 2007). The simplified diagrammatic scheme has been presented below (Fig. 7.1) to show steps of isolation, screening, and identification of endophytic microbes.

7.3 Diversity, Distribution, and Abundance of Endophytic Microbes

The endophytic microbe has been reported from different types such as archaea, eubacteria, and fungi. Among bacteria, endophytic bacteria were isolated from different phylum mainly: Actinobacteria, Firmicutes, proteobacteria, Bacteroidetes, and Deinococcus-Thermus. The Proteobacteria were further grouped as α-, β-, and γ-proteobacteria. Distribution of endophytic bacteria varied in all bacterial phyla. Proteobacteria were most dominant followed by actinobacteria. Least number of endophytic bacteria was reported from phylum Deinococcus-Thermus and Acidobacteria followed by Bacteroidetes (Fig. 7.2); such bacteria were both Gram-positive and Gram-negative bacteria (Lodewyckx et al. 2002; Verma et al. 2015b). Endophytic fungi were reported from phylum Ascomycota and Basidiomycota, in which Ascomycota were most dominant (Fig. 7.2).

Endophytic microbes were reported by both culture-dependent and culture in-dependent approaches. It is possible to assess only a small fraction of the bacterial diversity associated with plants using the isolation methods described above because few endophytic bacterial species can be cultivated using traditional laboratory methods. The sizes of bacterial communities as determined using culture-independent methods might be 100–1000-fold larger than communities uncovered via traditional isolation (Yashiro et al. 2011). Archaea were also reported as endophytes. There were only two reports of endophytic archaea from rice and maize plant. There was the first report on archaea that had to be identified as endophytes associated with rice by the culture-independent approach. *Methanospirillum* sp. and *Candidatus Methanoregula boonei* have been reported as endophytic archaea from rice (Sun et al. 2008). The archaea were isolated from phylum Euryarchaeota and belonged to different genera such as *Haloferax*, *Methanobacterium*, *Methanosaeta*, *Methanospirillum*, and *Thermoplasma* (Chelius and Triplett 2001).

Endophytic bacteria have been reported in almost every plant studied (Ryan et al. 2008). On review of 17 different plants (Figs. 7.2, 7.3, and 7.4), it was found that endophytic microbes that were most predominant and studied belong to three major phyla Actinobacteria, Proteobacteria, and Firmicutes. Among 116 reported genera from 17 different host plants, 23 microbes were reported as most predominant, namely, *Achromobacter*, *Acinetobacter*, *Agrobacterium*, *Bacillus*, *Brevundimonas*, *Burkholderia*, *Cladosporium*, *Clavibacter*, *Enterobacter*, *Flavobacterium*, *Herbaspirillum*, *Klebsiella*, *Methylobacterium*, *Microbacterium*, *Microbispora*, *Paenibacillus*, *Pantoea*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, *Staphylococcus*, *Stenotrophomonas*, and *Streptomyces* (Mcinroy and Kloepper 1995; Hallmann et al. 1997; Reinhold-Hurek and Hurek 1998; Rosenblueth and Martínez-Romero 2006; Ryan et al. 2008; Pageni et al. 2013; Verma et al. 2014, 2015b; Mercado-Blanco 2015).

Among 23 genera (most predominant), *Bacillus*, *Burkholderia*, *Enterobacter*, *Methylobacterium*, *Pantoea*, *Pseudomonas*, and *Streptomyces* were most dominant and reported in more than five host plants (Table 7.2). There were many endophytic bacteria found to be common in more than three host plants. Along with common endophytic microbial genera, there were many niche-specific microbial genera that have been reported from all 17 host plants such as *Mycobacterium*, *Planobispora*, *Planomonospora*, and *Thermomonospora* from wheat (*Triticum aestivum*); *Aeromonas*, *Alkanindiges*, *Azospirillum*, *Caulobacter*, *Chlamydomyces*, *Chryseobacterium*, *Clostridium*, *Comamonas*, *Coniothyrium*, *Curvibacter*, *Cytophagales*, *Exiguobacterium*, *Gallionella*, *Geobacter*, *Holophaga*, *Humicola*, *Hydrogenophaga*, *Kaistina*, *Methylophaga*, *Mitsuaria*, *Nigrospora*, *Novosphingobium*, *Phialophora*, *Plesiomonas*, *Rhizoctonia*, *Rhodopseudomonas*, *Sinorhizobium*, *Speiropsis*, *Stemphylium*, and *Trichoderma viride* from rice (*Oryza sativa* L.); *Bradyrhizobium* and *Raoultella* from sugarcane (*Saccharum officinarum*);

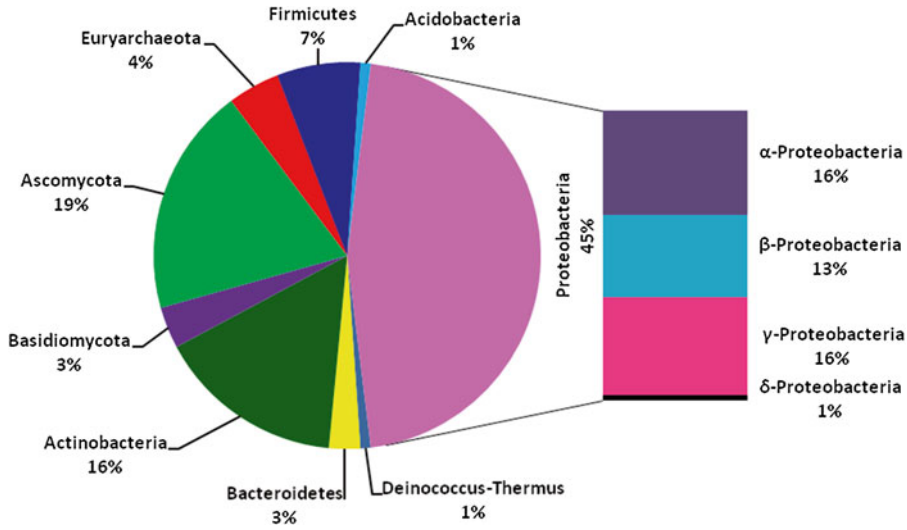

Fig. 7.2 Abundance of endophytic microbes belonging to diverse phylum and groups reported from 17 different plants

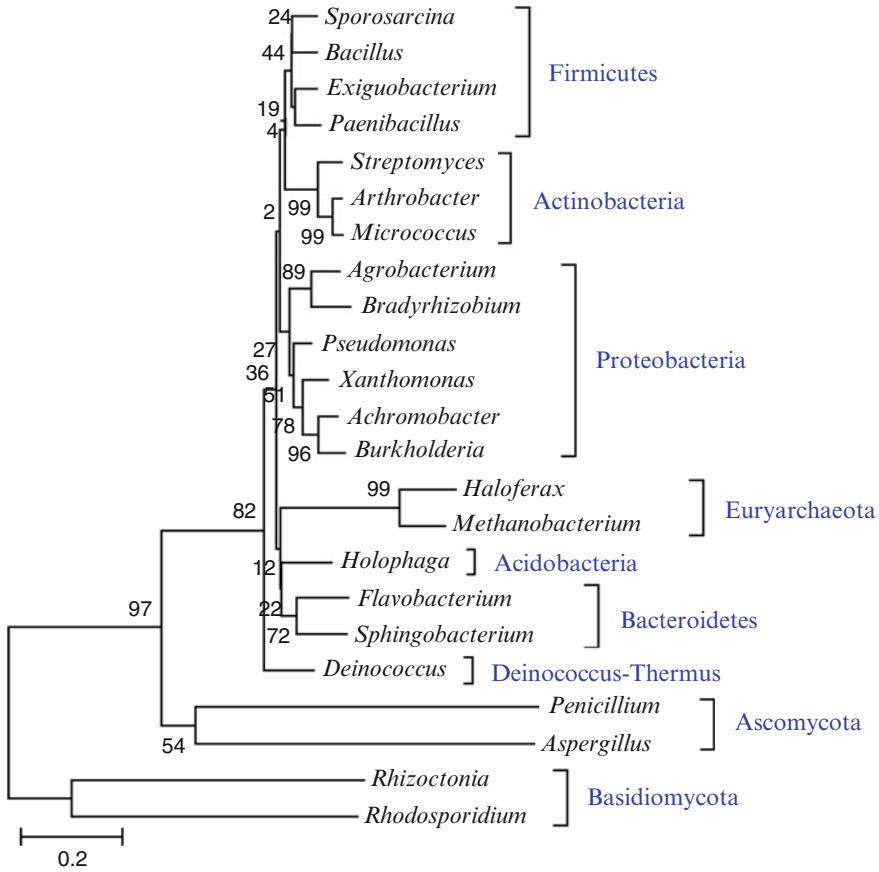

Fig. 7.3 Phylogenetic tree showed the relationship among different groups of microorganisms isolated from 15 different plants

Azoarcus, *Corynebacterium*, *Haloferax*, *Methanobacterium*, *Methanosaeta*, *Methanospirillum*, *Rhanella*, *Streptosporangium*, and *Thermoplasma* from maize (*Zea mays* L); *Curtobacterium* and *Guignardia* from citrus; *Frateuria*, *Janthinobacterium*, *Paracoccus*,

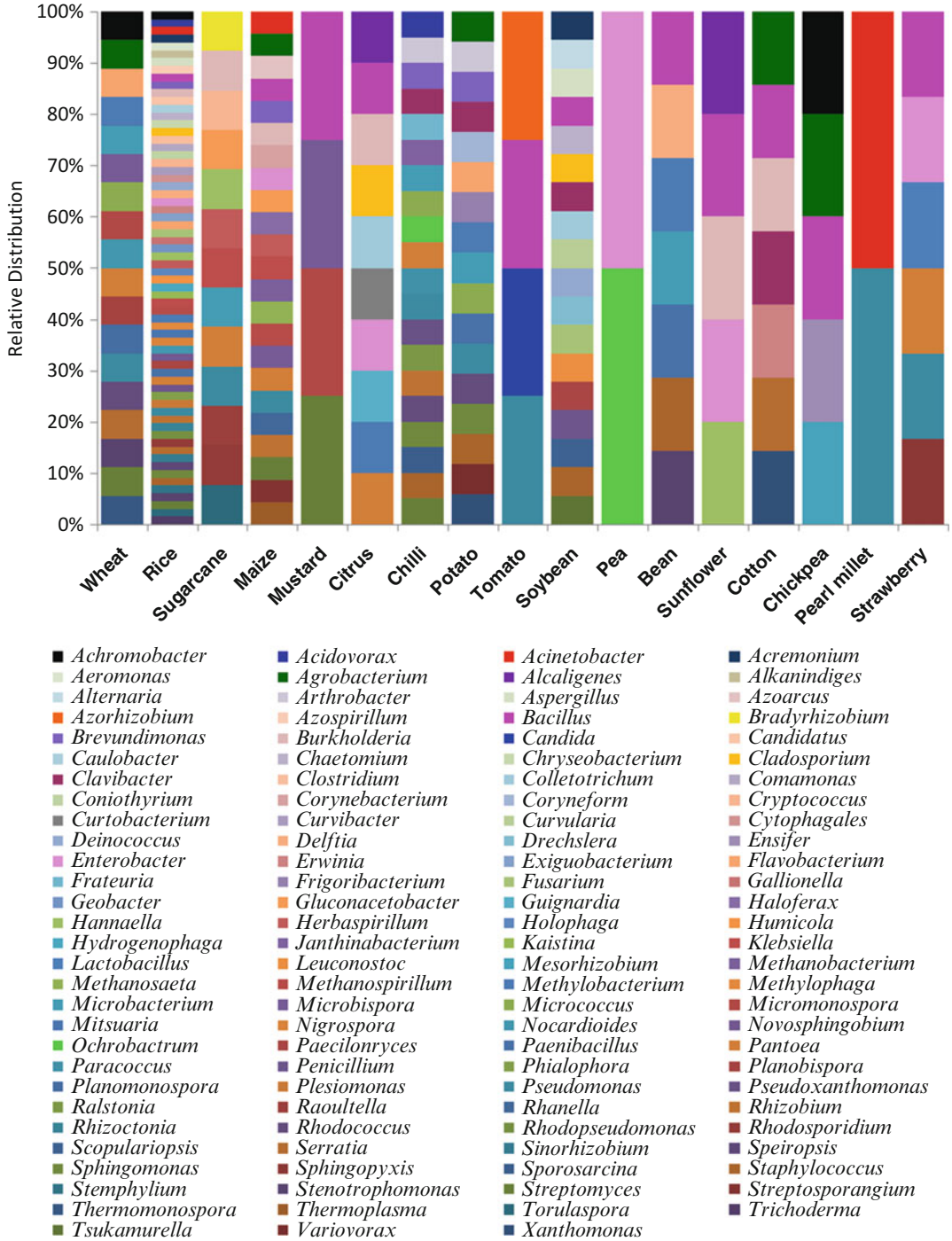

Fig. 7.4 Diversity and distribution of endophytic microbe reported form different 17 host plant

Table 7.2 Distribution of predominant genera in 17 different host plants

S.No.	Endophytes	Wheat	Rice	Sugarcane	Maize	Mustard	Citrus	Chilli	Potato	Tomato	Soybean	Pea	Bean	Sunflower	Cotton	Chickpea	Pearl millet	Strawberry
1.	*Achromobacter*																	
2.	*Acinetobacter*																	
3.	*Agrobacterium*																	
4.	*Bacillus*																	
5.	*Brevundimonas*																	
6.	*Burkholderia*																	
7.	*Cladosporium*																	
8.	*Clavibacter*																	
9.	*Enterobacter*																	
10.	*Flavobacterium*																	
11.	*Herbaspirillum*																	
12.	*Klebsiella*																	
13.	*Methylobacterium*																	
14.	*Microbacterium*																	
15.	*Microbispora*																	
16.	*Paenibacillus*																	
17.	*Pantoea*																	
18.	*Pseudomonas*																	
19.	*Rhizobium*																	
20.	*Rhodococcus*																	
21.	*Staphylococcus*																	
22.	*Stenotrophomonas*																	
23.	*Streptomyces*																	

Wheat (*Triticum aestivum*): (Coombs and Franco 2003; Larran et al. 2007; Jha and Kumar 2009; Verma et al. 2013, 2014, 2015b), rice (*Oryza sativa*): (Elbeltagy et al. 2000; Tian et al. 2007; Govindarajan et al. 2008; Sun et al. 2008; Naik et al. 2009; Rangjaroen et al. 2014; Piromyou et al. 2015), sugarcane (*Saccharum officinarum*): (Suman et al. 2001; Mendes et al. 2007; Govindarajan et al. 2007; Quecine et al. 2012; Nutaratat et al. 2014), maize (*Zea mays*): (Mcinroy and Kloepper 1995; Hallmann et al. 1997; Araújo et al. 2000; Chelius and Triplett 2001; Montanez et al. 2012; Thanh and Diep 2014; Matsumura et al. 2015), mustard (*Brassica campestris*): (Poonguzhali et al. 2006; Lee et al. 2008; Sheng et al. 2008), Citrus: (Araújo et al. 2001, 2002; Andreote et al. 2008; Lacava and Azevedo 2013), chili (*Capsicum annuum*): (Rasche et al. 2006; Kang et al. 2007; Yang et al. 2009)], potato (*Solanum tuberosum*): (Hallmann et al. 1997; Sessitsch et al. 2004; Berg et al. 2005; Manter et al. 2010; Pavlo et al. 2011; Rado et al. 2015), tomato (*Solanum lycopersicum*): (Hallmann et al. 1997; Li et al. 2014), soybean (*Glycine max*): (Hung and Annapurna 2004; Pimentel et al. 2006; Okubo et al. 2009; Selvakumar et al. 2013; Mingma et al. 2014), pea (*Pisum sativum*): (Tariq et al. 2014), common bean (*Phaseolus vulgaris*): (de Oliveira Costa et al. 2012; Suyal et al. 2015), sunflower (*Helianthus annuus*): (Forchetti et al. 2007; Forchetti et al. 2010; Ambrosini et al. 2012), cotton (*Gossypium hirsutum*): (Mcinroy and Kloepper 1995; Hallmann et al. 1997; Quadt-Hallmann et al. 1997), chickpea (*Cicer arietinum*): (Dudeja and Nidhi 2013; Saini et al. 2015), pearl millet (*Pennisetum glaucum*): (Hallmann et al. 1997; Rosenblueth and Martínez-Romero 2006; Gupta et al. 2013), strawberry (*Fragaria ananassa*): (Dias et al. 2009; de Melo Pereira et al. 2012; Hardoim et al. 2012)

Pseudoxanthomonas, *Ralstonia*, and *Sporosarcina* from chili; *Coryneform*, *Frigoribacterium*, and *Variovorax* from potato (*Solanum tuberosum*); *Candida* from tomato (*Solanum lycopersicum*); *Alternaria*, *Curvularia*, *Drechslera*, *Leuconostoc*, *Scopulariopsis*, and *Tsukamurella*; soybean (*Glycine max*); *Mesorhizobium* from chickpea (*Cicer arietinum)*; and *Lactobacillus* and *Sphingopyxis* from strawberry (*Fragaria ananassa*). There are no any reports for niche-/plant-specific endophytic microorganisms, but there were many reports on niche specificity of microbes from different extreme habitats (Kumar et al. 2014b; Verma et al. 2014, 2015a, 2015b; Yadav 2015; Yadav et al. 2015a, b, c, d, e).

Most studies on the occurrence of endophytic microbes have been performed using culture-dependent approaches. The genus *Bacillus* has been consistently described as culturable and endophytic, and these bacteria can colonize wheat (Verma et al. 2013, 2014, 2015b), rice (Sun et al. 2008), mustard (Sheng et al. 2008), chili (Rasche et al. 2006), citrus (Araújo et al. 2001), potato (Sessitsch et al. 2004), soybean (Hung and Annapurna 2004), common bean (Figueiredo et al. 2008), chickpea (Saini et al. 2015), and strawberry (Dias et al. 2009). The member *Bacillus* and *Bacillus*-derived genera (BBDG) associated with different plants showed multifarious plant growth-promoting attributes such as solubilization of phosphorus, potassium, and zinc; production of phytohormones; and biocontrol against different pathogens (Tilak et al. 2005; Verma et al. 2013, 2014, 2015b).

The genus *Burkholderia* has been reported as endophytic in different host plants but most dominant in sugarcane and associated mainly for nitrogen fixation (Suman et al. 2001, 2005, 2008; Castro-González et al. 2011). Additionally, other studies have described the importance of members of the genus *Burkholderia* in the cultivation of rice (Govindarajan et al. 2008; Rangjaroen et al. 2014), maize (Bevivino et al. 1998), citrus (Araújo et al. 2002), and cotton (Quadt-Hallmann et al. 1997). The member of *Enterobacter* bacteria has been reported as endophytic bacteria in different plants such as rice (Piromyou et al. 2015), maize (Montanez et al. 2012), citrus (Araújo et al. 2002), pea (Tariq et al. 2014), and strawberry (de Melo Pereira et al. 2012).

The pink-pigmented facultative methylotrophs (PPFMs) have been reported from diverse host plants such as wheat (Verma et al. 2013, 2014, 2015b), rice, citrus (Dourado et al. 2015), and bean (de Oliveira Costa et al. 2012). In plant colonization, the frequency and distribution may be influenced by plant genotype or by interactions with other associated microorganisms, which may result in increasing plant fitness. *Methylobacterium* have been reported to have a potential capacity to fix nitrogen, nodule the host plant, and produce cytokinins, auxin, and enzymes involved in the induction of systemic resistance, such as pectinase and cellulase, and therefore plant growth promotion. The different species of *Pantoea* have been described as cosmopolitan endophytes found in wheat (Verma et al. 2014), rice (Rangjaroen et al. 2014), maize (Ikeda et al. 2013), citrus (Araújo et al. 2002), chili (Kang et al. 2007), and potato (Reiter et al. 2002). Members of *Pantoea* are ubiquitous in plant tissue; they are able to influence plant growth through the production of auxins or cytokinins and induce systemic resistance against diseases.

Pseudomonas, a member of γ-proteobacteria, are ubiquitous in nature and have been also reported from different plant tissues of wheat (Verma et al. 2013, 2014, 2015b), rice (Sun et al. 2008), sugarcane (Suman et al. 2001, 2005, 2008), maize (Thanh and Diep 2014; Szilagyi-Zecchin et al. 2014), chili (Kang et al. 2007), tomato (Kumar et al. 2011), potato (Reiter et al. 2003; Sessitsch et al. 2004), pearl millet (Gupta et al. 2013), and strawberry (de Melo Pereira et al. 2012). *Streptomyces* has been reported from shoot, root, and seeds of different plant such as wheat (Coombs and Franco 2003), rice (Tian et al. 2004), maize (Araújo et al. 2000), and chili (Rasche et al. 2006) (Fig. 7.4).

7.4 Endophytic Microbes in Agriculture

Endophytic microbes are agriculturally important as they can enhance plant growth and improve plant nutrition through nitrogen fixation and other mechanisms (Sun et al. 2008; Araújo et al. 2000; Suman et al. 2001; Kumar et al. 2007; Lodewyckx et al. 2002; Yanni et al. 2011; Pavlo et al. 2011; Rangjaroen et al. 2014; Verma et al. 2015b). Endophytes may increase crop yields, remove contaminants, inhibit pathogens, and produce fixed nitrogen or novel substances (Quadt-Hallmann et al. 1997; Suman et al. 2001; Verma et al. 2015b). In endophytic relationships, growth-promoting microbes reside within the apoplastic spaces in the host plants. There is direct evidence for the existence of endophytes in the apoplastic intercellular spaces of parenchymal tissue (Dong et al. 1997) and the xylem vessels (James et al. 1994; Lacava and Azevedo 2013; Glick 2015). Endophyte-infected plants often grow faster than noninfected ones (Cheplick et al. 1989). The growth stimulation by endophytes can be a consequence of nitrogen fixation (de Bruijn et al. 1997; Suman et al. 2001; Iniguez et al. 2004; Taulé et al. 2012), production of phytohormones, such as IAA and cytokines (Rashid et al. 2012; Nath et al. 2013; Lin and Xu 2013; Jasim et al. 2014; Verma et al. 2015b), biocontrol of phytopathogens through the production of antifungal or antibacterial agents, siderophore production, nutrient competition and induction of acquired host resistance, or enhancement of the bioavailability of minerals. Several studies have indicated that endophytic colonization can also result in increased plant vigor, and it confers tolerance to biotic and abiotic stresses, enhanced drought tolerance, and improved phosphorus utilization (Verma et al. 2015b).

Sustainable agriculture requires the use of strategies to increase or maintain the current rate of food production while reducing damage to the environment and human health. The use of microbial plant growth promoters is an alternative to conventional agricultural technologies. Plant growth-promoting microbes (PGPM) can affect plant growth directly or indirectly. The direct promotion of plant growth by PGPM, for the most part, entails providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of certain nutrients from the environment. The indirect promotion of plant growth occurs when PGPM decrease or prevent the deleterious effects of one or more phytopathogenic organisms (Fig. 7.5).

7.4.1 Direct Plant Growth Promotion Activity

There are several ways in which different endophytic PGPM have been reported to directly facilitate the proliferation of their plant hosts. Endophytic PGPM can fix atmospheric nitrogen and supply it to plants; they synthesize siderophores that can solubilize and sequester iron from the soil and provide it to the plant; they synthesize several different phytohormones that can act to enhance various stages of plant growth; they may have mechanisms for the solubilization of minerals such as phosphorus, potassium, and zinc that will become more available for plant growth; and they may synthesize some less well-characterized, low-molecular mass compounds or enzymes that can modulate plant growth and development (Kloepper et al. 1989; Glick 1995; Quadt-Hallmann et al. 1997; Glick et al. 1999). A particular PGPB may affect plant growth and development by using any one or more of these mechanisms. It is probable that the same is true for endophytic bacteria. Direct evidence for the plant growth-promoting activity of endophytic bacteria was provided by Sturz (1995). According to this study, approximately 10 % of bacterial isolates recovered from within potato tubers promoted plant growth. Other experiments with clover and potatoes in a crop rotation revealed that 21 % of the isolated endophytic bacteria promoted plant growth, which was reflected by increased shoot height (63 %), shoot weight (66 %), and root weight (55 %) (Sturz et al. 1998).

7.4.1.1 Biological N_2 Fixation

Nitrogen is the major limiting factor for plant growth; the application of N_2-fixing endophytic bacteria as biofertilizer has emerged as one of the most efficient and environmentally sustainable methods for increasing the growth and yield of crop plants. For the sustainable agriculture, N_2 fixation by microbes can be one of the possible biological alternatives to N-fertilizers, without harming the environment. Nitrogen-fixing endophytic bacteria belonging to different genera *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas*, and *Serratia* have been reported and characterized for biological nitrogen fixation (James et al. 1994; Olivares et al. 1996; Elbeltagy et al. 2001; Suman et al. 2001, 2005; Boddey

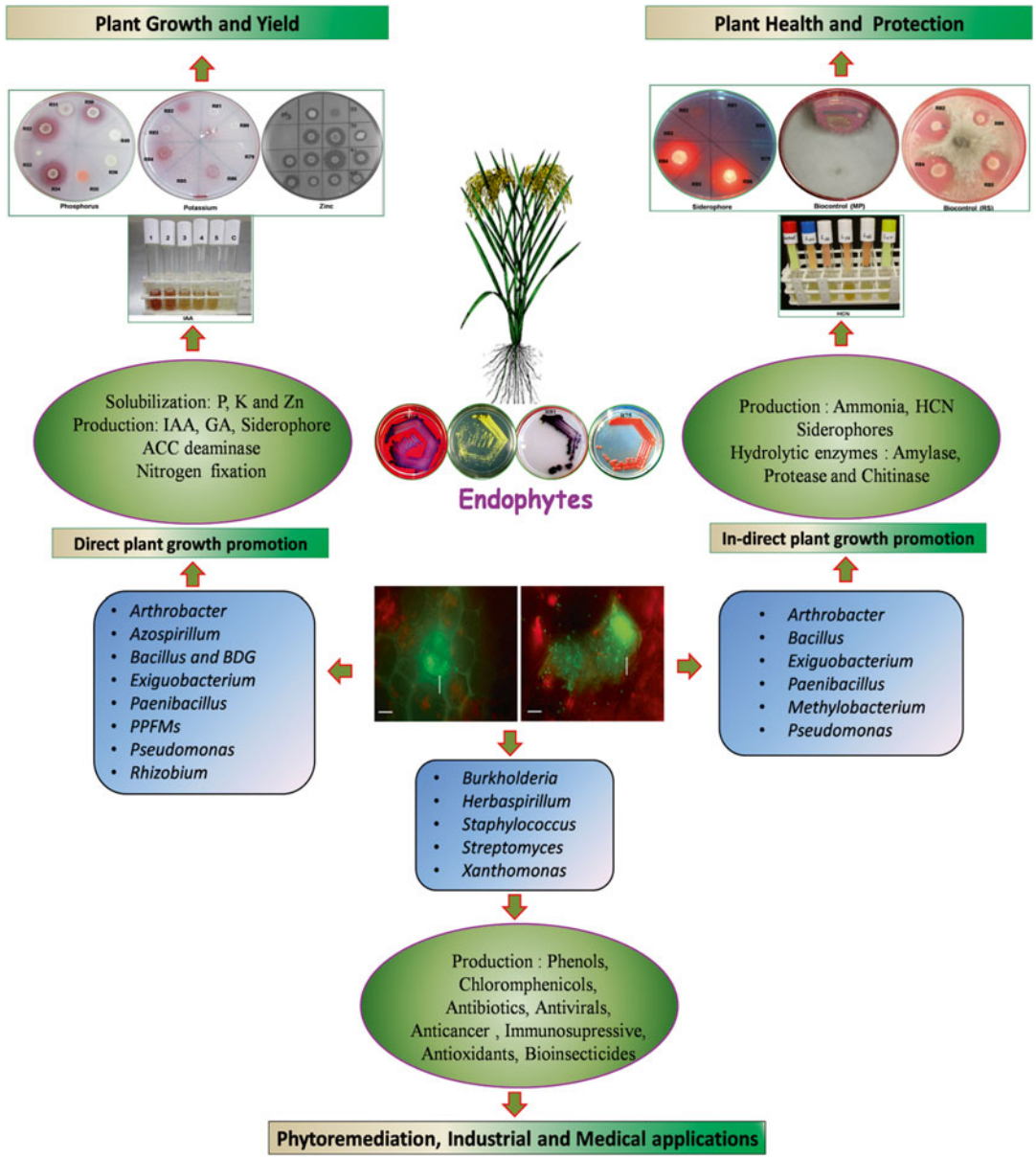

Fig. 7.5 Schematic diagram of the different plant microbial endophyte interactions and its applications

et al. 2003; White Jr et al. 2014; Wei et al. 2014; Verma et al. 2014, 2015b).

Application of N_2-fixing endophytic microbial inoculants for cereal crops has drawn attention for increasing yield. Endophytic microbes are considered to be better than that of rhizospheric one as they provide fixed nitrogen directly to their host plant and fix nitrogen more efficiently due to lower oxygen pressure in the interior of plants than that of soil. The concept of BNF by endophytes (Dobereiner 1992) has led to investigations on the potential uses of endophytic nitrogen-fixing bacteria that colonize graminaceous plants. *Gluconacetobacter diazotrophicus* is the main contributor of endophytic BNF in sugarcane, which according to nitrogen balance studies fix as high as 150 Kg N $ha^{-1}year^{-1}$ (Muthukumarasamy et al. 2005).

Suman et al. (2005, 2008) isolated *Gluconacetobacter diazotrophicus* strains from sugarcane roots and characterized for BNF in vitro. In vivo, these bacterial strains were screened for their efficiency to promote growth and nutrient uptake in sugarcane, and the inoculation by these strains resulted to improved germination, tiller number, and plant height. *Gluconacetobacter diazotrophicus* isolate IS100 was found to be the most efficient in promoting plant growth and nutrient uptake in sugarcane.

Studies of endophytes in sugarcane have focused on isolation and characterization using morphological and physiological studies of diazotrophic bacteria as well as molecular characterization of *nif* genes and 16S rRNA gene sequence analysis. Magnani et al. (2010) reported that endophytic bacteria live inside sugarcane plant tissues without causing disease. Isolated endophytic bacteria were identified using 16S rRNA gene sequencing, revealed that these bacteria belonged to five group Enterobacteriaceae, Bacilli, *Curtobacterium*, Pseudomonadaceae, and one from uncultured bacterium. Most of the bacteria isolated from the sugarcane stem and leaf tissues belonged to Enterobacteriaceae and Pseudomonadaceae and showed niche specificity.

Quecine et al. (2012) reported that sugarcane growth promotion by the endophytic bacterium *Pantoea agglomerans* 33.1 by nitrogen fixation. Tam and Diep (2014) characterized 27 isolates on LGI medium from sugarcane, and all of them have ability of nitrogen fixation and phosphate solubilization together with IAA biosynthesis, but there were 10 isolates having the efficient plant growth promoting attributes. All the endophytic bacteria belonged to Proteobacteria, and 3 isolates belonged to alpha-proteobacteria (30 %), 2 isolates belonged to beta-proteobacteria (20 %), and 5 isolates belonged to gamma-proteobacteria (50 %). *Enterobacter oryzae* LT7, *Achromobacter xylosoxidans* T16, *Achromobacter insolitus* R15b, and *Pantoea agglomerans* T12 revealed promising candidates with multiple beneficial characteristics, and they have the potential for application as inoculants or biofertilizer adapted to poor latosols and acrisols because they are not only famous strains but also are safety strains for sustainable agriculture. *Burkholderia*, *Herbaspirillum*, *Azospirillum*, and *Rhizobium leguminosarum* bv. *Trifolii* are contributor of endophytic BNF in rice (Biswas et al. 2000; Baldani and Baldani 2005; Govindarajan et al. 2008; Isawa et al. 2009; Doty 2011; Estrada et al. 2013; Choudhury et al. 2014; Aon et al. 2015). As all of these assumptions seem to support the conditions for nitrogen fixation by endophytic bacteria, there is still no direct evidence that endophytic bacteria actually are the responsible agents of biological nitrogen fixation. Although some agriculturally important crops, such as rice, wheat, and maize contain numerous endophytic bacteria such as *Acetobacter diazotrophicus*, *Herbaspirillum* sp., and *Azospirillum* sp., there is little evidence that these bacteria actually fix N_2 in their host plants (James et al. 1994).

Ji et al. (2014) have isolated 576 endophytic bacteria from the leaves, stems, and roots of rice. Endophytic bacteria were identified using *nif*H genes and 16S rRNA gene sequencing. *nif*H amplification occurred in different species of *Bacillus*, *Paenibacillus*, *Microbacterium*, and *Klebsiella.* These bacteria were used for enhancement of plant growth, increased height and dry weight, and antagonistic effects against fungal pathogens. For sustainable agriculture, the use of

biologically derived fertilizers would be ecologically important and economically viable. Inoculation with N-fixing endophytic bacterium may represent an alternative to the use of chemical N-fertilizers and is associated with decreased production costs as well as a considerable increase in crops production.

7.4.1.2 Solubilization of Phosphorus, Potassium, and Zinc

Phosphate (P) and potassium (K) are the major essential macronutrients for biological growth and development. However, the concentrations of soluble P and K in soil are usually very low, as the biggest proportions of P and K in soil are insoluble rocks, minerals, and other deposits (Goldstein 1994). Phosphorus is one of the major growth-limiting nutrients in plants. It is important for the plant growth and promotes root development, tillering, and early flowering and performs other functions like metabolic activities, particularly in synthesis of protein. Phosphorus is an essential element for the establishment and development of plants because it improves the entire root system, consequently improving the shoot. Lack of phosphorus can lead to atrophy and death of the plant and may also delay fruit maturation.

Phosphate solubilization is a common trait among endophytic bacteria. For instance, the majority of endophytic populations from wheat, rice, maize, peanut, legumes, and sunflower were able to solubilize mineral phosphates in plate assays, and a vast number of PGPB with phosphate-solubilizing property have been reported which include members belonging to *Burkholderia*, *Enterobacter*, *Pantoea*, *Pseudomonas*, *Citrobacter*, and *Azotobacter* (Forchetti et al. 2007; Puente et al. 2009; Verma et al. 2013, 2014, 2015b). Possible mechanisms for solubilization from organic-bound phosphate involve either enzymes, namely, C-P lyase and nonspecific phosphatases and phytases. However, most of the microbial genera solubilize phosphate through the production of organic acids such as gluconate, ketogluconate, acetate, lactate, oxalate, tartarate, succinate, citrate, and glycolate (Khan et al. 2009; Stella and Halimi 2015; Yadav et al. 2015e). The type of organic acid produced for P solubilization may depend upon the carbon source utilized as substrate. Highest P solubilization has been observed when glucose, sucrose, or galactose has been used as sole carbon source in the medium (Khan et al. 2009; Vyas and Gulati 2009; Park et al. 2010).

Vendan et al. (2010) investigated endophytic bacterial isolates from ginseng (*Panax ginseng*) for their phosphate-solubilizing ability by detecting extracellular solubilization of precipitated tricalcium phosphate with glucose as the sole source of carbon. Half of the endophytic isolates tested showed phosphate-solubilizing activity. Based on the solubilization zone, an endophytic isolate of *Lysinibacillus fusiformis* recorded higher solubilization of mineral phosphate. In the same study, endophytic isolates of *Bacillus cereus* and *B. megaterium* also showed notable solubilization activity.

Arora et al. (2014) isolated and characterized of endophytic bacterial colonizing halophyte and other salt-tolerant plant species from coastal Gujarat. They have reported two phosphorus-solubilizing endophytic halotolerant bacteria, identified as *Acinetobacter* sp., and *Bacillus aerius*.

Ji et al. (2014) isolated 576 endophytic bacteria from the leaves, stems, and roots of 10 rice cultivars and identified 12 of them as diazotrophic bacteria in which 4 isolates exhibited the phosphate-solubilizing activity by forming clear zones on NBRIP agar plates isolated from ten different rice cultivator. Four isolates solubilized variable amount of phosphates ranging from 1.3 to 3.3 μg/mL. P-solubilizing endophytic bacteria were identified as *Bacillus megaterium* and *Klebsiella pneumonia*.

The potassium-solubilizing microorganisms (KSMs) solubilized the insoluble potassium (K) to soluble forms of K for plant growth and yield. K-solubilization is carried out by a large number of bacteria (*Bacillus mucilaginosus*, *Bacillus edaphicus*, *Bacillus circulans*, *Acidithiobacillus ferrooxidans*, and *Paenibacillus* spp.) and fungal strains *(Aspergillus* spp. and *Aspergillus terreus).* Major amounts of K containing minerals (muscovite, orthoclase, biotite, feldspar, illite, mica) are

present in the soil as a fixed form which is not directly taken up by the plant. The main mechanism of KSMs is acidolysis, chelation, exchange reactions, complexolysis, and production of organic acid. Soil microbes have been reported to play a key role in the natural K cycle, and therefore, potassium-solubilizing microorganisms present in the soil could provide an alternative technology to make potassium available for uptake by plants. K-solubilizing bacteria (KSB) were found to resolve potassium, silicon, and aluminum from insoluble minerals (Aleksandrov et al. 1967). BBDG were best characterized for K-solubilization (Sheng et al. 2008). The K-solubilizing bacteria may have used in the amelioration of K-deficient soil in agriculture. There are only few reports on K-solubilization by endophytic bacteria isolated from wheat (Verma et al. 2013, 2014, 2015b).

Saravanan et al. (2007) reported *Gluconacetobacter diazotrophicus*, an endophytic diazotrophs that possess different plant growth-promoting characteristics along with zinc-solubilizing activity. *G. diazotrophicus* showed variations in their solubilization potential with the strains used and the Zn compounds tested. *G. diazotrophicus* PAl5 efficiently solubilized the Zn compounds tested, and ZnO was effectively solubilized than $ZnCO_3$ or $Zn_3(PO_4)_2$. The soluble Zn concentration was determined in the culture supernatant through atomic absorption spectrophotometer. Gas chromatography-coupled mass spectrometry analysis revealed 5-ketogluconic acid, a derivative of gluconic acid as the major organic acid produced by *G. diazotrophicus* PAl5 cultured with glucose as carbon source.

Natheer and Muthukkaruppan (2012) reported endophytic bacteria *Gluconacetobacter diazotrophicus* from sugarcane and screened for multifarious plant growth-promoting attributes. Among different PGP activities, zinc solubilization (different Zn compounds viz: ZnO, $ZnCO_3$, and $ZnSO_4$) by endophytic bacteria was characterized in vitro. Among different strains of *Gluconacetobacter diazotrophicus*, one strain GaD-1 isolate was found to be the most efficient strain in terms of zinc compounds solubilization and promotion of sugarcane plant growth when compared to other isolates. The use of *G. diazotrophicus* in the field might result in the solubilization of available zinc in the soil and increase zinc uptake by the plant, which in turn would lead to improved plant growth and yield.

Yaish et al. (2015) reported 85 endophytic bacteria from date palm tree (*Phoenix dactylifera* L.) in which 19 strains solubilized Zn from the insoluble form of zinc oxide (ZnO) after 5 days of incubation at 32 °C. These endophytic zinc-solubilizing bacteria belong to different genera of *Bacillus*, *Chryseobacterium*, *Paenibacillus*, *Rhodococcus*, *Staphylococcus*, *Achromobacter*, *Acinetobacter*, *Enterobacter*, *and Klebsiella.*

Verma et al. (2015b) reported psychrotolerant endophytic bacteria from wheat; there were three bacteria, namely, *Bacillus megaterium*, *Bacillus amyloliquefaciens*, and *Bacillus* sp., that solubilized P, K, and zinc. Phosphorus-solubilizing bacteria belonged to *Arthrobacter*, *Bacillus*, *Exiguobacterium*, *Bordetella*, *Providencia*, *Pseudomonas*, *Acinetobacter*, *and Stenotrophomonas.* Potassium-solubilizing bacteria belonged to different genera of *Achromobacter*, *Bacillus*, *Exiguobacterium*, *Stenotrophomonas*, and *Klebsiella.* According to Verma et al (2015b), *Achromobacter* and *Stenotrophomonas* were reported as K-solubilizers at low temperatures, for the first time, and the K-solubilizing bacteria may have used in the amelioration of K-deficient soil in agriculture at low temperatures. Zinc solubilizing endophytic bacteria belongs to different genera of *Arthrobacter*, *Achromobacter*, *Bacillus*, *Bordetella*, *Exiguobacterium*, *Flavobacterium*, *Kocuria*, *Pantoea*, *Providencia*, *Pseudomonas*, and *Staphylococcus.* These zinc-solubilizing bacteria solubilized insoluble form of different zinc compounds (ZnO, ZnS, $Zn_3(PO_4)_2$, and $ZnCO_3$). Diverse group of bacteria were characterized for nitrogen fixation, such as *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Bordetella*, *Providencia*, *Pseudomonas*, and *Stenotrophomonas.* Selected nitrogen-fixing and P- and K-solubilizing bacteria could be effectively used as biofertilizers at place of chemical fertilizers. NPK could be increased soil productivity to improve sustainability of agriculture production.

7.4.1.3 Phytohormones Production

Plant-associated bacteria typically produce plant growth hormones such as cytokinins, auxins, and gibberellins. The gibberellin production is most typical for the root-associated bacteria, cytokinins have been identified in some leaf isolates, and auxin production is common to all plant-associated microbes. Auxins are a group of indole derivatives that have various growth-promoting functions in plants, such as promotion of root formation, regulation of fruit ripening, and stimulation of cell division, extension, and differentiation. Indoleacetic acid (IAA) is the most-well known auxin. Auxins can promote the growth of roots and stems quickly (by increasing cell elongation) or slowly (through cell division and differentiation). The production of such growth regulators by endophytes provides numerous benefits to the host plant including the facilitation of root system expansion, which enhances the absorption of water and nutrients and improves plant survival.

There are several types of bacterial auxins, and the well-studied of these is indoleacetic acid. IAA does not function as a hormone in microbial cells; therefore, the ability of bacteria to produce IAA may have evolved as the plant-microorganism relationship developed. The ability to synthesize these phytohormones is widely distributed among plant-associated bacteria, and IAA may potentially be used to promote plant growth or suppress weed growth. Many studies have described the ability of endophytic bacteria to produce phytohormones and auxins, such as IAA (Hallmann et al. 1997), and the ability to produce IAA is considered to be responsible for plant growth promotion by beneficial bacteria, such as *Azospirillum*, *Alcaligenes faecalis*, *Klebsiella*, *Enterobacter*, *Acetobacter diazotrophicus*, and *Herbaspirillum seropedicae*.

Assumpção et al. (2009) investigated the endophytic bacteria in soybean seeds. The isolates that produced IAA were inoculated in soybean seeds to evaluate their ability to promote plant growth. There were 16 endophytic isolates: *Acinetobacter*, *Bacillus*, *Brevibacterium*, *Chryseobacterium*, *Citrobacter*, *Curtobacterium*, *Enterobacter*, *Methylobacterium*, *Microbacterium*, *Micromonospora*, *Pantoea*, *Paenibacillus*, *Pseudomonas*, *Ochrobactrum*, *Streptomyces*, *and Tsukamurella*. The results showed that all of the isolates synthesized IAA, and the strain 67A (57) of *Enterobacter* sp. significantly increased the dry root biomass

Vendan et al. (2010) investigated the IAA production of endophytic bacteria isolated from ginseng. Ginseng is one of the most important remedies in oriental medicine, and it is presently used as a health tonic and in adaptogenic, antiaging, prophylactic, and restorative remedies. Among 18 representative strains, amplification of *nifH* gene confirmed the presence of diazotrophy in only two isolates. Except four, all the other endophytic isolates produced significant amounts of indole acetic acid in nutrient broth. Isolates E-I-3 (*Bacillus megaterium*), E-I-4 (*Micrococcus luteus*), E-I-8 (*B. cereus*), and E-I-20 (*Lysinibacillus fusiformis*) were positive for most of the plant growth-promoting traits, indicating their role in growth promotion of ginseng.

Szilagyi-Zecchin et al. (2014) reported six endophytic bacteria of corn roots were identified as Bacillus sp. and as *Enterobacter* sp., by sequencing of the 16S rRNA gene. Two *Bacillus* strains (CNPSo 2477 and CNPSo 2478) showed outstanding skills for the production of IAA ranged values between 35.1 and 105.11 μg/mL. Thanh and Diep (2014) reported 301 endophytic bacteria in maize plant cultivated on acrisols of the eastern of South Vietnam. Isolates were isolated and all of them have the ability of nitrogen fixation and phosphate solubilization together with IAA biosynthesis, but there were 30 isolates having the best characteristics, and they were identified as maize endophytes and *nif*H gene owners. Endophytic bacteria were identified as *Bacillus*, A*zotobacter*, and *Enterobacter.*

Cytokinins are a group of compounds with the backbone of adenine having a substitution at the N-6 atom of the purine ring. These compounds are important in many steps of plant development, as they stimulate plant cell division, induce germination of seeds, activate dormant buds, and play a role in apical dominance. Cytokinins also induce the biosynthesis of chlorophyll, nucleic acids, and chloroplast proteins at the early stages

of leaf development. Both pathogenic and beneficial plant-associated bacterial species are capable of synthesizing cytokinins. Among plant-associated methylotrophs, species such as *Methylovorus mays* and *Methylobacterium mesophilicum* JCM2829 synthesize and excrete cytokinins (Ivanova et al. 2001, 2008).

Verma et al. (2015b) reported endophytic bacteria producing IAA at low temperatures. These bacteria belonged to different genera such as *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Bordetella*, *Brevundimonas*, *Enterobacter*, *Exiguobacterium*, *Klebsiella*, *Methylobacterium*, *Providencia*, *Pseudomonas*, and *Stenotrophomonas.* Strain IARI-HHS1-3 showed highest IAA production (70.8 ± 1.5 μg mg^{-1} protein day^{-1}) followed IARI-HHS1-8 (69.1 ± 0.5 μg mg^{-1} protein day^{-1}).

7.4.1.4 ACC Deaminase Activity

Plant growth-promoting endophytic microbes that contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase facilitate plant growth and development by decreasing plant ethylene levels at variety of abiotic stress such as drought, salinity, temperature water logging, heavy metals, and pH. ACC deaminase possessing microbe may play a role in regulating ethylene levels after such bursts, ensuring that ethylene levels stay below the point where growth is impaired (Glick 1995). Ethylene is a key regulator of the colonization of plant tissue by bacteria which in turn suggests that the ethylene-inhibiting effects of ACC deaminase may be a bacterial colonization strategy. Microbial strains exhibiting ACC deaminase activity have been identified in a wide range of genera such as *Arthrobacter*, *Achromobacter*, *Acinetobacter*, *Azospirillum*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, *Rhizobium*, *Serratia*, *Bacillus*, and *Bacillus*-derived genera (Glick 1995; Khalid et al. 2006; Xu et al. 2014; Verma et al. 2014, 2015b).

Xu et al. (2014) reported endophytic bacterial community in tomato (*Lycopersicon esculentum*) seeds and plant growth-promoting activity of ACC deaminase producing *Bacillus subtilis* (HYT-12-1) on tomato seedlings. Isolates exhibited multiple plant growth-promoting (PGP) traits: 37 % of IAA production, 37 % of phosphate solubilization, 24 % of siderophores production, 85 % of potential nitrogen fixation, and 6 % of 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. Isolate HYT-12-1 was shown to have highest ACC deaminase activity (112.02 nmol α-ketobutyrate mg^{-1} protein h^{-1}) among the five ACC deaminase-producing strains. This is the first study to describe endophytic *Bacillus* communities within tomato seeds, and the results suggest that *B. subtilis* strain HYT-12-1 would have a great potential for industrial application as biofertilizer in the future.

Verma et al. (2014, 2015b) reported psychrotolerant and drought-tolerant endophytic bacteria from wheat showing ACC deaminase activity by different genera of *Arthrobacter*, *Flavobacterium*, *Bacillus*, *Methylobacterium*, *Providencia*, *Pseudomonas*, *Stenotrophomonas*, and *Enterobacter.* These bacteria also possess solubilization of phosphorus, potassium, and zinc; produced IAA, siderophore, HCN, and ammonia; and showed antifungal activity against plant pathogens.

7.4.2 In-Direct Plant Growth Promoting Activity

The indirect mechanism of plant growth occurs when bacteria lessen or prevent the detrimental effects of pathogens on plants by production of inhibitory substances or by increasing the natural resistance of the host. Phytopathogenic microorganism can control by releasing siderophores, B-1, 3-glucanase, chitinases, antibiotics, and fluorescent pigment or by cyanide production. World agriculture faces a great loss every year incurred from infection by pathogenic organisms. The most promising way to increase crops productivity is application of microbe for control of disease. The biocontrol agents (bacteria and fungi) can be active at different conditions of pH, temperature, salinity, and drought. Biocontrol agents can inhibit the growth of diverse pathogens by producing different antagonistic substances.

7.4.2.1 Antifungal Activity

Recent studies have indicated that biological control of bacterial wilt disease could be achieved using antagonistic bacteria. Different bacterial species, namely, *Alcaligenes* sp., *Bacillus pumilus*, *B. subtilis*, *B. megaterium*, *Clavibacter michiganensis*, *Curtobacterium* sp., *Flavobacterium* sp., *Kluyvera* sp., *Microbacterium* sp., *Pseudomonas alcaligenes*, *P. putida*, and *P. fluorescens* have been reported as endophytes and were inhibitory to plant pathogens (Inderiati and Franco 2008; Ramesh et al. 2009; Nagendran et al. 2013; Gholami et al. 2014; Purnawati 2014; Verma et al. 2015b).

Coombs et al. (2004) reported endophytic microbes from cereal plants, belonged to phylum actinobacteria of different genera *Streptomyces*, *Microbispora*, *Micromonospora*, and *Nocardioides. These bacteria produced* antifungal compounds in vitro against *Gaeumannomyces graminis*, the causal agent of different diseases in wheat. Inderiati and Franco (2008) investigated actinomycetes; endophytes are promising biological control agents for use in agriculture and have been isolated from various plant species. Thirty-six endophytic actinomycetes were isolated from roots, stems, and leaves of healthy tomato plants. The identification revealed that the majority of the isolates were *Streptomyces* and the remaining belonged to genera *Microbispora* and *Nonomuraea*, which was the first time found as endophyte. To determine the antifungal activity of the isolates, 28 isolates were subjected to in vitro assay against six fungal pathogens. All the isolates tested inhibited the growth of at least one of the phytopathogenic fungi, and that five of the isolates inhibited the growth of all the fungal pathogens used in this assay. Selected isolates were tested for their activity in plants in pot experiments against *Rhizoctonia solani*. Of the 15 isolates tested, 14 isolates significantly reduced ($p<0.01$) the percentage of *Rhizoctonia solani*-infected plants from 30 to 76 %.

Nagendran et al. (2013) examined endophytic bacteria isolated from different plants and tested for biocontrol against *Xanthomonas oryzae.* Among all isolates, *Bacillus subtilis* found suitable biocontrol agent against pathogen causing leaf blight disease in rice. In vivo condition *Bacillus subtilis* suppressed bacterial leaf blight (2.80 %) compared to untreated control plots (19.82 %), which also recorded a higher grain and straw yield. Purnawati (2014) reported that 100 % lose in crop production of tomato due to *Ralstonia solanacearum* pathogen. Biological control using endophytic microbes is a control method to support agriculture sustainability. Endophytic bacteria were isolated from tomato stems and roots and screened for biocontrol against *Ralstonia solanacearum*. Two isolates (Ps1 and Ps8) inhibit *Ralstonia solanacearum* based on antagonistic test in vitro. In vivo condition, these endophytic bacteria suppressed 8.07–9.19 % pathogen attack.

Verma et al. (2015b) investigated assessment of genetic diversity and plant growth-promoting attributes of psychrotolerant bacteria allied with wheat (*Triticum aestivum*) from the northern hills zone of India. Of 121 representative, 14 bacteria, namely, *Arthrobacter methylotrophus*, *Achromobacter piechaudii*, *Bacillus altitudinis*, *Bacillus amyloliquefaciens*, *Bacillus horikoshii*, *Bacillus sp.*, *Bordetella bronchiseptica*, *Brevundimonas terrae*, *Exiguobacterium antarcticum*, *Exiguobacterium sp.*, *Pseudomonas fluorescens*, *Pseudomonas fragi*, *Pseudomonas mediterranea*, and *Staphylococcus arlettae*, were found to be antagonistic against three plant pathogens *Fusarium graminearum*, *Rhizoctonia solani*, and *Macrophomina phaseolina.* Cold-adapted isolates may have an application as inoculants for biocontrol agents for crops cultivating under cold climatic condition.

7.4.2.2 Production of Antibiotics and Lytic Enzymes

The production of antibiotics is considered to be one of the most powerful and studied biocontrol mechanisms of plant growth-promoting bacteria against phytopathogens and has become increasingly better understood over the past two decades (Shilev 2013; Gupta et al. 2015). A variety of antibiotics have been identified, including compounds such as amphisin, 2,4-diacetylphloroglucinol (DAPG), oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin,

tropolone, and cyclic lipopeptides produced by Pseudomonads and oligomycin A, kanosamine, zwittermicin A, and xanthobaccin produced by *Bacillus*, *Streptomyces*, and *Stenotrophomonas* sp. to prevent the proliferation of plant pathogens (Generally fungi). *Bacillus amyloliquefaciens* is known for lipopeptide and polyketide production for biological control activity and plant growth promotion activity against soil-borne pathogens (Ongena and Jacques 2008). Apart from the production of antibiotic, some bacteria are also capable of producing volatile compound known as hydrogen cyanide (HCN) for biocontrol of black root rot of tobacco, caused by *Thielaviopsis basicola* (Sacherer et al. 1994). Lanteigne et al. (2012) also reported the production of DAPG and HCN by *Pseudomonas* contributing to the biological control of bacterial canker of tomato.

Growth enhancement through enzymatic activity is another mechanism used by plant growth-promoting bacteria. Plant growth-promoting bacterial strains can produce certain enzymes such as chitinases, dehydrogenase, β-glucanase, lipases, phosphatases, proteases, etc., and exhibit hyperparasitic activity, attacking pathogens by excreting cell wall hydrolases. Through the activity of these enzymes, plant growth-promoting bacteria play a very significant role in plant growth promotion particularly to protect them from biotic and abiotic stresses by suppression of pathogenic fungi including *Botrytis cinerea*, *Sclerotium rolfsii*, *Fusarium oxysporum*, *Phytophthora* sp., *Rhizoctonia solani*, and *Pythium ultimum* (Arora 2013).

Quecine et al. (2011) evaluated chitinase production by endophytic actinobacteria and the potential of this for the control of phytopathogenic fungi. Actinobacteria are used extensively in the pharmaceutical industry and agriculture owing to their great diversity of enzyme production. In this study, endophytic *Streptomyces* strains were grown on minimal medium supplemented with chitin, and chitinase production was quantified. The strains were screened for any activity toward phytopathogenic fungi with a dual-culture assay in vitro. The correlation between chitinase production and pathogen inhibition was calculated and further confirmed on *Colletotrichum sublineolum* cell walls by scanning electron microscopy.

Castro et al. (2014) investigated endophytic bacteria mainly *Bacillus*, *Pantoea*, *Curtobacterium*, and *Enterobacter* from mangrove systems in Bertioga and Cananéia. Among isolated endophytic microbes, *Bacillus* was the most dominant genus. Isolated were screened for hydrolytic enzymes production, and it is found that more than 75 % isolated possess protease activity, whereas 62 % isolated showed endonucleases activity. Among different genera, *Bacillus* showed the highest activity of amylase and esterase and endoglucanase

7.4.2.3 Production of Siderophore

Iron is a necessary cofactor for many enzymatic reactions and is an essential nutrient for virtually all organisms. In aerobic conditions, iron exists predominantly in its ferric state (Fe^{3+}) and reacts to form highly insoluble hydroxides and oxyhydroxides that are largely unavailable to plants and microorganisms. To acquire sufficient iron, siderophores produced by bacteria can bind Fe^{3+} with a high affinity to solubilize this metal for its efficient uptake. Bacterial siderophores are low-molecular-weight compounds with high Fe^{3+} chelating affinities responsible for the solubilization and transport of this element into bacterial cells. Some bacteria produce hydroxamate-type siderophores, and others produce catecholate types. In a state of iron limitation, the siderophore-producing microorganisms are also able to bind and transport the iron-siderophore complex by the expression of specific proteins. The production of siderophores by microorganisms is beneficial to plants because it can inhibit the growth of plant pathogens. Siderophores have been implicated for both direct and indirect enhancements of plant growth by plant growth-promoting bacteria. The direct benefits of bacterial siderophores on the growth of plants have been demonstrated by using radio-labeled ferric siderophores as a sole source of iron and showed that plants are able to take up the labeled iron by a large number of plant growth-promoting bacteria including *Aeromonas*, *Azadirachta*, *Azotobacter*, *Bacillus*, *Burkholderia*,

Pseudomonas, *Rhizobium*, *Serratia*, and *Streptomyces* sp. (Vendan et al. 2010; Loaces et al. 2011; Verma et al. 2014, 2015b; Pedraza 2015).

Vendan et al. (2010) described the siderophore production by seven endophytic bacterial strains. These strains were classified as *Bacillus cereus*, *B. flexus*, *B. megaterium*, *Lysinibacillus fusiformis*, *L. sphaericus*, *Microbacterium phyllosphaerae*, and *Micrococcus luteus*. Siderophore production by endophytic bacteria has been investigated in only a few cases, mainly as a mechanism of certain bacteria to antagonize pathogenic fungi.

Loaces et al. (2011) described and characterized the community of endophytic, siderophore-producing bacteria (SPB) associated with *Oryza sativa*. Less than 10 % of the endophytic bacteria produced siderophores in the roots and leaves of young plants, but most of the endophytic bacteria were siderophore producers in mature plants. According to the results, 54 of the 109 endophytic SPB isolated from different plant tissues or growth stages from replicate plots of rice were unique. The relative predominance of bacteria belonging to the genera *Sphingomonas*, *Pseudomonas*, *Burkholderia*, and *Enterobacter* alternated during plant growth, but the genus *Pantoea* was predominant in the roots at tillering and in the leaves at subsequent stages. *Pantoea ananatis* was the SPB permanently associated with all of the plant tissues of rice.

Pedraza (2015) reported siderophores production by *Azospirillum* and its biocontrol attributes. Different species of plant growth-promoting bacteria produce siderophores which can be a competitive advantage for plant, not only for growth but also as biocontrol agent against phytopathogens.

7.5 Future Prospect

The need of today's world is high output yield and enhanced production of the crop as well as fertility of soil to get in an eco-friendly manner. Hence, the research has to be focused on the new concept of microbial (endophytic, epiphytic, and rhizospheric) engineering based on favorably partitioning of the exotic biomolecules, which create a unique setting for the interaction between plant and microbes. Future research in microbes will rely on the development of molecular and biotechnological approaches to increase our knowledge of microbes and to achieve an integrated management of microbial populations of endophytic. Fresh alternatives should be explored for the use of bioinoculants for other high-value crops such as vegetables, fruits, and flowers. The application of multi-strain bacterial consortium over single inoculation could be an effective approach for reducing the harmful impact of stress on plant growth. Research on nitrogen fixation and phosphate solubilization by plant growth-promoting rhizobacteria progresses, but little research can be done on potassium solubilization which is the third major essential macronutrient for plant growth. This will not only increase the field of the inoculants but also create confidence among the farmers for their use.

7.6 Conclusion

In the course of the past few decades, the human population has doubled. Food production has similarly increased. The use of man-made fertilizers has enabled much of the increase in the crop production. Concurrent with the escalating use of commercial fertilizers, the intensity of agricultural practices has increased, and a wide variety of fungicides, bactericides, and pesticides are utilized in large-scale crop production. Because of their close interaction with plants, attention has been focused on endophytes and their potential use in sustainable agriculture. An increasing number of researchers are attempting to elucidate the mechanisms of plant growth promotion, biological control, and bioremediation mediated by endophytes by examining species and conditions that lead to greater plant benefits. New information from transcriptome and proteome analyses will aid in the optimization of studies examining plant-microbe interactions. Research in this field is clearly very promising and will have significant economic and environmental impacts in the future.

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Exploiting PGPR and AMF Biodiversity for Plant Health Management

8

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Abstract

Indian subcontinent is one of the mega hotspots for biodiversity including microbes. So far, only very little microbial diversity has been harnessed for human and animal welfare. The importance of soil microorganisms in plant health management is well known. The interaction between bacteria and plant roots may be beneficial, harmful, or neutral for the plant, and sometimes the effect of a particular organism may vary as the soil conditions change. Among the diverse range of plant growth-promoting rhizobacteria (PGPR) identified, bacterial species such as *Pseudomonas* and *Bacillus* spp. have a wide distribution. The mechanisms by which PGPR enhance plant growth include plant growth promoters, resistance inducers, biochemicals, etc. The nitrogen-fixing bacteria are known to enhance growth of plants by means of symbiotic or free-living association with plants. Arbuscular mycorrhizal fungi (AMF) enhancing plant growth has been reported by several workers. These microbes could be inoculated either singly or in combinations to deliver maximum benefits to the plants. For instance, combined inoculation of AMF with other PGPR exerted positive effects on the growth of several crop plants. By exploiting the microbial biodiversity, the input cost in agricultural production systems could be reduced considerably and thereby make agriculture a sustainable venture especially for small and marginal farmers whose resources are limited. Microbes can supplement nutrients to the plants, induce resistance against biotic and abiotic stresses, protect from insect pests and plant pathogens, manage weeds and nematodes, etc. In this chapter, the rich

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microbial biodiversity, its systematic characterization, cataloguing, and evaluation for improving agricultural production in an economical and eco-friendly way have been discussed.

Keywords
PGPR • Mycorrhiza • Colonization • Diversity • Symbiosis • *Pseudomonas*

8.1 Introduction

Plant growth is a function of an interaction between plants and its immediate environment. The environment for roots is the soil or planting medium, which provide structural support as well as water and nutrients to the plant. The term "rhizosphere" was introduced in 1904 by the German scientist Hiltner to denote that region of the soil which is influenced by plant roots. Roots also support the growth and functions of a variety of microorganisms that can have a profound effect on the growth and survival of plants. However, they could be beneficial or neutral or deleterious with respect to root/plant health. Increased plant growth and crop yield can be obtained due to beneficial microbes which are also termed as plant growth-promoting rhizobacteria (PGPR). Kloepper and coworkers coined the term PGPR (plant growth-promoting rhizobacteria) in the late 1970s (Kloepper and Schroth 1978). PGPR improve plant growth by indirect or direct mechanisms although the difference between the two is not always distinct (Ashraf et al. 2013). Direct mechanisms include the improvement of nutrient availability to the plant by the fixation of atmospheric nitrogen, production of iron-chelating siderophores, organic matter mineralization (thereby meeting the nitrogen, sulfur, phosphorus nutrition of plants), and solubilization of insoluble phosphates. Another important direct mechanism involves the production of plant growth hormones and the stress-regulating hormone 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Indirect mechanisms include inhibition of microorganisms that have a negative effect on the plant (by niche exclusion), viz., hydrolysis of molecules released by pathogens, synthesis of enzymes that hydrolyze fungal cell walls, synthesis of HCN, improvement of symbiotic relationships with rhizobia and mycorrhizal fungi, and insect pest control (Das et al. 2013). Though the term PGPR strictly includes nitrogen-fixing and P-solubilizing organisms, scientists commonly refer those bacteria promoting plant growth directly through production of phytohormones or indirectly through suppression of pathogenic organisms, as PGPR.

Rhizosphere biology is approaching a century of investigations. PGPRs have attracted special attention on account of their beneficial activities. Bacteria that aggressively colonize roots are now referred to as "rhizobacteria." Strains of the genera such as *Aeromonas*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Klebsiella*, *Pseudomonas*, *Rhizobium*, and *Serratia* have been identified as PGPR (Dey et al. 2004; Raj et al. 2004; Tripathi et al. 2005). The diversity of PGPR in the rhizosphere largely varies according to the plant type, soil type, and nutrients available (Tilak et al. 2005). According to the mode of action, PGPR have been divided into two groups: biocontrol PGPRs that indirectly benefit the plant growth and PGPRs that directly affect plant growth and seed emergence or improve crop yields (Glick et al. 1999).

The importance of soil microorganisms in nutrient cycling and fertility maintenance is well known. The six billion world population today consumes about 25 million tonnes of protein nitrogen each year. By 2050, it is expected to reach 40–45 million tonnes. To meet such enormous nitrogen requirements through chemical

fertilizers would not only be expensive but also could severely degrade soil health. Biofertilizers are preparations containing live microorganisms that help in nutrient availability through fixation, solubilization, or mobilization. Annually, about 170 million tonnes of nitrogen is contributed through biological nitrogen fixation. However, the biofertilizer production in 2000–2001 was about 13,000 tonnes. This huge gap between production and demand coupled with the increasing interest in the usage of microorganisms for crop health management needs development of good strains that fix nitrogen. Biofertilizers are an important component of the integrated plant nutrient management systems, particularly in rainfed areas, where farmers tend to rely either on "no-cost" or "low-cost" inputs. Species of *Azotobacter* and *Azospirillum* are known to fix nitrogen in a nonsymbiotic mode mainly in cereal crops. Similarly, strains of *Bacillus*, *Pseudomonas*, *Aspergillus*, and AMF have been commercialized for phosphorus mobilization. Beneficial plant–microbe interactions in the rhizosphere are primary determinants of plant health and soil fertility. Not much attention has been paid to the effects of plant–microbe interactions, on ecosystem variability, productivity, and plant biodiversity. In the last few decades, strains of microbes have been identified for mobilization of important plant nutrients like nitrogen, phosphorus, potassium, zinc, etc.

The potential negative environmental impacts of large-scale use of chemical fertilizers together with their increased cost have prompted a public outcry for alternatives to replace chemical fertilizers. Bacterial inoculants capable of facilitating plant growth have been considered reasonable substitutes but, in the past, suffered from a lack of consistency when used under field conditions. However, it is plausible that in the future, the use of bacterial inoculants will become part of everyday agricultural practice, as tools of molecular biology are available for better understanding of the mechanisms utilized by these organisms and to improve their efficacy in agriculture.

The interaction between bacteria and plant roots may be beneficial, harmful, or neutral for the plant, and sometimes the effect of a particular bacterium may vary as the soil conditions change (Lynch 1990). For example, a bacterium that facilitates growth by providing plants with fixed nitrogen, which is usually present in only limited amounts in the soil, is unlikely to provide any benefit to plants when large amounts of chemical nitrogen fertilizer are added to the soil. The importance of PGPR was realized as an offshoot of biological control of soilborne pathogens. But, according to the mode of action, PGPRs were divided into two groups, viz., biocontrol PGPBs and PGPBs (Bashan and Holguin 1997). Protection of bacterial-inoculated seedlings against soilborne pathogens was observed inseparable from the plant growth-promoting activity of several of the reported PGPR (Manjula and Podile 2001). As a consequence, PGPR were more emphasized as protectants of soilborne pathogens.

8.2 PGPR Diversity

Among the diverse range of PGPR identified, *Pseudomonas* and *Bacillus* spp. have a wide distribution and are the most extensively studied. *Azospirillum*, a N_2-fixing genus, is an important group of PGPR, since treatment with almost all strains and species of this genus positively affect the root biomass and surface area (Bashan et al. 2004). Recent developments in metagenomics, i.e., the study of collective genome of an ecosystem, provide insights of bacterial diversity in the rhizosphere including the non-culturable organisms. Though, by definition, PGPR are free-living plant-associated bacteria, few *Rhizobium* strains that colonize the roots of nonlegume plants such as *Gramineae* and crucifers and promote root growth by mechanisms other than biological N_2 fixation are also considered as PGPR (Antoun et al. 1998). In this chapter, we report the exploration of various genera of PGPR and AMF on their plant growth-promoting activities with a detailed note on *Pseudomonas* spp., *Bacillus* spp., and AMF.

8.2.1 Symbiotic N_2-Fixing Bacteria

Nitrogen is required for cellular synthesis of enzymes, proteins, chlorophyll, DNA, and RNA and is therefore important in plant growth and production of food and feed. For nodulating legumes, nitrogen is provided through symbiotic fixation of atmospheric N_2 by nitrogenase in rhizobial bacteroids. This process of biological nitrogen fixation (BNF) accounts for 65 % of the nitrogen currently utilized in agriculture. Rhizobia (species of *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, and *Sinorhizobium*) form intimate symbiotic relationships with legumes. Nodules, the sites for symbiotic nitrogen fixation, are formed as a result of series of interactions between rhizobia and leguminous plants. However, there are number of factors which affect the nodulation on legume roots including host–microsymbiont compatibility, physicochemical conditions of the soil, and the presence of both known and unknown biomolecules such as flavonoids, polysaccharides, and hormones (Zafar-ul-Hye et al. 2007). *Rhizobium/Bradyrhizobium* is one of the widely studied organisms relevant to a number of pulse crops, groundnut, and soybean in the rainfed production systems. Contribution of the legume–*Rhizobium* symbiosis to the production system varies depending on a number of physical, environmental, nutritional, and biological factors. Most cultivated tropical soils in India are reported to have relatively large populations (>100 g^{-1} dry soil) of rhizobia capable of nodulating the legumes (Nambiar et al. 1988).

8.2.2 Free-Living Nitrogen-Fixing PGPR

Free-living nitrogen-fixing bacteria belong to a wide array of taxa; among the most relevant bacterial genera are *Azospirillum*, *Azotobacter*, *Burkholderia*, *Herbaspirillum*, and *Bacillus* (Vessey 2003). Azotobacteraceae is the most representative of bacterial genera able to perform free nitrogen fixation. Various reports describe the benefits of Azotobacteraceae on several crops (Mayea et al. 1998). Nitrogen-fixing *Pseudomonas* was also characterized in studies carried out by Sazzad Mirza et al. (2006). *Azotobacter* is the genus most used in agricultural trials. As previously suggested, the effect of *Azotobacter* and *Azospirillum* is attributed not only to the amounts of fixed nitrogen but also to the production of plant growth regulators (indole acetic acid, gibberellic acid, cytokinins, and vitamins), which result in additional positive effects to the plant (Rodelas et al. 1999). Application of inoculants in agriculture has resulted in notable increases in crop yields, especially in cereals, where *Azotobacter chroococcum* and *Azospirillum brasilense* have been very important. These two species include strains capable of releasing substances such as vitamins and plant growth regulators, which have a direct influence on plant growth (Velazco and Castro 1999).

The association of diazotrophic rhizobacteria with grasses is well documented (Baldani et al. 1997) and includes several bacterial genera and many important agricultural plants (Table 8.1). Free-living diazotrophs are frequently the predominant culturable bacteria in the rhizosphere of wheat (Heulin et al. 1994). Under certain circumstances, free-living diazotrophic bacteria that associate with roots of nonleguminous plants can increase the growth and yield of crops (Abbass and Okon 1993). However, nitrogen fixation by free-living rhizobacteria is thought to contribute only a small proportion of the nitrogen assimilated directly by plants (Wood et al. 2001) with the observed growth responses being attributed to secretion of plant growth-promoting substances (Dobbelaere et al. 2001).

8.2.3 *Pseudomonas* spp.

Fluorescent pseudomonads have been predominantly recovered from the rhizoplane and rhizosphere of not only crop species but also from woody tree seedlings and fruit trees. Among the Gram –ve soil bacteria, *Pseudomonas* is the most abundant genus in the rhizosphere, and the PGPR activity of some of these strains has been known for many years, resulting in a broad knowledge of the mechanisms involved (Lucas-García et al.

Table 8.1 Examples of the diversity of associations between nitrogen-fixing (diazotrophic) rhizobacteria and grasses

Bacterium	Principal association	Reference
Azospirillum amazonense	Many grasses in Amazonia	Boddey and Dobereiner (1988), Reis et al. (2001)
Azospirillum lipoferum	Many grasses and cereals	Boddey and Dobereiner (1988)
Azospirillum halopraeferans	Kallar grass	Boddey and Dobereiner (1988)
Azospirillum irakense	Rice	Baldani et al. (1997)
Azospirillum spp.	Guinea grass, sugarcane	Bilal et al. (1990), Ghai and Thomas (1989)
Azotobacter chroococcum	Maize	Martinez-Toledo et al. (1988)
Azotobacter vinelandii	*Paspalum*, grasses	Dobereiner and Pedrosa (1987)
Gluconacetobacter diazotrophicus	Sugarcane	Michiels et al. (1989)
Bacillus azotofixans	Wheat, sugarcane, grasses	Boddey and Dobereiner (1988)
Bacillus circulans	Maize	Berge et al. (1991)
Bacillus polymyxa	Prairie grasses, xeric grasses, wheat	Nelson et al. (1976), Dobereiner and Pedrosa (1987)
Erwinia herbicola	Wheat, sorghum	Pedersen et al. (1978)
Herbaspirillum seropedicae	Maize, cereals, elephant grass, forage grasses	Boddey and Dobereiner (1988), Indira and Bagyaraj (1996)
Klebsiella pneumoniae	Wheat, sorghum	Pedersen et al. (1978)
Pseudomonas sp.	Wetland rice	Boddey and Dobereiner (1988)
Saccharobacter nitrocaptan	Sugarcane	Graham (1988)

2004). *Pseudomonas* strains show high versatility in their metabolic capacity. Antibiotics, siderophores, and hydrogen cyanide are among the metabolites generally released by these strains (Charest et al. 2005). These metabolites strongly affect the environment, both because they inhibit growth of other deleterious microorganisms and because they increase nutrient availability for the plant. Members of the genus are rod shaped, Gram –ve with one or more polar flagella providing motility, aerobic, and nonspore forming. Important type species of the genus is *Pseudomonas aeruginosa*, and other important species are *P. alcaligenes*, *P. putida*, *P. fluorescens* group, *P. antarctica*, *P. marginalis*, *P. syringae*, *P. alcaliphila*, *P. psychrophila*, *P. rhizosphaerae*, and *P. nitroreducens*.

8.2.3.1 *Pseudomonas* for Plant Growth Promotion

The mechanisms by which *Pseudomonas* and *Bacillus* spp. are known to enhance plant growth include nutrient mobilization, secretion of phytohormones and exopolysaccharides, production of siderophores, volatiles, antibiosis, etc. *Pseudomonas* spp. produce cytokinins and gibberellins (gibberellic acid), in addition to IAA (Gaudin et al. 1994). A few PGPR strains were reported to produce cytokinins (Vessey 2003) and gibberellins (gibberellic acid, GA; Gutiérrez Mañero et al. 2003). A mutant strain of *Pseudomonas putida* with fourfold increase in IAA production lost its ability to induce root elongation in canola seedlings, though its growth rate and production of siderophores and 1-amino cyclopropane-1-carboxylate (ACC) deaminase remained unaltered (Xie et al. 1996).

The solubilization of P in the rhizosphere is the most common mode of action implicated in PGPR that increases nutrient availability to host plants (Richardson 2001). Examples of some of the studied associations include *Pseudomonas chlororaphis* and *P. putida* and soybean (Cattelan et al. 1999). *Pseudomonas* spp. are the potent siderophore producers among Gram –ve PGPR, and they produce pseudobactin, pyochelin, pyoverdine, quinolobactin, and salicylic acid, and the structure of the outer membrane receptor proteins complementary to some of these siderophores has been determined (David et al. 2005). The majority of fluorescent pseudomonads produce complex fluorescent peptidic siderophores called

pyoverdines or pseudobactins, which are very efficient iron scavengers (Cornelis and Matthijs 2002). Different types of siderophores produced by *Pseudomonas* spp. are listed in Table 8.2.

8.2.4 *Bacillus* spp.

Bacillus is the most abundant bacterial genus in the rhizosphere, and the PGPR activity of some of these strains has been known, resulting in a broad knowledge of the mechanisms involved (Gutiérrez et al. 2003). There are a number of metabolites that are released by these strains (Charest et al 2005), which strongly affect the environment by increasing nutrient availability of the plants (Barriuso and Solano 2008). Multiple species of *Bacillus* and *Paenibacillus* are known to promote plant growth. The principal mechanisms of growth promotion include production of growth-stimulating phytohormones, solubilization and mobilization of phosphate, siderophore production, antibiosis, inhibition of plant ethylene synthesis, and induction of plant systemic resistance to pathogens (Richardson et al. 2009). It is very likely that plant growth promotion by rhizosphere bacilli may be a result of combined action of two or more of these mechanisms. *Bacillus*–plant interactions fall into three general groups: beneficial, neutral, and detrimental to the host plants' health.

Growth promotion in lodgepole pine seedlings inoculated with auxin-producing *Paenibacillus polymyxa* L6 was supported by the enhanced levels of auxin in roots (Bent et al. 2001). *Bacillus megaterium* from tea rhizosphere is able to solubilize phosphate, and thus it helps in the plant growth promotion (Chakraborty et al. 2006). The PSB inoculation with mineral phosphorus raises the efficiency of P fertilizer and decreases the required P rate to plants. The zinc can be solubilized by microorganisms, viz., *B. subtilis*, *Thiobacillus thiooxidans*, *and Saccharomyces sp.* These microorganisms can be used as biofertilizers for solubilization of fixed micronutrients like zinc (Raj 2007).

Bacillus megaterium from tea rhizosphere is able produce siderophore, and thus it helps in the plant growth promotion and reduction of disease intensity (Chakraborty et al. 2006). Volatiles produced by *Bacillus subtilis* and *B. amyloliquefaciens* stimulated the growth of *Arabidopsis thaliana* in vitro as observed by an increase in the total leaf area. Volatiles produced by these bacteria were 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol. Choudhary and Johri (2008) have reviewed ISR by *Bacillus* spp. in relation to crop plants and emphasized the mechanisms and possible applications of ISR in the biological control of pathogenic microbes. Various strains of species *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus* are known as potential elicitors of ISR and exhibit significant reduction in the incidence or severity of various diseases on diverse hosts (Choudhary and Johri 2008).

Table 8.2 Secondary siderophores from fluorescent pseudomonads

Siderophore	Species	Characteristics	Reference
Pyochelin	*P. aeruginosa*	Binds other metals	Cox et al. (1981)
	P. fluorescens CHAO	Redox-active	Britigan et al. (1997)
	P. fluorescens Pf-5		Paulsen et al. (2005)
Pseudomonine	*P. fluorescens* WCS374	Lower affinity siderophore	Mercado-Blanco et al. (2001)
Quinolobactin/thioquinolobactin	*P. fluorescens* ATCC17400	Repressed by cognate pyoverdine	Mossialos et al. (2000)
		Anti-*Pythium* activity	Matthijs et al. (2007)
PDTC	*P. stutzeri* KC	Binds several metals	Stolworthy et al. (2001)
	P. putida	Antimicrobial activity	Lewis et al. (2004)
		CCl_4 degradation	

Table 8.3 Antibiotics of PGPR in the management of soilborne diseases

Antibiotics	PGPR	Pathogen	Crop	Reference
DAPG	*Pseudomonas* sp.	*P. ultimum*	Sugar beet	Shanahan et al. (1992)
Aerugine	*P. fluorescens*	*Phytophthora*	Pepper	Lee et al. (2003)
Phenazine	*Pseudomonas* sp.	*F. oxysporum*	Tomato	Chin-A-Woeng et al. (1998)
Pyrrolnitrin	*P. fluorescens*	*R. solani*	Cotton and Cucumber	Hammer et al. (1997)
Pyrrolnitrin	*P. cepacia*	*Sclerotinia sclerotiorum*	Sunflower	McLoughlin et al. (1992)
Viscosinamide	*P. fluorescens*	*P. ultimum* and *R. solani*	Sugar beet	Nielsen et al. (1998)
Pyoluteorin	*P. fluorescens*	*Pythium* spp.	Cotton and Sugar beet	Howell and Stipanovic (1980)

Table 8.4 Commercially available PGPR strains, those essentially act through direct plant growth-promoting mechanisms

Trade name	PGPR strain	Manufacturer	Recommended application
Bioboost	*Delftia acidovorans*	Brett-Young Seeds Ltd., Manitoba	Seed treatment in canola
Bioplin	*Azotobacter* spp.	Kumar Krishi Mitra Bioproducts Pvt. Ltd., Pune, India	Soil drenching for sunflower, tomato, and other vegetable crops
Bioyield	*Bacillus* spp.	Gustafson, LLC, Plano, Tx	Seed treatment in tomato, tobacco, cucumber, and pepper
Compete	*Bacillus*, *Pseudomonas*, and *Streptomyces* spp.	Plant Health Care BV, CA Vught	Soil drenching for turfgrass, nursery, and greenhouse plantations
Kodiak	*B. subtilis* GB03	Gustafson, LLC, Dallas, Tx	Seed treatment in fruits and vegetables

Suppression of *Pythium* root rot of cucumber was improved by enhancing the production of DAPG and pyoluteorin in *P. fluorescens* strain CHA0 (Fenton et al. 1992) (Table 8.3). Seed bacterization of tomato and chili with a talc-based consortia comprising of *P. fluorescens* and *P. chlororaphis* performed better in reducing the incidence of damping-off (Kavitha et al. 2003). Gnanamanickam et al. (1998) showed that a strain of *P. fluorescens* afforded 79–82 % control of rice blast and sheath blight and simultaneously enhanced the grain yield in rice.

8.2.5 Effect of PGPR on Plant Growth

Numerous examples of plant growth stimulation by fluorescent *Pseudomonas* spp. have been reported. Significant increases in growth and yield of potatoes by up to 367 % were reported in greenhouse experiments by Burr et al. (1978) with specific fluorescent *Pseudomonas* strains. Van Peer and Schippers (1988) documented increases in root and shoot fresh weight for tomato, cucumber, lettuce, and potato as a result of bacterization with *Pseudomonas* strains. Improvement of seed germination by rhizobacteria has been reported in other cereals such as sorghum (Raju et al. 1999), pearl millet (Niranjana et al. 2004), wheat, and sunflower (Shaukat et al. 2006). Inoculation of *Burkholderia cepacia*, *Pseudomonas fluorescens*, and *Enterobacter* sp. consortium on *Sorghum bicolor* enhanced root colonization and plant growth promotion (Chiarini et al. 1998). A list of commercially marketed strains of PGPR is depicted in Table 8.4.

The PGPR influence crop growth and development by releasing plant growth regulators

(Glick and Bashan 1997) and improving morphological characteristics of inoculated roots (Biswas 1998), which favored nutrient uptake (Praveen Kumar et al. 2013). Bacteria-mediated increases in root weight are commonly reported responses to PGPR inoculations (Praveen Kumar et al. 2012a). More importantly, increases in root length and root surface area are reported (Holguin and Glick 2001). When *P. putida* GR12-2, a nitrogen-fixing strain isolated from the rhizosphere of an arctic grass, was applied to seeds of canola, it increased root length in sterile growth pouches (Lifshitz et al. 1986).

The tripartite association composed of legume plant, rhizobia, and *Pseudomonas* spp. has been reported to increase root and shoot weight, plant vigor, nitrogen fixation, and grain yield in various legumes. Sindhu et al. (2002) showed that dual inoculation of chickpea with *Pseudomonas* and *Mesorhizobium* enhanced plant growth. Dual inoculation increased yields in sorghum (Praveen Kumar et al. 2012b), soybean (Abdalla and Omer 2001), and wheat (Galal 2003). Sahin et al. (2004) documented increased sugar content in sugar beet by co-inoculation of N-fixing and PSB. Parmar and Dadarwal (1999) also observed that co-inoculation of *Pseudomonas* and *Bacillus* sp. with *Rhizobium* strains enhanced the nodule weight, root length, shoot biomass, and total plant nitrogen in chickpea, when grown in sterilized jars or under pot culture conditions. Studies carried out by Stijn Spaepen et al. (2008) on wheat plants showed that inoculation with IAA producing *A. braziliense* leads to stimulation of early plant development and significant increase in dry weight of plants and also increased uptake of nitrogen by wheat plants. However, PGP is a complex phenomenon that often cannot be attributed to a single mechanism and, as outlined above, PGPR may typically display a combination of mechanisms (Ahmad et al. 2008).

Seed bacterization temporarily changes the balance of the rhizosphere populations, and such changes may sometimes enhance the plant growth, yield, and uptake of nutrients depending upon the establishment of the introduced cultures. The co-inoculation of *B. subtilis*, *Bradyrhizobium*, and AMF enhanced the growth, nutrient uptake, and yield of green gram in the study conducted by Almas Zaidi and Saghir Khan (2006). The fact that plant growth and nutrient uptake increased in the presence of AMF suggested a strong synergistic relationship between root colonization, P uptake, and growth promotion. Vladimir et al. (2001) reported that *B. japonicum* co-inoculated with rhizosphere-competent *Pseudomonas* sp. enhanced nitrogen fixation in soybean. A two-year field study conducted on pigeon pea using *Pseudomonas fluorescens* showed an increase in grain yield of pigeon pea, maize, and wheat by 23.3 %, 194 %, and 16.7 %, respectively, over uninoculated control (Tilak and Srinivasa Reddy 2006).

8.2.6 Synergistic Interaction between PGPR

Improvement in the efficiency of nitrogen fixation has been reported when plants are co-inoculated with rhizobia and strains of *Pseudomonas* and *Bacillus* (Parmar and Dadarwal 1999). Combined inoculation of PGPR and rhizobia was observed to exert positive effects on the growth of legumes including pea, clover, common bean, cowpea, and soybean, by increased nodulation. For example, co-inoculation of rhizobacteria with rhizobia increased the number of nodules and nodule occupancy (Cattelan et al. 1999).

8.3 Arbuscular Mycorrhizal Fungi (AMF)

The term "mycorrhiza" was coined by A. B. Frank (1885) literally means "fungus root." It is a symbiotic association between plant roots and fungi. Though there are different kinds of mycorrhiza, the most common mycorrhizal association occurring in crops important in agriculture and horticulture is the arbuscular type. These fungi in soil are ubiquitous throughout the world and form symbiotic relationships with the roots of

most terrestrial plants. AMF occurs over a broad ecological range from aquatic to desert environments (Bagyaraj 1992).

AMF belong to the phylum Glomeromycota, which has three classes (Glomeromycetes, Archaeosporomycetes, and Paraglomeromycetes) with 5 orders (Glomerales, Diversisporales, Gigasporales, Paraglomerales, and Archaeosporales), 14 families, and 26 genera (Sturmer 2012). The commonly occurring genera of AMF are *Glomus*, *Gigaspora*, *Scutellospora*, *Acaulospora*, and *Entrophospora.* In the soil, AMF produce large thick-walled resting spores called extramatricular chlamydospores, which can survive adverse soil conditions and germinate when conditions are favorable. The hyphae penetrate the root and ramify in the cortex producing highly branched hyphal structures called arbuscules and round to oval structures called vesicles. The presence of vesicles and arbuscules is the criteria for identifying AMF in the roots (Dar 2010).

These fungi are obligate symbionts and have not been cultured on nutrient media. AMF are not host specific although evidence is growing that certain endophytes may form preferential association with certain host plants (Rivera et al. 2007; Bagyaraj 2011). It is now proved beyond doubt that AMF greatly enhance plant growth. The improved growth is mainly attributed to uptake of diffusion-limited nutrients such as P, Zn, Cu, etc. from soil. The other beneficial effects are their role in the biological control of root pathogens, hormone production, greater ability to withstand water stress, and synergistic interaction with nitrogen fixers, P solubilizers, and plant growth-promoting rhizo-microorganisms (PGPRs) (Bagyaraj 2011).

The role played by these fungi in improving plant growth is much more significant in tropical soils compared to temperate soils. This is mainly because most of the soils of the tropics are of low inherent fertility. They are deficient in phosphorus. In addition to being deficient in phosphorus, they also get fixed in the soil and are not readily available over the crop period necessitating fresh additions. In acidic soils, they are fixed as iron and aluminum phosphates, while in neutral soils they are fixed as calcium phosphates. Continuous application of P fertilizers will result in increased concentration of total phosphorus in the soil over times, resulting in large reserves of fixed P. According to Ozanne (1980), less than 10 % of soil P enters the plant–animal cycle. Experiments with P32-labeled phosphorus conclusively proved that AMF cannot solubilize unavailable inorganic phosphorus sources but draw extra phosphate only from the labile pool in soil solution (Raj et al. 1981). The improved P nutrition in plants has been explained mainly by the extension of AMF hyphae beyond the root system which allows for the exploration of spatially unavailable nutrients (Smith et al. 2000). In exchange, the AMF receive carbohydrates from its host plant (Smith and Read 1997).

8.3.1 Plant Growth Response to Inoculation

Earlier experiments conducted in sterilized soil showed that AMF inoculation could improve plant growth. Since most of the natural soils usually harbor AMF, it was felt that plants may not respond to mycorrhizal inoculation in unsterile soils. But later investigations indicated that even in unsterile soils, plants do respond to inoculation with efficient strains of AMF. Now it is proved beyond doubt that AMF improve plant growth. The growth increase is favored in soils with low to moderate fertility, especially phosphorus in limiting concentrations (Dodd and Jeffries 1986).

8.3.2 Selection of Efficient AMF for Inoculation

It is well known that AMF are not host specific. Though a particular AMF can infect and colonize many host plants, it has a preferred host, which exhibits maximum symbiotic response when colonized by that particular AMF (Abbott and Robson 1982). This led to the concept of "host preference" in AMF and in turn the procedure for screening and selecting an efficient fungus for a particular host. This in turn led to the selection of

inoculant AMF for many crops important in agriculture, horticulture, and forestry (Gianinazzi et al. 1990; Bagyaraj and Kehri 2012).

AMF are obligate symbionts. Attempts to culture AMF on artificial media have met with little or no success. At present time, the only method to produce these fungi is in association with the host plant root. Novel techniques to produce AMF inoculum in almost sterile environment through nutrient film technique, circulatory hydroponic culture system, root organ culture, and tissue culture are available. However, for large-scale field trials, the only convenient method to produce large quantities of inoculum is by the traditional pot culture technique (Bagyaraj and Kehri 2012).

8.3.3 Use of AMF for Plant Growth

AMF inoculum of suitably selected strains can be used for inoculation in the nursery bed (Palazzo et al. 1994). Growers only need to incorporate inoculum in the nursery beds or seedling trays at the appropriate rate by hand. Seedlings thus raised will be colonized by the introduced fungus and then can be planted out in the field. There are several reports of increased growth and yield of food, fodder, and fuel crops because of inoculation with efficient AMF (Bagyaraj 2014). These studies also brought out that because of inoculation, nearly 50 % of phosphate fertilizer application could also be reduced. Some horticultural plants are propagated through cuttings. In such cases, rooting of cuttings is important. Enhanced rooting of cuttings through inoculation with AMF has been reported. AMF-inoculated plants withstanding transplant stock have also been reported in avocado (Menge et al. 1980). Later studies showed high percentage of grafting success in cashew. Inoculation of micropropagated plantlets with AMF after hardening also improved plantlet vigor and growth in coffee, grapevine, apple, avocado, pineapple, kiwi fruit, strawberry, raspberry, asparagus, and banana (Yao et al. 2002; Bagyaraj 2014). The information available as to whether perennial plants already established in the field respond to AMF inoculation is very meager. In a study, it was found that ten-year-old mulberry plants and one-and-half-year-old papaya trees positively responded to mycorrhizal inoculation (Mamatha et al. 2002). Thus, use of AMF can be considered as an alternate strategy to more rational and sustainable agriculture.

8.4 Interaction between AMF and PGPR

8.4.1 Interaction of AMF with Symbiotic Nitrogen Fixers

There are several reports on the interaction between AMF and the legume–bacterium *Rhizobium* species. These studies suggest that the interaction is synergistic, improving nodulation and AMF colonization, with consequential benefit to plant growth. Legumes have repeatedly been shown to require high levels of phosphate for effective nodulation and growth (Gibson 1976). It is also known that nitrogen fixation has a high phosphate requirement. While the principal effect of mycorrhiza on nodulation is undoubtedly phosphate mediated, mycorrhiza may also have other secondary effects. Such potentially limiting factors include supply of photosynthates, trace elements, and plant hormones, which play an important role in nodulation and nitrogen fixation. Colonization by AMF has been found to increase the amount of phytoalexins in certain legume roots, which are isoflavanoid substances. Flavones are known to induce nod gene expression. These findings have paved new way for a line of research in understanding the role of AMF in the expression of nodulation gene in rhizobia (Suresh and Bagyaraj 2002).

8.4.2 Interaction of AMF with Asymbiotic Nitrogen Fixers

Nearly 25 genera of free-living bacteria can fix atmospheric nitrogen. Species of *Azotobacter*, *Azospirillum*, *Beijerinckia*, *Clostridium*, and

Derxia are well known among these. Bagyaraj and Menge (Bagyaraj and Menge 1978) studied the interaction between *Azotobacter chroococcum* and the AMF *Glomus fasciculatum* in tomato and found a synergistic effect on plant growth. Mycorrhizal infection increased the *A. chroococcum* population in the rhizosphere, which was maintained at a high level for a longer period, and *A. chroococcum* enhanced colonization and spore production by the mycorrhizal fungus. Similar interactions have also been observed between *A. paspali* and AMF in *Paspalum* and between *A. chroococcum* and *G. fasciculatum* in tall fescue (Ho and Trappe 1979). Synergistic interaction between AMF and associative nitrogen-fixing bacteria *Azospirillum* spp. and *Acetobacter diazotrophicus* has been reported (Bagyaraj et al. 2015). The studies conducted so far reveal a definite positive interaction between free-living nitrogen-fixing bacteria and AMF in the rhizosphere with consequential improvement in plant growth.

8.4.3 Interaction of AMF with Phosphate Solubilizers

Many soil bacteria termed as phosphobacteria solubilize unavailable forms of phosphorus, and they have been used as bacterial fertilizers on crop plants (Bagyaraj et al. 2015). Interaction between AMF and phosphate-solubilizing microorganisms and their effect on plant growth have been studied by several workers. Phosphate-solubilizing bacteria (*Agrobacterium* sp. and *Pseudomonas* sp.) inoculated onto the seeds and/or seedlings maintained higher populations for longer duration in the rhizosphere of mycorrhizal than nonmycorrhizal roots of lavender and maize plants. Dual inoculation also resulted in increased plant dry matter and phosphorus uptake in soils. The phosphate-solubilizing bacteria also produced plant growth hormones, which enhanced plant growth (Dar 2010). Studies conducted with neem and *Pennisetum* grass and many other hosts further confirmed a synergistic interaction between AMF and phosphate-solubilizing bacteria (Bagyaraj et al. 2015). Recent studies have shown that inoculation with microbial consortia consisting of an efficient AMF together with a nitrogen fixer, P solubilizer, and PGPR carefully screened and selected for a particular crop plant or forestry species is more beneficial than AMF alone in improving the growth, biomass, and yield (Hemalatha et al. 2010).

8.4.4 Interaction of AMF with PGPR other than N-Fixers and P-Solubilizers

In the recent past, much research is addressed for better understanding of the diversity, dynamics, and significance of rhizosphere microbial populations and their cooperative activities (Barea et al. 2005). The potential impacts of rhizobacteria on mycorrhizal fungi include changes in root fungus signaling, recognition, and receptivity, as well as effects on fungal growth and germination (Johansson et al. 2004). The synergistic effect of PGPR and AMF on plant growth promotion is well documented (Bagyaraj 2014). Combined application of *P. fluorescens* and *Glomus mosseae* resulted in improved growth of chickpea compared to the application of the two bioinoculants separately and also reduced the galling and multiplication of the nematode pathogen *Meloidogyne javanica* (Siddiqui and Mahmood 2001). Co-inoculation of *Pseudomonas* sp. F113 and *G. mosseae* stimulated spore germination by *G. mosseae* in soil and hence better colonization of tomato roots (Barea et al. 1998). Two rhizobacterial isolates *Enterobacter* sp. and *B. subtilis* promoted the establishment of *Glomus intraradices*, which in turn increased biomass, N, and P accumulation in plant tissues (Toro et al. 1997). *Paenibacillus* spp. have been found to be associated with ectomycorrhiza and AMF (Giese et al. 2002). *Paenibacillus validus* (DSM ID617 and ID618) stimulated the growth of the AMF *Glomus intraradices* and led to the formation of fertile spores, which recolonized carrot roots (Hildebrandt et al. 2006). Li et al. (2008) reported that different species of *Paenibacillus* differentially affect cucumber mycorrhizal fungi *Glomus intraradices* or *Glomus mosseae*.

8.5 Conclusion

Soil is a habitat of huge variety of organisms. Soil organisms make significant contributions to not only production function but also to regulatory functions of ecosystems. While species and genetic diversity of soil organisms is important from the point of inventory, their functional qualification is more useful in evaluating and managing their functions. Some organisms such as PGPR and AMF directly benefit plant growth. It is very well established that inoculation with PGPR and efficient AMF improves the growth and yield of crop plants and saves use of chemical fertilizer. In summary the PGPR and AMF discussed above have unique abilities that can be exploited for further research and commercial use. Recent studies have shown that co-inoculation of AMF with PGPR is more useful in improving plant growth, thus suggesting the need for development of suitable microbial consortia for inoculating different crop plants. The fundamental means of assessing the effectiveness of inoculating the nursery seedlings are their survival and growth in the field. Such studies with AMF alone or as microbial consortia are meager (Bagyaraj 2014). In a recent study, chilly seedlings inoculated in the nursery with microbial consortia consisting of a selected AMF (*Funneliformis mosseae*) and a PGPR (*Bacillus sonorensis*) were planted in a farmer's field. Inoculated plants with 50 % of NPK fertilizer performed equal to uninoculated plants receiving 100 % NPK fertilizer (Thilagar and Bagyaraj, unpublished). Therefore, the need of the hour is to develop microbial consortia for inoculating different crop plants and the promotion of these consortia in eco-friendly sustainable production of crop plants for the benefit of mankind.

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Biopesticides: An Eco-Friendly Approach for the Control of Soilborne Pathogens in Peanut

9

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Abstract

The peanut (*Arachis hypogaea* L.) is a widespread oilseed crop of great agricultural significance. Argentina is one of the major peanut producers in the world, and about 90 % of its production takes place in the province of Córdoba. During the last 20 years, peanut production has not only been increasing in yield but also in the quality of the harvested product because consumers tend to require high-quality products. Therefore, research and dissemination of technologies constitute essential elements for growing peanuts.

Peanut is susceptible to several diseases which are caused by the confluence of a susceptible cultivar, a pathogen (fungus, bacteria, or virus), and a favorable environment. Soilborne fungal diseases of peanut are spreading throughout Argentina, causing such losses that they are being considered as one of the most important factors in the decrease of peanut yield. Fungicides are the main tool for controlling such diseases, but their use has been shown to bring important ecological adverse consequences for human health and the natural balance of the soil microflora. An alternative disease management option is biological control. It consists mainly in using microorganisms to control harmful microorganisms that cause plant diseases without disturbing the ecological balance. Several scientists around the world have described different *Pseudomonas* and *Trichoderma* strains that are able to significantly control a number of fungal diseases. Here, we review the main researches conducted using these organisms as well as the mechanisms involved in their biocontrol activity. We hope that

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this work will contribute to future research programs that aim to promote strains of *Pseudomonas* and/or *Trichoderma* as potential biopesticides for biological control of many diseases of agricultural relevance.

Keywords
Biopesticides • *Arachis hypogaea* • *Trichoderma* • *Pseudomonas* • Soilborne pathogen

9.1 Introduction

The peanut (*Arachis hypogaea* L.) is an annual legume, which is also known as groundnut, earthnut, monkey-nut, and goobers. It is native to the Western Hemisphere, probably originated in South America and spreads throughout the New World as Spanish explorers discovered their versatility. Peanut is now grown throughout the tropical and warm temperate regions of the world. It is the 13th most important food crop and 4th most important oilseed crop in the world. World peanut production totals approximately 39.9 million metric tons per year, China being the world's largest producer, followed by India and the United States (USDA 2012). Argentina, the United States, Sudan, Senegal, and Brazil are the major exporters of peanuts, accounting for 71 % of the total world exports. Countries such as India, Vietnam, and several African countries periodically enter the world market depending on their crop quality and world market demand. Three areas, the European Union, Canada, and Japan, account for 78 % of the world's imports (Integrated Breeding Platform 2015). All parts of the peanut plant can be used. It contains about 48–50 % oil, 25–28 % proteins, and 20–26 % carbohydrates, making this oilseed a rich source of energy. Also, peanut occupies a unique position among oilseeds, because it can be consumed directly as food. In Europe as well as in North and South America, about 75 % of the peanut produced is used as a foodstuff (Birthal et al. 2010). Peanut kernels also contain many other important nutrients, 13 essential vitamins particularly vitamin E, folic acid, and niacin, seven essential minerals, and antioxidants (Bishi et al. 2015). Peanut is a source of biologically active polyphenolics, flavonoids, and isoflavones such as p-coumaric acid and resveratrol (Francisco and Resurrección 2008). The phenolic compounds have beneficial effects which have been attributed to their antioxidant capacity (Heim et al. 2002). Peanuts contain healthy monounsaturated and polyunsaturated fatty acids and do not contain any trans-fatty acids (Sanders 2001). For this reason, the consumption of peanuts has beneficial biological effects such as help in weight loss (Alper and Mattes 2002), inhibition of cancer (Awad et al. 2000), contribution to a reduction of serum lipid levels and blood pressure, and, consequently, prevents cardiovascular diseases (Lopes et al. 2011). In addition, Higgs (2003) reported that peanut could exert protection against the Alzheimer disease as well as anti-inflammatory effects. For all these reasons, peanut is not only an oilseed crop but nowadays is gaining importance as a functional food. The multiple uses of groundnut plants make this oilseed a very good alternative crop for domestic markets and foreign trade, both in developing and developed countries.

Peanut is essentially a tropical plant and requires a long and warm growing season. Favorable climatic conditions for growing peanuts include a well-distributed rainfall of at least 500 mm during the growing season, an abundant sunshine, and a relatively warm temperature. Temperatures in the range of 25–30 °C are optimal for plant development (Prasad et al. 2003). Once established, this oilseed is tolerant to drought and can tolerate flooding. Although this crop can be produced on as little as 300–400 mm of rainfall, the rainfall should be of 500–1000 mm for commercial production. The best soils for cultivating peanut are well-drained sandy loam

soils, because these light soils facilitate penetration of pegs and their development and harvesting. The best soil pH for a higher peanut productivity is between 6.0 and 6.5.

9.2 Peanut Diseases

This crop is susceptible to many diseases which are caused by the confluence of a susceptible cultivar, a pathogen (fungus, bacteria, or virus), and a favorable environment. Within the latter, we include not only the weather but also the production system developed by man (March and Marinelli 1998).

Diseases can be classified into two groups: phylloplane diseases (mainly those affecting foliage) and rhizoplane diseases (caused primarily by soil fungi). In the first group, we find pox, scabies, wet spot, and rust, while among the latter are blights, wilts, and root rot.

Several soilborne pathogens that affect peanut are important to Argentina, including *Botrytis cinerea*, *Rhizoctonia solani*, *Sclerotinia minor* and *S. sclerotiorum*, *Sclerotium rolfsii*, *Fusarium solani*, *Aspergillus niger*, *Cercospora arachidicola*, *Cercosporidium personatum*, *Sphaceloma arachidis*, and *Tecaphora frezii*.

9.2.1 *Botrytis cinerea*

It is an Ascomycota pathogen of many plants, animals, and bacteria. *Botrytis cinerea* is characterized by abundant conidia (asexual spores) oval at the end of gray-branched conidiophores. The fungus is usually referred to by its anamorph name, because the sexual phase is rarely observed, the teleomorph is *Botryotinia fuckeliana*, also known as *Botryotinia cinerea*. The fungus also produces highly resistant sclerotia as forms of resistance in old cultures. Overwinters as sclerotia or intact mycelia, both forms germinate in spring to produce conidiophores. Conidia of the fungus are 9–12 × 6.5–10 μm and ellipsoid to ovoid, pigmented, and single-celled. These conidia are produced abundantly in a botryoid habit on the ends of conidiophores, which cause the lesions to appear gray and moldy. The conidia are dispersed by wind and rain and cause new infections. There has been a considerable genetic variability in different strains of *Botrytis cinerea* (polyploidy).

This fungus prefers temperatures below 20 °C and conditions of high humidity for colonization and, under favorable conditions, may cause the wilt and death of plant tissues or even the entire plant (Porter 1997). Although all parts of the peanut plant are susceptible to this pathogen, the most affected parts are those in direct contact with the soil, especially when injured by frost damage or other pathogens.

9.2.2 *Rhizoctonia solani*

Rhizoctonia solani Kühn (anamorph) is a basidiomycete fungus that does not produce any asexual spores and only occasionally will the fungus produce sexual spores (basidiospores). In nature, *R. solani* reproduces asexually and exists primarily as vegetative mycelium and/or sclerotia. The sexual fruiting structures and basidiospores (i.e., teleomorph) were first observed in 1891. The sexual stage of *R. solani* has undergone several name changes since 1891 but is now known as *Thanatephorus cucumeris*.

The hyphae of *R. solani* are pigmented and septate and display 90° hyphal branching. The fungus also produces nondifferentiated sclerotia that survive on plant debris. *Rhizoctonia solani* is capable of surviving saprophytically on a wide host range, including rotated crops and various weed species (Brenneman 1997).

Rhizoctonia solani is a ubiquitous fungus with a wide host range; the differentiation from other pathogens causing seed decay is often difficult, making the management of *R. solani* diseases difficult. *Rhizoctonia solani* causes seed decay, damping-off, root rot, limb rot, and pod rot. The disease could be serious and reduce yields when conditions are unfavorable for seedling development. The losses caused by *R. solani* are also difficult to ascertain because pod rot may be caused by several soilborne pathogens and shows no aboveground symptoms. Germinating sclerotia

or hyphae in the soil or on plant debris constitute a potential inoculum that may infect the host tissue. Hyphae penetrate the new tissue through appressoria or through wounds and natural openings of the plant. *R. solani* may cause seed decay prior to emergence and may infect plants at any stage of development. On emerged seedlings, dark, sunken lesions just below the soil line become present, and under favorable disease conditions, the fungus will cause plant death. *Rhizoctonia* limb rot is described as dark-brown target-patterned lesions on stems and lower branches (Bell and Sumner 1984a). *Rhizoctonia* pod rot is differentiated by a dry, brown- or russet-colored rotted pod and contrasted with the dark, greasy-appearing lesions characteristic of *Pythium* spp. Peanut shells decay slowly and have been observed in soil 1–3 years after peanuts were harvested and other crops were grown. Colonized shells may serve as primary reservoirs for pathogens (Bell and Sumner 1984b; Sumner and Bell 1994).

9.2.3 *Sclerotinia minor* and *S. sclerotiorum*

Sclerotinia minor and *S. sclerotiorum* are ascomycetes that produce white aerial mycelia and black, irregularly shaped sclerotia. The sclerotia from *S. sclerotiorum* are large and less abundant, similar to the sclerotia produced by *B. cinerea*, while the *S. minor* sclerotia are small and abundant. One or several apothecia, pale orange to white, may originate from sclerotia. The apothecium contains ascospores produced in the asci with a range of 8–17 × 5–7 µm. *Sclerotinia minor* overwinters as sclerotia, which, under favorable environmental conditions, germinate producing mycelia. *Sclerotinia* blight is caused by *Sclerotinia minor* Jagger and, on rare occasions, may be caused by *Sclerotinia sclerotiorum* (Lib.) de Bary (Porter and Melouk 1997). Plant tissue in contact with soil infested with *S. minor* becomes infected. Infected plants rapidly wilt and show chlorotic, water-soaked lesions; as the disease progresses, the surface of the affected tissue is invaded by white fluffy mycelia.

With the passage of days, these spots reach greater size and light brown color, clearly being marked separation between diseased and healthy tissues. Under conditions of high humidity on the diseased tissues, cottony white mycelium is formed. After 10–12 days, the tissue death occurs.

The fungus eventually causes branches; these branches, due to oxalic acid produced by *S. minor*, begin to have a shredded appearance. When the disease progresses, the infected tissues are degraded and sclerotia are produced. These resistant structures are shed into the soil, where they overwinter until optimum conditions exist to germinate (Marinelli et al. 1998).

9.2.4 *Sclerotium rolfsii*

Sclerotium rolfsii is a basidiomycete and does not produce conidia. It has the teleomorph *Athelia rolfsii* (Curzi) Tu and Kimbrough, which forms basidiocarps and has hyphal strands emerging from germinating sclerotia (Tu and Kimbrough 1978). It has a host range of more than 200 plant species and may colonize living or dead plant tissue. The fungus is characterized by white mycelia, and round, brown sclerotia, which range from 0.5 to 2 mm in diameter. The mycelia of *S. rolfsii* survive best in sandy soils, whereas the sclerotia survive best in moist, aerobic conditions found at the soil surface (Punja 1985).

Wilt white fungus, also known as the white mold, southern stem rot, and *Sclerotium* rot, is caused by the fungus *Sclerotium rolfsii* Sacc. The fungus is ubiquitous and has a wide host range. This disease is found in all major peanut-growing areas of the world. In extreme cases, the disease may cause up to 80 % yield loss; however, losses less than 25 % are more typical (Backman and Brenneman 1997).

Initial symptoms of this disease include a yellowing and wilting of the main stem, the lateral branches, or the entire plant. White mycelium may be observed at the base of the plant near the soil line, branches in contact with the soil, and even on the same soil and detached leaflets.

Under favorable conditions, warm temperatures and high humidity, during the growing season, the mycelia rapidly spread to other branches and peanut plants.

Sclerotia are spherical and are initially white but later become light brown to dark brown in color and serve as the initial inoculum. Temperature fluctuations, fungal isolate, and nutrient availability may affect sclerotial formation size and shape (Punja 1985).

If the pathogen infects the pods, the pods exhibit a brown rot with a water-soaked and mashed appearance. Often, when infected pods are removed from the ground, the mycelium-covered pods show soil adhering to the fungal hyphae.

9.2.5 *Fusarium solani*

Fusarium solani (Mart.) Sacc. is a name that has been applied broadly to what is now known as the *F. solani* species complex (FSSC, O'Donnell 2000). Members of the FSSC, which includes several additional named species and currently corresponds to approximately 50 phylogenetic species (Zhang et al. 2006; O'Donnell et al. 2008; Nalim et al. 2012), are ubiquitous in soil, in plant debris, and in other plant and animal substrata and can be serious plant and human pathogens (Booth 1971). The FSSC contains both heterothallic and homothallic strains and species, as well as strains that have no known sexual stage. Peanut brown root rot (PBRR) was first observed in the Córdoba province in 1992 (March and Marinelli 1998) and is now widespread in Argentina peanut-growing regions. The pathogen kills adult plants resulting in large economic losses. In seasons with long drought stress periods, this disease is the most important of peanut and may reach a 95 % disease incidence in some fields (March and Marinelli 2005). As for other diseases caused by soilborne pathogens, PBRR may be influenced by tillage practices and crop rotation (Bockus and Shroyer 1998). In a 2-year rotation with soybean and maize in which a paratill subsoiler was used before peanut seeding in a no-till system, root growth was improved, water deficits were reduced, and increases in native populations of biocontrol agents were observed as well as a reduction in PBRR (Oddino et al. 2008). The species of *F. solani* associated with the brown root rot in peanut share many morphological and physiological characteristics with other species of the complex, mainly the formation of multiple types of conidia. Casasnovas et al. (2013) showed that amplified fragment length polymorphism (AFLP) banding patterns obtained for strains isolated from diseased roots were clearly distinguishable from other members of the FSSC. However, the similarities observed between the isolates from peanuts and other strains in the FSSC were in an indeterminate range (40–60 %). Thus, it can be concluded that although these strains were closely related, their species status remained not resolved.

9.2.6 *Aspergillus niger*

Aspergillus niger is one of the most common species of the genus *Aspergillus*. It causes a disease called black mold on certain fruits and vegetables such as grapes, apricots, onions, and peanuts and is a common contaminant of food. It is ubiquitous in soil and is commonly reported from indoor environments, where its black colonies can be confused with those of *Stachybotrys* (Samson et al. 2001).

Crown rot disease of peanut caused by *Aspergillus niger* van Tieghem is an important disease in several temperate countries (Carina et al. 2006). Annual world yield loss caused by the disease is more than 10 % and is more prevalent in soils with low moisture content and high temperature, approximately 30 °C. The fungus attack very devastatingly on stem tissues near ground surface and causing rot, witling, and plant death (Pande and Rao 2000; Kishore et al. 2007).

A. niger is present in most peanut soils and is a common contaminant of peanut seed. However, outbreaks of the disease are sporadic and appear to be related to the prior occurrence of one or more stresses. Extreme heat or fluctuations in

soil moisture during the seedling stage, poor seed quality, seedling damage from pesticides or cultivation, and feeding by the root and stem boring insects are stresses thought to aggravate the disease.

While the fungus may cause seed rot and pre-emergence damping-off of seedlings, the most obvious symptom is the sudden wilting of young plants. The crown area of infected plants just below the soil line may be swollen and eventually becomes covered with a black, sooty mass of fungus growth. Most affected plants die within 30 days of planting. Later in the season, individual branches or entire plants may develop similar symptoms. Splitting the crown and tap root of affected plants reveals an internal discoloration of the vascular system that is dark gray in color.

9.2.7 *Cercospora arachidicola* y *Cercosporidium personatum*

Cercospora arachidicola y *Cercosporidium personatum* are ascomycetes, class *Dothideomycetes. Cercospora arachidicola* is the anamorph, and *Mycosphaerella arachidis* is the teleomorph. They have septate mycelia. During plant tissue infection, initially, it is intercellular then it becomes intracellular. The mycelium penetrates directly into plant cells without the formation of haustoria. Conidiophores are supported by dark-brown stromata. Conidiophores are yellowish brown in color, fasciculated and geniculated with several septations. Secondary conidiospores and conidia are seen on slide made from host tissue kept under extremely favorable environmental conditions. Perithecia are scattered mostly along margins of lesions produced by spores of imperfect state. These are amphigenous, somewhat embedded in the leaf tissue, erumpent, ovate to nearly globose, and black in color. Ascospores (uniscriate to biscriate in the ascus) are bicellular with upper cell slightly curved and hyaline (Kolte 1985).

Cercosporidium personatum in its imperfect state is a *Hyphomycetes*, and the perfect state is a *Loculoascomycetes*, order *Dothideales*. Mycelium of *C. personatum* is septate and exclusively intercellular. The infection is caused by haustoria into the palisade and mesophyll tissue. In later stages of disease development, conidiophores arise in clearly concentric tufts from heavy stromatic base. The conidiophores are fasciculated and geniculated, reddish brown in color with mostly hyaline tips and non- or severally septate. Conidia are obclavated with attenuated tips and pale brown dilutely olivaceous color with one to nine septa and bluntly rounded top cells. Secondary conidia and conidiophores are not reported in *C. personatum*. Perithecia, asci, and ascospores of teleomorphic stage of *C. personatum* only differ from *C. arachidicola* in size (Kolte 1985).

Early leaf spot, caused by C*ercospora arachidicola* Hori and *Cercosporidium personatum* (Berk. & Curt.) Deighton, is the major foliar disease of peanut in Argentina (March and Marinelli 1998). Significant losses occur in the absence of control measures (Ghuge et al. 1981; Mercer 1976). Cultural practices offer only a partial control, fungicide spray programs are generally expensive, and all cultivated varieties of peanuts are susceptible to the pathogens (Porter et al. 1982). Therefore, the development of peanut cultivars resistant to leaf spot is essential. All aerial parts of the plant are affected, but in the leaflets, symptoms are seen more easily. In the leaflets, brown spots are observed, light to dark and circular or irregular with a diameter between 2 and 7 mm. These spots are usually surrounded by a yellow halo. On the underside of the leaflets, stains due to early smallpox are usually light brown and have smooth texture, while the late smallpox are dark brown to almost black and have rough texture. The final symptom is defoliation.

In the cycle of the disease, both species spend the unfavorable period (winter) as mycelium in tissues and stubble. In the spring, conidia are produced and dispersed by the action of rain and wind causing the first infections. Subsequently, under favorable conditions of humidity and temperature on the spots appear, fruiting and new conidia which will now disperse and initiate new infections are generated. The cycle may be repeated several times during cultivation if environmental conditions permit. The favorable envi-

ronmental conditions for epidemics include periods of 5 or more hours with relative humidity above 95 % and above minimum temperature at 16 ° C during these hours. The rate of development of disease increases as temperature increases (March and Marinelli 1998).

9.2.8 *Sphaceloma arachidis*

Within fungal diseases frequently observed, peanut scab caused by *Sphaceloma arachidis* Bit. & Jenk. has become increasingly important (March and Marinelli 1999). This fungus is a member of the *Ascomycota* phylum, class *Dothideomycetes*. It causes the disease known as peanut scab, which was first described in Brazil (Bitancourt and Jenkins 1940); later, it was observed on germplasm collections in the Argentine-producing region (Ojeda 1966). Since then, peanut scab has been observed with variable intensity in isolated commercial fields (Giorda et al. 1985) becoming epidemic and causing severe yield losses on peanut crops in Argentina.

The spread of scab in this peanut-producing region raises questions concerning inoculum sources and dissemination. *Sphaceloma arachidis* forms acervuli with two types of conidia, microconidia (1 urn) and macroconidia (3–4×9–20 urn) in the affected plant parts (Bitancourt and Jenkins 1940). Since the disease affects leaves, petioles, stems, pegs, and shells, crop residues and seeds may be a source of inoculum for the onset of scab epidemics in the field, and infected peanut debris was an efficient inoculums. Furthermore, there is a general agreement that scab is more serious in monoculture of peanut. The importance of infested peanut debris as inoculum sources may depend on several variables, including intensity in the preceding peanut crop, rate of residue decay, competitive saprophytic ability, sporulation potential of the pathogen, and weather variables (Buchwaldt et al. 1996; Bockus and Shroyer 1998). Debris from peanut plants infected with *S. arachidis* from the previous agricultural year was an inoculum source in initiating disease epidemics. The severity index was higher when infected debris was applied over the seeded rows compared to mixing it with seeds at planting. This may be attributed to differences in inoculum quantity and/or to a microbial decomposition of peanut residues when it was grounded and buried (Kearney et al. 2002).

9.2.9 *Thecaphora frezii*

The fungus was described by Carranza and Lindquist in 1962 infecting the wild peanut (*Arachis* sp.) from Brazil, but smut was first detected in peanut plants cultivated during the agricultural cycle 1994/1995, and since then, it has been observed in some commercial peanut fields. *T. frezzi* is the causal agent of peanut smut, causing severe yield losses in peanut-growing areas. *T. frezii* causes local infections, producing hypertrophy in pods and grains. The infection occurred in the peg when it penetrates the soil. The colonized organs (fruits and seeds) are replaced with a smutted mass formed by the teliospores, i.e., the dispersal and survival structures of the pathogen and the source of disease inoculum (March and Marinelli 2005). Since the earliest detection in samples from commercial fields in the central northern region of Córdoba, the disease has spread throughout the peanut-growing area of Córdoba province, with records being reported also for Salta province (Conforto et al. 2013).

The species mentioned above that cause disease in phyloplane and rhizoplane can also affect the fruits. Other diseases in fruits are attributed to *Fusarium equiseti* (Corda.) Sacc., *Leptosphaerulina crassiasca* (Séchet.) Jackson & Bell, *Verticillium* sp., *Thielaviopsis basicola* (Berk. & Broome), *Phomopsis* sp., and *Phoma* sp. The management of diseases in fruits is very complex. In general, grasses are recommended rotations and tillage modes used to improve the physical properties of soil. The use of controlled seeds is also advised and applies broad-spectrum fungicides (March and Marinelli 1998).

9.3 Role of Potential Biocontrol Agents in the Management of Peanut Diseases

Soilborne fungal peanut diseases are becoming increasingly widespread in Argentina, causing such losses that they are being considered as one of the most important factors in the decrease of peanut yield. Fungicides are the main tool for controlling such diseases, but their use has been shown to bring important ecological adverse consequences for human health and the natural balance of the soil microflora (Andrés et al. 1998, 1999). The indiscriminate use of chemical compounds has led to several environmental problems such as the development of resistance to pesticides in pests, pesticide residues in the environment, and the destruction of beneficial parasites and predators of pests. The high cost associated with the use of fungicides to control fungal diseases is a limiting factor in the profitability of peanut production (Partridge et al. 2006).

The use of management strategies for the control of fungal diseases can reduce the use of chemicals, their high cost, and pollution by soil fungicides. Rotation is important for managing soilborne fungal pathogens in peanut (Melouk and Backman 1995). Long-term rotation with maize or cotton, neither of which is a host of *S. minor*, has been shown to help reducing the incidence of peanut blight (Phipps et al. 1997).

Other alternative disease management options were considered among which biological control appears promising. Biological methods consist mainly in using microorganisms to control harmful microorganisms that cause plant diseases without disturbing the ecological balance.

Biological control involves the use of suppressing microorganisms to improve the health of crops and involves interactions between the plant, the pathogen, the biocontrol organism, the rhizosphere microbial community, and the physical environment (Handelsman and Stab 1996).

Rhizosphere bacteria that exhibit root colonization and exert beneficial effects on plants are termed plant growth-promoting rhizobacteria (PGPR, Kloepper et al. 1989). It has been reported that PGPR can elicit plant defenses (Van Loon and Glick 2004) and antagonize or prevent phytopathogens or deleterious microorganisms (Kloepper et al. 2004). Therefore, the use of peanut seeds coated with rhizobial inoculants exerting an antagonistic ability will reduce the use of chemical substances in agriculture. Moreover, such rhizobial inoculants will be able to promote plant growth and control plant diseases. Incorporating rhizobia with selected PGPR traits increases nitrogen fixation and reduces fungicide application in peanut, providing an appropriate approach for sustainable agriculture (Yuttavanichakul et al. 2012).

In addition, there are several researches with strains of *Pseudomonas* and *Trichoderma* as potential biopesticides and/or biofertilizers. Some species of the genus *Trichoderma* have been used as effective biocontrol agents against soilborne, foliar, and postharvest fungal pathogens (Cortes et al. 1998) in several plant crops, including peanut (Podile and Kishore 2002). *Trichoderma viride* and *T. harzianum* reduced the collar rot incidence in groundnut caused by *A. niger* in a pot culture study (Gajera et al. 2011). There is evidence on the successful control of peanut blight through the inoculation of nonspecific fungal antagonists such as *Trichoderma* and *Gliocladium* species (Budge et al. 1995). Shanmugam et al. (2002) conducted a study to test the effect of *P. fluorescens* Pf1 in peanut to control root rot, a severe soilborne disease caused by *Macrophomina phaseolina*. Also, the leaf shoot disease caused by *Cercosporidium personatum* was reduced when the groundnut seeds were treated with *P. fluorescens* Pf1. *P. fluorescens* strain FDP-15, isolated from peanut roots, was proven to be an efficient and ecologically fit strain. FDP-15 improved seed germination, nodulation, dry weight, and pod yield as well as protected plants from sclerotial infection, as compared with captan (Patil et al. 1998).

At the present time, our research group is devoted to the study of microbial agents from the genera *Pseudomonas* and *Trichoderma* as potential antagonists of fungal pathogens of

high economic impact on peanut production in Argentina, such as *Fusarium solani* and *Thecaphora frezii*, causal agents of root rot and smut, respectively. *Trichoderma harzianum* ITEM 3636 controlled PBRR in fields naturally or artificially infested with *F. solani* PBRR, decreasing disease severity, increasing the frequency of healthy plants, and boosting plant yield (Rojo et al. 2007).

The following sections provide a summary of researches performed by several authors on physiological and biochemical characteristics of *Pseudomonas* and *Trichoderma* strains and their behavior as beneficial microorganisms under different ecological and environmental conditions. Their traits encourage the interest in further studies aimed at achieving biotechnological formulations applicable to agronomically important crops, particularly peanuts.

9.3.1 Application of *Pseudomonas* for an Improved Biocontrol Activity against Phytopathogens

Members of the genus *Pseudomonas* are rod-shaped gram-negative bacteria characterized by a metabolic versatility, aerobic respiration (some strains also have anaerobic respiration with nitrate as the terminal electron acceptor and/or arginine fermentation), and motility as a consequence of one or several polar flagella. Fluorescent pseudomonads belong to a major group of rhizosphere bacteria known as plant growth-promoting rhizobacteria (PGPR). These bacteria are involved in the stimulation of plant growth and in the control of diseases. Several *Pseudomonas* strains represent a promising alternative for disease management since they can inhibit plant fungal pathogens. Several researchers have shown that fluorescent *Pseudomonas* is abundant in the rhizosphere of different crops. Effectively, they produce a variety of biologically active substances of interest. Several biological control mechanisms have been studied, including an effective root colonization, production of antifungal metabolites, interference with pathogenicity factors, and elicitation of induced systemic resistance in plants. Siderophore-producing *Pseudomonas* sp. plays an important role in stimulating plant growth and in controlling several plant diseases (O'Sullivan and O'Gara 1992). The antagonistic effect of PGPR is influenced by a number of environmental and genetic factors. Biotic and abiotic factors play an important role in the regulation of genes involved in biocontrol, e.g., repression of siderophore biosynthesis. Siderophore production by PGPR is influenced by amino acids, sugars, minerals, and other components present in root exudates (Deelip et al. 1998). Those factors affecting PGPR growth and/or production of siderophores affect the effectiveness of the growth-promoting effect or disease control.

Fluorescent pseudomonads owe their fluorescence to an extracellular diffusible pigment called pyoverdine (Pvd) or pseudobactin (pigment with high affinity for Fe^{3+}), which is a siderophore that participates in the transport of Fe^{3+} into the cytoplasm. Under conditions where Fe^{3+} is poorly soluble (in aerated, neutral, or alkaline soils), Pvd-producing *Pseudomonas* spp. inhibit the growth of bacteria and fungi with less potent siderophores (Kloepper et al. 1980). Pyoverdines differ from other siderophores in their exceptionally strong affinity for iron (III) ions and the high stability of the complexes formed. The synthesis of siderophores by these bacteria is one of the main factors inhibiting the growth and development of bacterial and fungal pathogens (Sharma and Johri 2003; Bano and Musarrat 2004).

Another pseudomonad siderophore, pyochelin, has been identified as an antifungal antibiotic. Pyochelin is the condensation product of salicylate and two molecules of cysteine, and it requires two gene clusters, *pchDCBA* and *pchEFGHI* (Youard et al. 2011). Since pyochelin is a relatively weak Fe^{3+} chelator but a strong Cu^{2+} and Zn^{2+} chelator (Cuppels et al. 1987; Visca et al. 1992), it has the potential to deprive some fungi of copper and/or zinc. This example shows that the distinction between siderophores and typical antibiotics is blurred.

Pseudomonas aeruginosa produces three types of siderophores under iron-limiting conditions, pyoverdine, pyochelin, and its precursor salicylic acid, and induces resistance to plant diseases caused by *Botrytis cinerea* on bean and tomato and by *Colletotrichum lindemuthianum* on bean (Höfte and Bakker 2007).

Several strains of *Pseudomonas* produce antifungal metabolites, among which phenazines, pyrrolnitrin (PRN), 2,4-diacetylphloroglucinol (DAPG), and pyoluteorin are the most frequently detected classes (Thomashow et al. 1990; Iavicoli et al. 2003). However, new substances belonging to the class of cyclic lipopeptides such as viscosinamide and tensin have been described (Nielsen et al. 1999, 2000). The ability to produce multiple classes of antibiotics that differentially inhibit different pathogens is likely to enhance biological control. *Pseudomonas putida* WCS358r, genetically engineered to produce phenazine and DAPG, showed improved capacities to suppress plant diseases in wheat (Glandorf et al. 2001). *P. chlororaphis* subsp. *aurantiaca* SR1 inhibits a wide range of phytopathogenic fungal species including *Macrophomina phaseolina*, *Rhizoctonia* spp. T11, *Fusarium* spp., *Alternaria* spp., *Pythium* spp., *Sclerotinia minor*, and *Sclerotium rolfsii*. SR1 shows the ability to produce indole-3-acetic acid (IAA), phenazine-1-carboxylic acid (PCA), PRN, and hydrogen cyanide (HCN). This strain is also able to colonize the root system of several crops, maintaining appropriate population densities in the rhizosphere area (Rosas et al. 2005, 2011).

Micolytic enzymes produced by antagonistic microorganisms are other types of substances that may be involved in the biocontrol of pathogens (Gohel et al. 2004). *Pseudomonas* may also produce different types of cell wall-degrading enzymes like chitinases, proteases/elastases, and β-1,3 glucanases. These enzymes are supposed to degrade the cell wall of several bacterial and fungal plant pathogens. It has also been proved that an extracellular chitinase and an extracellular laminarinase synthesized by *Pseudomonas stutzeri* digest and lyse the mycelium of *F. solani* (Lim et al. 1991).

Pseudomonas rhizobacteria have the ability to induce a state of systemic resistance (ISR) in plants, which provides protection against a broad spectrum of phytopathogenic organisms. ISR mediated through bacterial antagonists has been well documented in bean, carnation, cucumber, radish, tomato, and *Arabidopsis thaliana*. The bacterial antagonists confer resistance to a broad spectrum of pathogens (van loon et al. 1998). For instance, van Peer et al. (1991) reported that *Pseudomonas* WCS417 induced resistance in carnation against *Fusarium* wilt caused by *Fusarium oxysporum* f.sp. *dianthi* when the roots were inoculated with the bacterium 1 week prior to inoculation of the stem with the pathogen. Bacterial determinants that are claimed to cause ISR include siderophores, the *O*-antigen of lipopolysaccharides, and salicylic acid. *P. chlororaphis* strain PA23 and *B. amyloliquefaciens* strain BS6 significantly reduced the stem rot caused by *S. sclerotiorum*. In addition, ascospore germination was inhibited, and plant defense enzymes were triggered by *P. chlororaphis* PA23.

Voisard et al. (1989) observed that the suppression of black rot of tobacco was due to the production of HCN by *P. fluorescens*. HCN also induced resistance in the host plant. Manjula et al. (2004) selected four isolates of *P. fluorescens* and observed the production of HCN, which might have contributed to their biocontrol ability in addition to antibiotics. These authors suggested that the four *P. fluorescens* isolates differed in their biocontrol ability possibly due to differences in root colonization and production of antifungal metabolites in natural environments.

The isolates of *P. aeruginosa* GSE 18 and GSE 19 have broad-spectrum antifungal activity against several fungal pathogens of groundnut: *Aspergillus flavus*, *A. niger*, *Cercospora arachidicola*, *Puccinia arachidis*, *Rhizoctonia bataticola*, *R. solani*, and *Sclerotium rolfsii*. Strain GSE 18, tolerant to thiram, showed an improved control of collar rot in groundnut in combination with the fungicide (Kishore et al. 2005).

A number of studies have recently shown that inoculation with some plant growth-promoting bacteria increased growth and yield of several

plants including legumes (Shaharoona et al. 2006; Pirlak and Kose 2009). Seed bacterization with plant growth-promoting *P. fluorescens* isolates PGPR1, PGPR2, and PGPR4 suppressed soilborne fungal diseases such as collar rot of peanut caused by *A. niger* and the stem rot caused by *S. rolfsii* (Dey et al. 2004).

The occurrence of fluorescent *Pseudomonas* characterized by production of antimicrobial compounds was evidenced in the environments of some important agricultural plants, and their abundance and prevalence in suppressive soils have been demonstrated. The host plant species has a significant influence on the dynamics, composition, and activity of specific indigenous antagonistic *Pseudomonas* spp. (Bergsma-Vlami et al. 2005). Wang et al. (2013) researched on the effect of the application of the herbicide chlorimuron-ethyl on soil microorganisms, particularly *Pseudomonas* spp., in soybean fields from China. These authors observed a negative effect on the abundance and diversity of soil *Pseudomonas* spp., including species with different antifungal activities against pathogens. Siderophores and HCN rather than lytic enzymes constituted the antifungal metabolites of *Pseudomonas* spp., and the number of antifungal *Pseudomonas* that produced siderophores and HCN decreased markedly after application of chlorimuron-ethyl, especially after a 10-year application. The investigation on the functional diversity of bacterial communities from the rhizosphere has a great practical importance since application of promising bacterial strains as potent biofertilizers and/or biocontrol agents leads to an improved productivity of crops.

At the present time, application of biofungicides has its limitations. It is not simple to implement feasible biocontrol products against soilborne diseases. Nevertheless, there are enough reasons to encourage and promote their use. There are many possibilities for combining several biocontrol agents with each other or with agronomical, physical, or chemical control methods. The bioproducts must represent an applicable strategy, combined with other management practices, for the protection and health of plants.

9.3.2 *Trichoderma* as Biocontrol Agents

Trichoderma is a genus of asexually reproducing filamentous fungi and widely distributed in the soil, plant material, decaying vegetation, and wood. Nearly all temperate and tropical soils contain 10^1–10^3 culturable propagules per gram (Harman et al. 2004a). Their dominance in soil may be attributed to their rapid growth, diverse metabolic capability, and aggressive competitive nature (Elad 2000). These fungi are opportunistic, avirulent plant symbionts and function as parasites and antagonists of many phytopathogenic fungi, thus protecting plants from disease. So far, *Trichoderma* spp. have been widely studied, and are presently marketed as biopesticides, biofertilizers, and soil amendments, due to their ability to protect plants, enhance vegetative growth, and contain pathogen populations under numerous agricultural conditions (Harman 2000; Vinale et al. 2008).

The benefits of using *Trichoderma* in agriculture are multiple, and depending upon the strain, the advantages for the associated plant can include: (1) colonization of the rhizosphere by rhizosphere competence, allowing rapid establishment within the rhizosphere of a stable microbial community; (2) control of phytopathogenic and competitive microflora or fauna by using a variety of mechanisms; (3) overall improvement of the plant health; (4) plant growth promotion, by stimulation of above and below ground parts; (5) enhanced nutrient availability and uptake; and (6) induced systemic resistance (ISR) similar to that stimulated by beneficial rhizobacteria (Howell 2003; Harman et al. 2004a; Woo and Lorito 2007).

Fungi from the genus *Trichoderma* are soilborne saprophytes with a high growth rate. Most of these fungi produce lytic enzymes such as cellulases, chitinases, and glucanases, antibiotics, and several diffusible or volatile metabolites (Harman et al. 2004a). The main biocontrol mechanisms used by *Trichoderma* species in the direct antagonism of fungal pathogens are

mycoparasitism (Papavizas 1985; Howell 2003) and antibiosis (Sivasithamparam and Ghisalberti 1998).

Mycoparasitism is a complex process that includes several stages from recognition of the host to a tropic growth toward it. The mycoparasite penetrates the mycelium partially degrading the fungal pathogen's cell wall (Harman et al. 2004a). During this process, *Trichoderma* biocontrol agents secrete hydrolytic enzymes whose degrading activity releases different oligomers from the cell wall of host fungal pathogens (Howell 2003; Woo et al. 2006). It is widely presumed that *Trichoderma* biocontrol agents constitutively secrete degrading enzymes and detect other fungi by sensing the molecules that were released from the host after degradation by the hydrolytic enzymes (Woo and Lorito 2007). The molecular biology of mycoparasitic interactions between *Trichoderma* species and pathogens has been widely studied (Woo et al. 2004).

The chitinolytic and glucanolytic enzymes are especially significant because of their cell wall-degrading activity on phytopathogenic fungi, cleaving complex compounds not found in plant tissues (Woo et al. 1999). Each of these sets of enzymes contains diverse ensembles of proteins with distinctive enzymatic activity, and some were purified and characterized. Once purified, several *Trichoderma* enzymes have been proven to have a strong antifungal activity against a wide range of phytopathogenic fungi (i.e., species of *Rhizoctonia*, *Fusarium*, *Alternaria*, *Ustilago*, *Venturia*, and *Colletotrichum*, as well as Oomycetes, *Pythium*, and *Phytophthora*), and they can hydrolyze not only the early hyphal tips of the target fungal host, but they can also degrade endurable structures such as sclerotia (Lorito et al. 1993, 1994).

Trichoderma spp. are prolific producers of secondary metabolites, and the genomes of the mycoparasitic *Trichoderma* spp. are especially enriched in genes for secondary metabolism (Reino et al. 2008; Kubicek et al. 2011). Most *Trichoderma* strains produce volatile and non-volatile toxic metabolites that impede colonization by antagonized microorganisms. These metabolites are harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-pentyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, and heptelidic acid (Vey et al. 2001; Mukherjee et al. 2012). These metabolites may act by directly inhibiting the growth of pathogens or by indirectly triggering the defense system in the host plant, thus increasing disease resistance, and by promoting plant growth (Vinale et al. 2012). For instance, strains of *T. virens* with the best efficiency as biocontrol agents are able to produce gliovirin (Howell 1998). Also, the most effective isolates of *T. harzianum* against *Gaeumannomyces graminis* var. *tritici* produce pyrone antibiotics, and the success of the strains was clearly related to the pyrones they produced.

The combination of hydrolytic enzymes and antibiotics results in a higher level of antagonism than that obtained by either mechanism alone (Monte 2001). When combinations of antibiotics and several kinds of hydrolytic enzymes were applied to propagules of *Botrytis cinerea* and *Fusarium oxysporum*, synergism occurred, but it was lower when the enzymes were added after the antibiotics, indicating that cell wall degradation was needed to establish the interaction. In tobacco plants, exogenous applications of peptaibol triggered a defense response and reduced the susceptibility to the tobacco mosaic virus. A peptaibol synthetase from *T. virens* has been purified, and the corresponding gene, which has been cloned, will facilitate studies of this compound and its contribution to biocontrol (Wiest et al. 2002).

Competition for carbon, nitrogen, and other growth factors, together with competition for space or specific infection sites, may be also used to control plant pathogens. Under iron starvation conditions, most fungi excrete low molecular weight ferric-iron specific chelators, termed siderophores, to mobilize environmental iron (Eisendle et al. 2004). All three *Trichoderma* spp. whose genomes have been sequenced have a single gene for ferricrocin synthesis, belonging to a secondary metabolism gene cluster (Kubicek et al. 2011). Preliminary investigations revealed that this gene is indeed involved in the synthesis of ferricrocin and protection against oxidative

stress in *T. virens* (Mukherjee et al. 2012). Some *Trichoderma* isolates produce highly efficient siderophores that chelate iron and stop the growth of other fungi (Benítez et al. 2004). For this reason, soil composition influences the biocontrol effectiveness of *Pythium* by *Trichoderma* according to iron availability. The competition for iron was indeed shown to be important for control of *Fusarium* wilt of tomato by *T. asperellum* (Segarra et al. 2010).

In controlling pathogens such as *B. cinerea*, competition plays an important role (Latorre et al. 2001). This activity involves several mechanisms at the same time, thus making it practically impossible for resistant strains to appear. Among these mechanisms, the most important is nutrient competition, since *B. cinerea* is particularly sensitive to the lack of nutrients. *Trichoderma* has a superior capacity to mobilize and take up soil nutrients compared to other organisms. The efficient use of available nutrients is based on the ability of *Trichoderma* to obtain ATP from the metabolism of different sugars, such as those derived from polymers widespread in fungal environments: cellulose, glucan and chitin, and others, all of them rendering glucose (Chet et al. 1997).

The presence in soil of compounds released by other microorganisms, plants, and from agricultural activities influences the activity and survival of fungal species. *Trichoderma* strains grow rapidly when inoculated in the soil, because they are naturally resistant to many toxic compounds (herbicides, fungicides, insecticides, and phenolic compounds) and because the strains recover very rapidly after the addition of sublethal doses of some of these compounds (Chet et al. 1997). Resistance to toxic compounds may be associated with the presence in *Trichoderma* strains of ABC transport systems (Harman et al. 2004a). ABC transporters are probably necessary for the establishment of mycoparasitic interactions with plant pathogenic fungi. Knockout mutants of *T. atroviride* P1, lacking specific ABC transporters, were inhibited by the presence of various plant fungal pathogens (*B. cinerea*, *Rhizoctonia solani*, and *Pythium ultimum*) in the culture medium, and they exhibited reduced capacity as effective fungal parasites (Ruocco et al. 2009).

In addition to the beneficial effects that occur in direct interactions with plant disease agents, some *Trichoderma* species are also able to colonize root surfaces and cause substantial changes in plant metabolism. It is well documented that some strains promote plant growth, increase nutrient availability, improve crop production, and enhance disease resistance (Harman et al. 2004b).

Trichoderma spp. can colonize plant roots, both externally and internally. Colonization involves an ability to recognize and adhere to roots, penetrate the plant, and withstand toxic metabolites produced by the plant in response to invasion (Hermosa et al. 2012). Root colonization by *Trichoderma* spp. also frequently enhances the root growth and development, crop productivity, resistance to abiotic stresses, and uptake and use of nutrients. Several studies have shown that root colonization by *Trichoderma* strains results in increased levels of defense-related plant enzymes, such as the enzymes phenylalanine ammonia-lyase and chalcone synthase, involved in the biosynthesis of phytoalexins, chitinases, and glucanases. These comprise pathogenesis-related proteins (PR proteins) and enzymes involved in the response to oxidative stress (Yedidia et al. 1999, 2003; Howell et al. 2000; Harman et al. 2004b).

Trichoderma spp., and other beneficial root-colonizing microorganisms, also enhance plant growth and productivity. In general, applications of *T. harzianum* to seed or the plant resulted in improved germination, increased plant size, augmented leaf area and weight, and greater yields (Inbar et al. 1994; Altomare et al. 1999; Harman 2000; Vinale et al. 2008). Therefore, *Trichoderma* spp. recently were suggested as a plant growth-promoting fungi (PGPF) due to their ability to produce siderophores, phosphate-solubilizing enzymes, and phytohormones (Doni et al. 2013). These species were reported to be able to increase growth in plants such as strawberries, tomatoes, soya beans, apples, cotton, and gray mangroves (Porras et al. 2007; Morsy et al. 2009;

Shanmugaiah et al. 2009; John et al. 2010; Saravanakumar et al. 2013).

Vinale et al. (2004) demonstrated the ability of *T. harzianum* T22 and *T. atroviride* P1 to improve the growth of lettuce, tomato, and pepper plants under field conditions. Crop productivity was increased up to 300 %, as determined by comparing the treated plots with the untreated controls and measuring fresh/dry root and aboveground biomass weights, height of plants, and number of leaves and fruits. In addition, a yield increase was also observed when plant seeds were exposed to *Trichoderma* conidia, suggesting that *Trichoderma* metabolites can influence the plant growth (Benítez et al. 2004).

Increased plant growth induced by *Trichoderma* species has also been reported for many arable crops such as maize (Harman et al. 1989), wheat (Shivanna et al. 1996), rice (da Silva et al. 2012), and peanut (Rojo et al. 2007). Numerous studies indicated that metabolic changes occur in the root during colonization by *Trichoderma* spp., such as the activation of pathogenesis-related proteins, which induce in the plant an increased resistance to subsequent attack by numerous microbial pathogens. The induction of systemic resistance (ISR) observed in plants determines an improved control of different classes of pathogens (mainly fungi and bacteria), which are spatially and temporally distant from the *Trichoderma* inoculation site. This phenomenon has been observed in many plant species, both dicotyledons (tomato, pepper, tobacco, cotton, bean, cucumber) and monocotyledons (corn, rice). For example, *T. harzianum* strain T-39, the active ingredient of the commercial product TricodexTM, induces resistance toward *B. cinerea* in tomato, tobacco, lettuce, pepper, and bean plants, with a symptom reduction ranging from 25 to 100 % (De Meyer et al. 1998).

9.4 Conclusion

Environmental and consumer concerns have focused interest in the development of biological control agents as an alternative, environmentally friendly strategy for the protection of agricultural crops against phytopathogens. The microbial antagonism in combination with fungicides has sometimes improved the efficacy of disease control. Many encouraging reports by several scientists around the world have described different *Pseudomonas* and *Trichoderma* strains that are able to significantly control a number of fungal diseases.

The management practices should protect the soil biodiversity. The soil environment that allows the development of populations of beneficial microorganisms that antagonize pathogens causing diseases will reduce populations of pathogens to a manageable level. The soil microbial populations can be used as indicators of soil quality and crop health, with a leading role in the development of a sustainable agriculture, minimizing the impact of chemicals and maintaining the intrinsic characteristics of the soil.

The present review contributes to future research programs that aim to promote strains of *Pseudomonas* and/or *Trichoderma* as potential biopesticides for biological control of many diseases of agricultural relevance. However, a better understanding of the factors involved and the signaling interaction among antagonists, pathogens, soil, and plants are yet to be revealed to promote the biocontrol agents as wide applicable biopesticides in the future.

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Strategies for High-Density Cultivation of Bio-inoculants in Submerged Culture with Special Reference to Pseudomonads

10

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Abstract

This chapter provides a brief overview of submerged cultivation methodologies of *Pseudomonas*-based microbial inoculants, which when delivered as a formulation improves the health of the host plants. The classifications of bio-inoculant delivery systems in various agronomical applications are discussed in the initial section of the chapter. Among various modules in the supply chain of bio-inoculant development, the chapter deals with medium development and cultivation strategies for successful production of active ingredients. The chapter specifically explores the mass propagation strategies of two potential *Pseudomonas* strains in submerged cultivation with emphasis on fed-batch mode of cultivation.

Keywords

Pseudomonas • High-density cultivation • Microbial inoculants • Agronomy

10.1 Introduction

Microbial inoculants/biofertilizers are considered as a step towards achieving sustainable agriculture systems. Bio-inoculants are ready to use live formulates of beneficial microorganisms, which when applied to soil, roots or seeds enhance the availability of different nutrients to the plant by their inherent metabolic activities (Bashan 1998). These delivery systems to host plants essentially consist of plant growth-promoting rhizobacteria (PGPR); the term coined

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by Kloepper and Schroth (1981) encompasses those bacteria that are able to colonize plant root systems and promote plant growth. It includes all bacteria of rhizosphere origin that promote plant growth. However, PGPR were further subclassified into two groups based on their mechanisms of action according to Bashan and Holguin (1998):

Plant growth-promoting bacteria (PGPB): These are bacteria whose metabolites or their precursors are used as growth regulators.

Biocontrol PGPB: The metabolites released from these bacteria are involved in imparting antagonistic action against microorganisms that are detrimental to plants by means of different direct and indirect modes.

The above definition does not include fungi and class of bacteria that are not essentially rhizobacteria. In the last decade, several fungi have been reported to exert beneficial mode of action on host plants in improving their health. Hence, the definition has been updated to plant growth-promoting microorganisms (PGPM) according to Owen et al. (2015). As this term seems to be more generic, it will be used in this chapter for referring to PGPR. PGPM are classified as follows (Owen et al. 2015):

PGPM bacteria: Further classified as intracellular (belonging to genera of *Rhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Allorhizobium*, *Mesorhizobium*, *Sinorhizobium*, etc.) and extracellular (belonging to genera of *Bacillus*, *Burkholderia*, *Paenibacillus*, *Erwinia*, *Stenotrophomonas*, *Micrococcus*, *Flavobacterium*, *Streptomyces*, *Serratia*, *Azospirillum*, *Agrobacterium*, *Actinomycetes*, *Pseudomonas*, *Arthrobacter*, etc.). Intracellular bacteria, primarily all rhizobium species, function as nutrition suppliers, whereas extracellular bacteria play the roles of bio-stimulants, bio-protection and bioremediation apart from being nutrient suppliers.

PGPM fungi: These are classified as root-associated fungi (RAF) and mycorrhizae. RAF includes *Aspergillus*, *Trichoderma*, *Penicillium*, *Saccharomycetes*, etc. Although *Piriformospora indica* (Varma et al. 1999) has not been mentioned in Owen et al. (2015), this potential endophytic fungus seems to be fitting in RAF category for its PGP activities. Mycorrhizae are subclassified as (EcM) ectomycorrhiza (*Thelephora*, *Pisolithus*, *Rhizopogon* and *Scleroderma*) and (AM) arbuscular mycorrhiza (*Rhizophagus*, *Glomus*, *Funneliformis*, *Claroideoglomus*, *Gigaspora* and *Scutellospora*). RAF and mycorrhizae also span all the functional abilities as extracellular bacteria except that mycorrhizae do not act as bio-stimulants.

There are several bio-inoculant products sold across the globe, which contain either products (including viable cells) derived from pure culture or consortium of different inoculants marketed by companies. Some of the trademark names with active ingredient (*ai*) composition are listed in Table 10.1.

As the chapter focuses on fluorescent pseudomonads, the microbial description of traits for plant growth promotion is described here. *Pseudomonas* is the most important genus in the order *Pseudomonadales*, family *Pseudomonadaceae*. A group of bacteria among genus *Pseudomonas*, which produces yellow-green fluorescent water-soluble pigments, is termed as fluorescent pseudomonads. The exhaustive list of mechanisms and role of PGPM in phyto-promotional activities is well detailed in reviews by Lugtenberg and Kamilova (2009) and Podile and Kishore (2006). Figure 10.1 depicts some of the direct and indirect mechanisms exhibited by fluorescent pseudomonads for improving the plant health.

The culture broth of the pseudomonad or other PGPM containing potential metabolites, which form the active ingredients listed in Fig. 10.1, is usually formulated into a deliverable form (either liquid or carrier-based formulations) that is applied to host plants to exert growth promotion. The entire supply chain of such bio-inoculant development process is depicted in Fig. 10.2. In brief, an organism of interest is screened from the isolates obtained from the rhizosphere of a certain plant as a first step. Later, the screened organisms

Table 10.1 A few commercially available bio-inoculant products[a]

S. No.	Product name	Company	Active ingredient (*ai*)	PGPM category
1	Promot	JH Biotech Inc, USA	30 cfu/g *Pisolithus tinctorius*	Ectomycorrhiza
2	Mycormax	JH Biotech Inc, USA	25 cfu/g *Glomus intraradices* 25 cfu/g *Glomus mosseae* 15,300 cfu/g *Pisolithus tinctorius*	AMF
3	Endomycorrhizal Inoculant (BEI)	BioOrganics, USA	*Glomus aggregatum*, *G. etunicatum*, *G. clarum*, *G. deserticola*, *G. intraradices*, *G. monosporus*, *G. mosseae*, *Gigaspora margarita* and *Paraglomus brasilianum*	AMF
4	Viva Roots	AgBio Inc, USA	Endomycorrhizae mix	Mycorrhiza
5	Bactofil A 10	Agro. Bio Hungary	*Azospirillum brasilense*, *Azotobacter vinelandii*, *Bacillus megaterium*, *Bacillus polymyxa*, *Pseudomonas fluorescens*, *Streptomyces albus* as well as other agents. 4.3×10^9 cells/mL	Bacteria (extracellular)
6	Bactvipe	International Panaacea Ltd., India	*Pseudomonas fluorescens*	Bacteria (extracellular)
7	Microbion UNC	Syn-Bio-Tech Ltd, Hungary	*Azotobacter vinelandii-B 1795*, *Bacillus megaterium B1091*, *Clostridium pasteurianum*, *Azospirillum* sp., *Bacillus subtilis*, *Rhodobacter* sp., *Lactobacillus* sp., *Trichoderma reesei*, *Saccharomyces cerevisiae*, *Streptomyces* sp. 4.0×10^{10} cells/g	Bacteria (extracellular)
8	Nodulator XL	BASF	*Rhizobium leguminosarum* biovar *viceae*	Bacteria (intracellular)
9	Vault SP	BASF	*Bradyrhizobium* sp. (*Arachis*), 2.0×10^9 cells/g	Bacteria (intracellular)
10	Rhizo-Flo	BASF	Consortium of rhizobium	Bacteria (intracellular)
11	Primo	Verdesian Life Sciences	High load of *Rhizobium*	Bacteria (intracellular)
12	Accolade-L	Verdesian Life Sciences	*Azospirillum brasilense* strains	Bacteria (extracellular)
13	Kodiak HB	Chemtura	*Bacillus subtilis*, 6.0×10^9 spores/g	Bacteria (extracellular)
14	Poncho/Votivo	Bayer	*Bacillus firmus* + Clothianidin, 2.0×10^9 cfu/ml	Bacteria (extracellular)

(continued)

Table 10.1 (continued)

S. No.	Product name	Company	Active ingredient (*ai*)	PGPM category
15	Grandevo	Marrone Bio Innovations	*Chromobacterium subtsugae* strain PRAA4-1 + spent medium	Bacteria (extracellular)
16	Jumpstart	Novozymes BioAg	*Penicillium bilaiae*, 7.2×10^8 cfu/g	RAF (extracellular)
17	TagTeam MultiAction	Novozymes BioAg	*Penicillium bilaiae*, 3.7×10^6 cfu/g *Rhizobium leguminosarum*, 7.4×10^8 cfu/g	RAF (bacteria consortium)
18	BlightBan A506	Nufarm, Australia	*Pseudomonas fluorescens* A506, 71 % of *ai*	Bacteria (extracellular)

[a]Data collected from Owen et al. (2015), product lists of various companies and other web sources such as www.seedquest.com

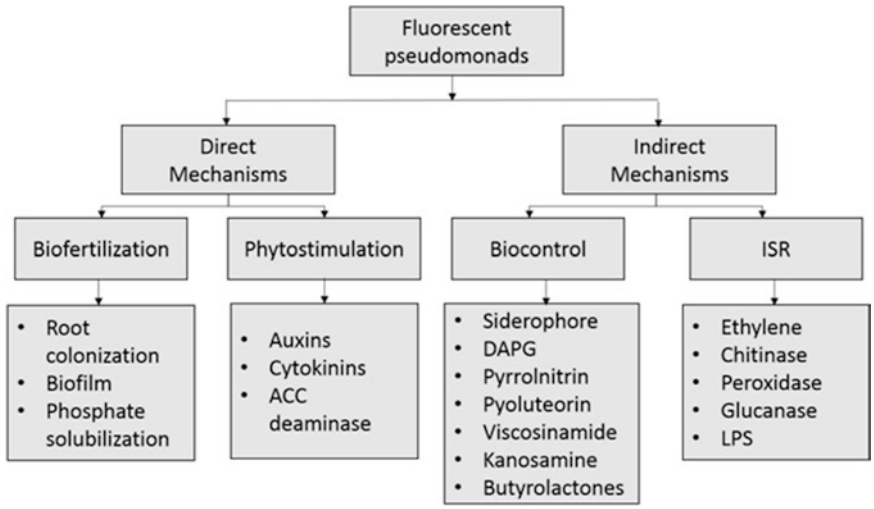

Fig. 10.1 Various mechanisms by which fluorescent pseudomonads influence plant health (Adapted from Podile and Kishore 2006). *ISR* induced systemic resistance, *LPS* lipopolysaccharide, *DAPG* diacetylphloroglucinol, *ACC deaminase* 1-aminocylopropane-1-carboxylate deaminase

are established by microbiological and genetic tools for their plant growth-promoting characteristics. Usually the secondary metabolites whose traits exert growth promotional activities (direct or indirect) in the host plant are qualitatively screened by analyzing the gene sequence responsible for their synthesis in the metabolic pathways (Gaur et al. 2004). In the process development stage, nutritional medium is designed for cultivation of the organism. Here in this stage, efforts should be made to design a synthetic medium instead of complex medium as complex sources would act as bait for contamination if any residual components are present during formulation and storage (Saharan et al. 2011). Fluorescent pseudomonads enjoy the advantage

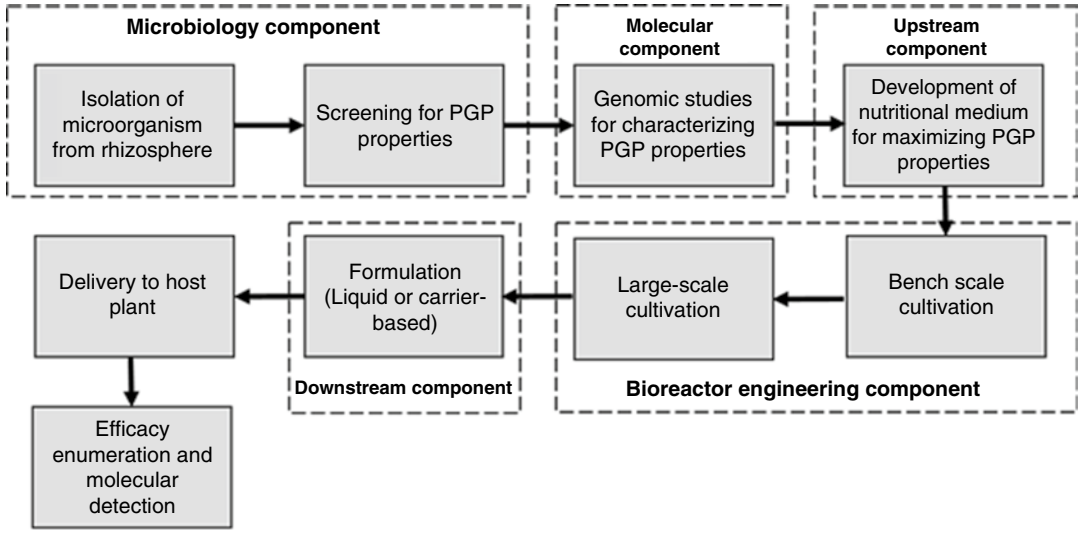

Fig. 10.2 Supply chain for bio-inoculant production and delivery to host plant

of assimilating glycerol as the primary carbon source, which is selective to some extent. Once the medium is designed, process conditions for submerged cultivation need to be standardized in a bench-scale reactor, and later the process is scaled up to higher volumes. The culture broth obtained is subsequently formulated for storage and delivery to host plants. Finally the supply chain ends with enumeration of target growth parameters in controlled or open field conditions and also their possible detection from the test plant rhizosphere using molecular tools (Mathimaran et al. 2008).

The chapter focuses on methodologies used for development of nutritional medium for cultivation of bio-inoculants (the upstream component of Fig. 10.2), and the process strategies that are used for their mass multiplication in bioreactors with a view to get their high colony-forming units (cfu) count along with the desired metabolites having PGP properties (the bioreactor engineering component of Fig. 10.2) with special reference to *Pseudomonas* spp.

10.2 Medium Development and Cultivation Strategies for Pseudomonas-Based Bio-inoculant Production

10.2.1 Medium Development

Development of medium for cultivating potential PGPM is critical during production and formulation with respect to contamination. In most cases, the obtained bio-inoculant broth post submerged cultivation is formulated into a delivery system and later stored under shelf. This gestation during storage may allow room for contaminants to proliferate on nutritional components present in the residual cultivation medium. Especially when complex sources such as peptone and yeast extract are used, such contamination risk would be pronounced. Hence, a simple synthetic medium should be designed and optimized for submerged cultivation. One of the functional needs for optimizing medium components is to improve the production levels of biocontrol

agents in the active ingredient so that they not only keep check on microbial contaminants during shelf storage but also initiate defence mechanisms even before the colonization by the strain of specific root niches. For instance, siderophore and 2,4-diacetylphloroglucinol (DAPG) are the compounds released by fluorescent pseudomonads contributing to their defensive mode of action against antagonists (Saharan et al. 2011). If the synthesis level of these compounds is increased during submerged cultivation by manipulating the medium components, then it would serve its intended purpose.

Most of the medium development strategies for extracellular PGPM rely on existing synthetic medium that has been used for some purpose other than that of bio-inoculant production. This would perhaps be the starting point for medium design, and the challenge would be the inclusion of nutritional components for production of compounds which exert the effects of bio-stimulation, bioremediation and bio-protection. The following section will focus on medium development for two such as PGP traits of fluorescent pseudomonads, namely, siderophore (iron-chelating agent) and DAPG (antifungal polyketide).

The production of siderophores by strains of *Pseudomonas* spp. depends on several nutritional and environmental factors as detailed by Elena and de Villegas (2007): ferric ion concentration (Meyer and Abdallah 1978; Budzikiewicz 1993; Laine et al. 1996; de Villegas et al. 2002), carbon and nitrogen source (Albesa et al. 1985; Park et al. 1988; Duffy and Défago 1999) and phosphate concentration (Barbahaiya and Rao 1985; Défago and Haas 1990). The critical factor among these is the concentration of ferric ion in the culture medium. The concentration of iron in the vicinity of 10 μM is considered good enough to yield biomass with modest levels of siderophores (Neilands 1984). In the strain *Pseudomonas fluorescens* 94, the siderophore levels were high even at 50 μM of Fe^{+3} concentration (Manninen and Mattila-Sandholm 1994). Co^{+2}, fructose, mannitol and glucose increased in vitro production of pyochelin by *P. fluorescens*, while NH_4Mo^{+2}, glycerol and glucose increased the production of its precursor salicylic acid (Duffy and Défago 1999). Bultreys and Gheysen (2000) found that the strains of *Pseudomonas syringae* produced pronounced levels of siderophore when amino acids were used as the sole source of both carbon and nitrogen. In studies conducted by Meyer and Abdallah (1978), citric and succinic acids were used as sole carbon sources for producing siderophore in *Pseudomonas fluorescens* strains. As described earlier, glycerol has been widely used as the carbon source for *Pseudomonas* spp. in different media, including the standard King's B medium (Nowak-Thompson and Gould 1994). The effect of nature of carbon source is remarkable on growth during mass multiplication of the *Pseudomonas* strains. Sugars like glucose and sucrose caused digression of pH from near neutral to below 6.4, while organic acids like citric or succinic acid cause upward pH digression to 7.4 and above, while the use of glycerol causes relatively small digressions (Saharan et al. 2010). The extreme digressions slow down the growth rate considerably leading to low productivities. During inoculum development in shake flask, such digressions in pH may result in very low optical density and larger lag periods, thereby necessitating the use of a larger inoculum size. In addition to carbon source, nitrogen source also causes digression in pH substantially upwards or downwards. A synthetic medium was developed (Saharan et al. 2010) using glycerol (as a carbon source) and urea and ammonium sulphate (as dual nitrogen sources) which was able to contain pH digressions within 7.0±0.2 during fermentation. The use of such a medium is desirable in shake-flask cultures where pH control is not possible. Glycerol metabolizes very slowly due to very low level of key enzymes, glycerol kinase and glycerol phosphate dehydrogenase, involved in its catabolism. The presence of citrate or succinate at low levels (0.05 %) in the medium increased activity of the glycerol kinase almost 15-fold causing rapid utilization of glycerol (Saharan et al. 2010).

In submerged cultivation, antibiotic production by many organisms is influenced by the type and abundance of carbon and nitrogen sources. Phosphate, iron and micronutrients modulate antibiotic production (Weinberg 1977; Slininger

and Jackson 1992). Production of idiolites (secondary metabolites) is usually inhibited by concentrations of phosphate that can support optimum biomass production. Phosphate regulates the syntheses of several classes of antibiotics such as peptide antibiotics, polyene macrolides, tetracyclines and biosynthetically complex antibiotics (Martin 1977). Industrial production of these antibiotics is carried out at growth-limiting concentrations of inorganic phosphate. Phosphate in concentrations ranging from 0.3 to 300 mM generally supports extensive cell growth, but concentrations of 10 mM and above suppress the biosynthesis of many antibiotics (Martin 1977). The soil and the rhizosphere also have profound effects on the production of antimicrobial compounds by root-associated bacteria (Thomashow et al. 1990; Clarke et al. 1992). It was reported that *P. fluorescens* usually produces 0.1–20 µg/ml of antibiotics (DAPG, pyoluteorin, phenazine and pyrrolnitrin) extracellularly when grown on nutritional liquid media. Environmental factors that regulate the biosynthesis of antimicrobial compounds by disease-suppressive strains of *Pseudomonas fluorescens* have been extensively studied by Défago and coworkers (Duffy and Défago 1999). They screened minerals and carbon sources for stimulation or repression of biosynthesis of several antibiotics (PHL, PLT and pyrrolnitrin) and siderophores (pyochelin and salicylic acid) by *Pseudomonas fluorescens*. The amendments of minerals and carbon sources during liquid fermentation of bio-inoculant were observed to improve the biocontrol activity of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* strains (Siddiqui and Shaukat 2002).

The active ingredient (*ai*) used for formulation and delivery of bio-inoculant usually comprises viable cell fraction and metabolite/s synthesized by the organism. Hence, the '*ai*' is a multi-product formulation mixture wherein each response requires different nutritional requirement for its optimal production. To design and optimize such medium for multi-response objective requires nontraditional medium design tools. In one such study by Sarma et al. (2009), the medium was optimized after screening the medium components based on a multi-objective genetic algorithm (GA). The problem statement was defined as follows, where both dry cell weight and siderophore concentration were selected as responses.

$$\text{maximize } F(x) = \sum_{m=1}^{M} w_m y_m$$

$$y_m = f(x_i)$$

$$x_i^{(L)} \le x_i \le x_i^{(U)} \quad i = 1, 2, \ldots . n.$$

$$w_m \in [0,1] \text{ and } \sum_{m=1}^{M} w_m = 1$$

Here $F(x)$ is an objective function to be maximized with $m = 2$. y_1 and y_2 are the dry cell weight (g/L) and siderophore concentration (g/L), respectively, for individual chromosome of GA. x_i refers to the various medium components with concentration ranging between lower limit, $x_i^{(L)}$, and upper limit, $x_i^{(U)}$. A total of $n = 10$ medium components varied to 32 levels were optimized within 80 experiments only, in comparison to 32^{10} experiments that were required if one variable at a time (OVAT) was conducted or 2^{10} (=1020) if response surface methods (RSM) were employed. In this way, there was significant increase in both viable cell fraction and siderophore concentration when GA was applied in comparison to RSM. Moreover, GA being a stochastic algorithm, it spans the entire search space to obtain a near global optimum. For instance, carbon to nitrogen (C/N) ratio which is critical in designing medium for growth responses is optimized automatically by GA and prevents the requirement for separate experimentation. In this study (Sarma et al. 2009), the GA-based optimization has also rendered good buffering to the medium composition, thereby alleviating the requirement of pH control during batch cultivation in a 14 L bioreactor.

10.2.2 Cultivation Strategies

Similar to the rule of thumb adopted in medium development, the cultivation strategy of the potential bio-inoculant relies on existing strate-

gies for the test microorganism evolved for applications other than bio-inoculant development. This heuristic may not be applicable to all PGPM categories but can be widely applied to *bacteria-extracellular* classification (cf. Table 10.1).

A wide range of physical parameters, including cultivation pH, temperature, aeration and agitation, shear stress, etc. determines optimal growth of the microorganism. These factors can be optimized/manipulated in a controlled environment such as in a bioreactor. In the case of *Pseudomonas* spp., the optimal settings for different applications are discussed below.

10.2.2.1 Temperature

Optimal temperature during cultivation determines the growth rate of microorganism. Most bacteria are mesophilic, growing at temperatures between 10 and 40 °C. Temperature is considered as key environmental factor for siderophore production by *Pseudomonas* strains (Elena and de Villegas 2007). Most of *Pseudomonas* spp. grow well in the temperature range of 28–30 °C (Todar 2004). In the studies conducted by Loper and Schroth (1986), the siderophore synthesis in fluorescent pseudomonads was inhibited above 33 °C.

10.2.2.2 pH

The pH is difficult to control in shake-flask cultures as the growth of the bacteria itself results in a change of the pH of the medium. Moon and Parulekar (1991) have reported that culture pH strongly affected many intracellular enzymatic reactions and transportation of compounds across the cell membrane. Ammonium ions tend to make the medium acidic, while the consumption of organic nitrogen sources such as amino acids and peptides makes the medium alkaline. Usually fluorescent pseudomonads grow well in the neutral pH range (6.7–7.2). Moreover, most of the enzymes inside the cell show maximum activity around pH 7.0 in *Pseudomonas* spp. (Gummadi et al. 2009). Rahman et al. (2005) reported neutral pH maximized protease production of organic solvent-tolerant protease in *P. aeruginosa* strain K. Production of some metabolites from fluorescent pseudomonads has been reported to have adverse effect on pH digressions beyond the range 6.5–7.5 (Budzikiewicz 1993).

10.2.2.3 Agitation and Aeration

The growth of microorganisms and metabolite synthesis is significantly affected by agitation and aeration (de Fernando et al. 1991). In many situations, it has been reported that agitation increases the rate of oxygen and nutrient transfer from the liquid medium to the cells and also prevents cell clumps formation (Brown et al. 1987). Fluorescent pseudomonads being obligate aerobes require oxygen throughout the cultivation time. Usually the oxygen concentration in terms of dissolved % saturation should be above 20 % for aerobic cultures. Aeration and agitation during the cultivation had an influence on bacterial growth and consequently on product formation (Akhurst 1982; Chen et al. 1996). Turbulent flow regime is required for high cell density cultivations in bioreactor for uniform distribution of air bubbles (Doran 1995). This could be possible with optimal aeration and agitation values. Moreover, in bacterial fermentations, high aeration rates beyond 2 vvm inside the bioreactor would lead to foam generation (Yeh et al. 2006). The caffeine demethylase production was maximized at 0.27 vvm and 700 rpm using *Pseudomonas* spp. by Gummadi et al. (2009). During cultivation of *P. aeruginosa* PAO1 under iron-limited conditions, the oxygen transfer rate of the culture, characterized by volumetric oxygen transfer coefficient, k_La, was found to decrease significantly (Kim et al. 2003).

10.2.2.4 Mode of Bioreactor Operation

Bio-inoculant formulations usually contain a population of pure culture or a consortium of potential microorganisms along with the compounds released by the pure culture or the consortia (cf. Table 10.1). Thus, the challenge during cultivation is to have

(a) Good amount of viable cells (which perform the designated growth promotional activities)

(b) Compounds (which are usually secondary metabolites) released in the culture medium which control the contamination risk under shelf storage and also thwart disease incidence during germination stage

To a significant extent, the cultivation strategies could be exploited as a tool to address above challenges. Usually in a batch mode of cultivation, obtaining cell concentrations above 10 g/L is quite difficult as the cells suffer from substrate inhibition and catabolite repression (Yamane and Shimizu 1984; Yee and Blanch 1992). Fed-batch cultivations can be successfully used to get higher cell density and desired metabolites such as of organic acids, antibiotics, vitamins and enzymes (Gummadi and Kumar 2008; Nath et al. 2008). During fed-batch cultivation, one or more nutrients are added, either continuously or intermittently, to the bioreactor, while the cells and the products remain in the bioreactor until the end of operation (Stanbury and Whitaker 1989). Fed-batch cultivation is, therefore, an efficient cultivation methodology to achieve high cell density, which is often necessary for getting high yield and productivity of the desired product (Lee et al. 1999; Riesenberg and Guthke 1999). The cultivation constraints for which fed-batch operation may be an effective strategy are substrate inhibition, high cell concentrations, catabolite repression, auxotrophic mutants, extension of operation time, etc. (Yamane and Shimizu 1984).

Fed-batch microbial processes can be classified according to the feeding mode. Figure 10.3 illustrates the classification by Yamane and Shimizu (1984). It is broadly classified into processes without feedback control and with feedback control. Fed-batch processes without feedback control are further subdivided with reference to feed rates as intermittent, constant, exponential and optimized. The fed-batch processes with feedback control can be carried out by using an indirect feedback parameter such as pH, DO, RQ and redox.

(a) *Intermittent/pulse feeding*

In this feeding strategy, the nutrients are added to the bioreactor intermittently at predefined times or based on output signals from dissolved oxygen (DO) or CO_2 sensor from exit gas analyzer. The addition of feed can be a discrete event or in semi-continuous mode. In certain cases, the time of addition and the duration are predetermined (Elias et al. 2000). Pulse feeding can also be combined with other strategies (Yee and Blanch 1992).

(b) *Constant feed rate*

As the name suggests, in this fed-batch strategy, the nutrients are fed at a constant rate. Achieving quasi-steady state using this strategy was reported to be highly ineffective (Yamane and Shimizu 1984).

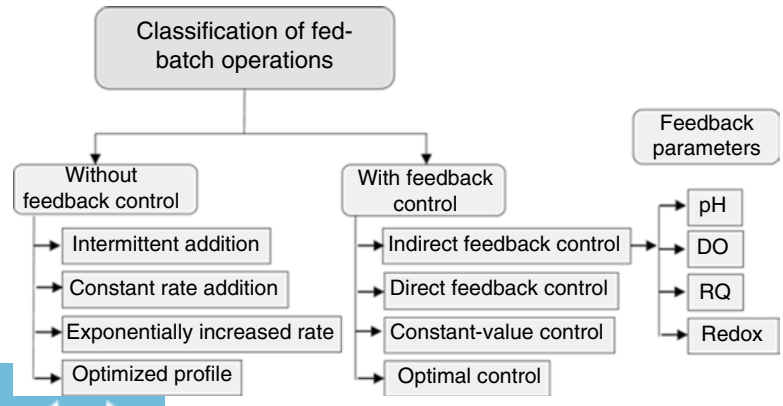

Fig. 10.3 Classification of fed-batch operations (Adapted from Table 1 of Yamane and Shimizu 1984)

(c) *Exponential feeding*

Exponential feeding falls in the category of predetermined feeding strategy. It is a robust method that allows cells to grow closely to a constant specific growth rate. High cell density cultures of *E. coli* have been extensively carried out using this method (Kleman and Strohl 1994; Yoon et al. 1994; Lee 1996). As acetate production predominantly inhibits cell growth as well as product formation in *E. coli* cultivations, it could be minimized by adopting this strategy at lower specific growth rates (Lee 1996). This feeding strategy does not use a measured variable for manipulating the nutrient feed rate. Instead, the feed rate is manipulated either in automated mode or manual mode by using fed-batch model equation as below.

$$F(t)=\frac{\left(\left(\mu / Y_G\right)+m_s\right)x_i V_i e^{\mu t}}{S_o}$$

where $F(t)$ is the exponential feed profile at any time t, μ is the specific growth rate (h^{-1}), t is the elapsed time (h), S_o is the substrate concentration in feed (g/L), x_i is the biomass concentration at the start of feed (g/L), V_i is the working volume at the start of feeding (L), Y_G is the true growth yield coefficient for substrate (g biomass produced/g substrate consumed), and m_s is the maintenance coefficient (g/(g.h)).

(d) *Indirect feedback methods*

Indirect signals such as respiratory quotient (Wang et al. 1977), pH (Nishio et al. 1977) or dissolved oxygen (DO) concentration (Yano et al. 1978; Mori et al. 1979) have been used to control the substrate feeding to the culture. The block diagram for such feedback control is illustrated in Fig. 10.4. The DO is an interesting alternative since whenever the substrate in the culture broth is about to be exhausted and becomes a limiting factor, the DO increases rapidly. However, when a certain amount of substrate is added to the culture medium, the DO level increases (Yamane and Shimizu 1984). In case of pH-based feeding, the pH rises due to excretion of ammonium ions when the principal carbon substrate is depleted (Suzuki et al. 1990). In the case of *Pseudomonas* spp., both DO- and pH-based indirect feedback feeding strategies have been reported (Sun et al. 2006; Chen et al. 2007). pH-based feeding was coupled with fill-and-draw methodology to achieve stable repeated fed-batch technique for enhancing rhamnolipid production in *Pseudomonas aeruginosa* S2 strain (Chen et al. 2007). Sun et al. (2006) observed that pH- and DO-based feeding strategies were superior to exponential feeding during fed-batch cultivation of *Pseudomonas putida* KT2440 for achieving high cell densities. Both of these feeding strategies, which are based on similar indirect feedback control scheme of keeping the substrate concentration below a non-inhibitory level by pulse feeding, were implemented in *Pseudomonas* fed-batch cultivations (Lee et al. 2000; Kim 2002). Suzuki et al. (1988) used CO_2 evolution rate as an indirect signal for feeding limiting substrate during fed-batch cultivation of *Pseudomonas fluorescens* for enhanced production of lipase enzyme.

Although fed-batch cultivation of *Pseudomonas putida* has been widely studied for several applications (Kim et al. 1996; Lee et al. 2000; Thuesen et al. 2003; Sun et al. 2006), there have been very few studies on high cell density cultivation of fluorescent pseudomonads for bio-

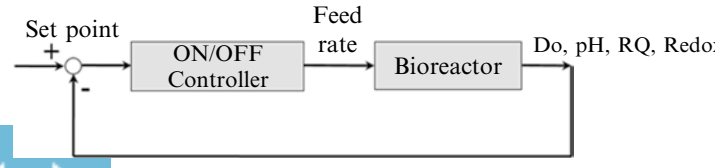

Fig. 10.4 Block diagram of indirect feedback methods

Table 10.2 Various fed-batch strategies used for cultivation of *Pseudomonas* spp.

Pseudomonas spp.	Carbon source	Fed-batch strategy	Biomass productivity (g/L.h)	Product	Ref.
Pseudomonas putida KT2440	Glucose	Exponential feeding	3.0	Biomass	Sun et al. (2006)
Pseudomonas putida BM014	Glucose	DO based	0.25	Cis,cis-muconic acid	Bang and Choi (1995)
Pseudomonas aeruginosa MB 5001	Glucose	Constant rate	NA	Lipase	Chartrain et al. (1993)
Pseudomonas putida	Glucose	Exponential feeding	0.77	Di-heme cytochrome c4	Thuesen et al. (2003)
Pseudomonas oleovorans ATCC 29347	Octanoic acid	pH-based feeding	1.50	Polyhydroxy alkanoates	Kim (2002)
Pseudomonas putida KT2442	Oleic acid	DO based and pH based	3.80	Polyhydroxy alkanoates	Lee et al. (2000)
Pseudomonas fluorescens	Olive oil	Constant rate	1.20	Lipase	Suzuki et al. (1988)
Pseudomonas aeruginosa S2	Glucose	pH based	0.014	Rhamnolipid	Chen et al. (2007)
Pseudomonas aeruginosa S2	Glucose	Constant rate	0.016	Rhamnolipid	Chen et al. (2007)
Fluorescent pseudomonad R81	Glycerol	pH based	0.54	Biomass, DAPG	Sarma et al. (2013)
Fluorescent pseudomonad R62	Glycerol	pH based	0.51	Biomass, DAPG	Sarma et al. (2013)

inoculant formulations. Some of the feeding strategies applied by researchers for fed-batch cultivation of *Pseudomonas* spp. are tabulated in Table 10.2.

10.3 Case Study on Fed-Batch Cultivation of *Pseudomonas* Strains R62 and R81

Fluorescent pseudomonad strains R62 and R81 were established as potential PGPM (Gaur et al. 2004), and field applications implicated significant improvement in assessed growth parameters (Mäder et al. 2011). The genome sequences of these strains revealed maximum similarity of R62 and R81 with *Pseudomonas fluorescens* strain Pf0-1 and *Pseudomonas fluorescens* strain SBW25, respectively. By homology searching, it could also be established that the strains possessed genes encoding enzymes for functional secondary metabolites such as ferric siderophores, DAPG, hydrogen cyanide, orfamide A, phenazine, pyoluteorin and pyrrolnitrin, which are important for growth promotional mechanisms via biocontrol activities (Mathimaran et al. 2012).

DAPG produced by both strains, R62 and R81, is the most potent and most extensively studied antibiotic produced by PGPM (Raaijmakers et al. 2002). The purified polyketide DAPG has broad antiviral, antibacterial, antifungal, antihelminthic and phytotoxic properties (Keel et al. 1992; Bangera and Thomashow 1999). DAPG being a nongrowth-associated metabolite, its production depends on the number of cells entering the stationary phase. Therefore, a process strategy was realized where high cell mass could be achieved in the initial phase of growth, and later this culture could be subjected to stationary phase for improved production of DAPG. In order to attune the above hypothesis,

Table 10.3 Effect of various feeding strategies on PGP traits of fluorescent pseudomonads R81 and R62 (Sarma et al. 2010, 2013)

Strain	Mode of cultivation[a]	Observed specific growth rate, μ (h^{-1})	Biomass conc. (g/L)	Biomass productivity g/(L.h)	DAPG conc. (mg/L)
R81	Batch	0.22	7.0	0.19	20
	Intermittent feeding	0.24	25.0	0.25	70
	Exponential feeding[b]	0.015	18.0	0.44	25
	DO-based feeding	0.02	25.0	0.29	250
	pH-based feeding	0.10	27.0	0.54	342
R62	Batch	0.20	7.6	0.21	25
	DO-based feeding	0.02	20.9	0.24	220
	pH-based feeding	0.07	25.5	0.51	298

[a]The cultivation times for batch, intermittent feeding, exponential feeding, DO-based feeding and pH-based feeding were 23, 100, 41, 87 and 50 h, respectively

[b]Feeding exponentially with $\mu = 0.10\ h^{-1}$

some of the above-mentioned fed-batch cultivation strategies were applied on these strains under controlled conditions. Both open-loop feeding strategy such as exponential feeding of nutrients and intermittent feeding and closed-loop feedback strategies using dissolved oxygen (DO) and pH signals were studied for cultivation of fluorescent pseudomonad strains R62 and R81 (Sarma et al. 2010, 2013). The control algorithms for pH- and DO-based fed-batch cultivation of these *Pseudomonas* strains are detailed in Sarma et al. (2013).

The results obtained on mass cultivation of pseudomonads R62 and R81 under different feeding strategies are shown in Table 10.3. The intermittent feeding in case of R81 cultivation resulted in significant increase in cell population in comparison to batch cultures, but the productivity was low. It was observed that the predetermined exponential feeding did not converge to expected specific growth rate and led to the accumulation of primary carbon source (glycerol). This eventually caused severe substrate inhibition in the fed-batch cultivation and resulted in lower biomass concentration (compared to intermittent feeding). The DO-based feeding was also not found to be very effective, as the cultures grew at lower specific growth rates (in comparison to pH-based feeding) resulting in low biomass productivities. The pH-based feeding successfully improved both cell population and DAPG (compound of interest) in the culture broth for both strains. Here a brief description of the cultivation process designed by Sarma et al. (2013) for pH-based feeding is given.

Using pseudomonads R62 and R81 in a 5 L bioreactor, the pH was found to rise in batch cultivation when glycerol (carbon source) was limiting and started to decline when a pulse of glycerol was added to the reactor at this juncture (Fig. 10.5). This variation of pH signal on addition/depletion of glycerol was exploited to design a control system for feeding. This control algorithm was invoked only in the dead band of the actual pH controller for the bioreactor. Dead band for a controller signifies the region around the set point for the controller where the control action does not take place. In these studies, pH of 6.9 was selected as the set value for controller with dead band of 0.05, which means that the control action does not take place in the region 6.85–6.95. A user-defined pH set point of 6.92 was selected within the dead band, and whenever the pH rose above this value due to glycerol limitation, the feeding was initiated for a fixed amount of time based on the flow rate of feed pump (Fig. 10.5). During pH-based feeding till 44 h, the glycerol concentration in the bioreactor at any given time was always well below the inhibitory level. During the subsequent second phase of fed-batch cultivation (i.e. the idiophase), the

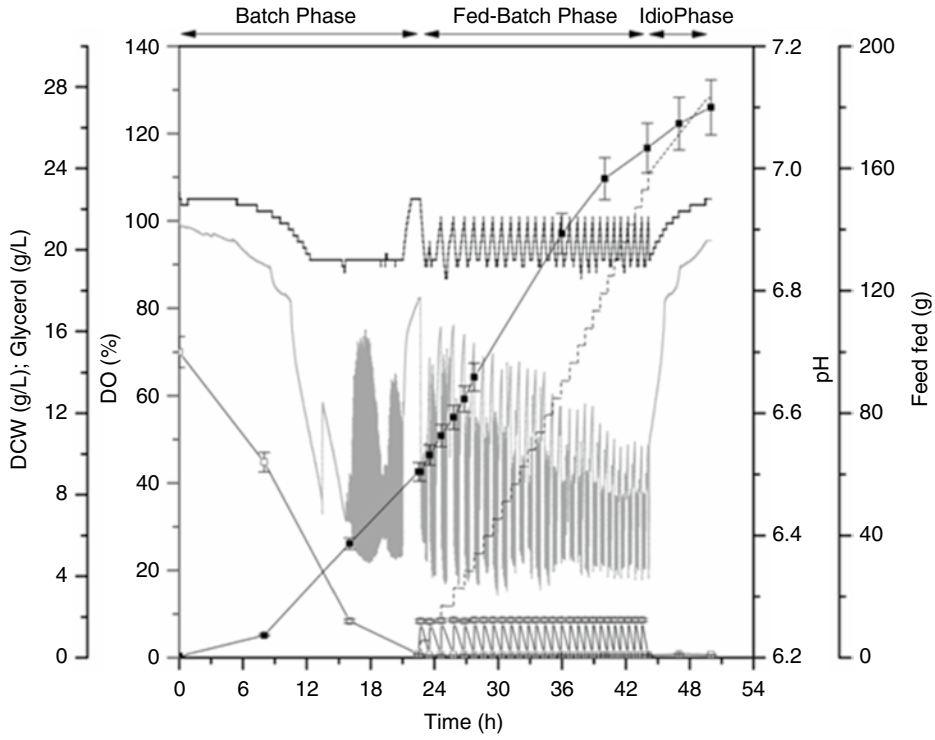

Fig. 10.5 Time course of the pH signal-based fed-batch cultivation of fluorescent pseudomonad R81 in a 5 L bioreactor. The feeding commenced at 22.5 h after batch cultivation and continued up to 44 h followed by constant feeding for 6 h up to 50 h. *Solid grey line* denotes dissolved oxygen (% saturation), the *solid black line* denotes pH profile. *Filled square* (■) denotes dry cell weight (g/L) and *hollow square* denotes (□) glycerol (g/L) profile. Total feed medium fed into the bioreactor is shown by *stepped line* (Sarma et al. 2013)

feeding was done for the maintenance of the culture and also to enhance the synthesis of DAPG. During this phase (44–50 h), the onset of secondary metabolism in the culture resulted in DAPG production. This strategy enabled the addition of the feed in a robust manner, alleviating the substrate inhibition and improving both biomass and DAPG concentrations at end of fed-batch. The major advantage of pH-based feeding strategy was that the feed addition rate slows down with decrease in growth rate due to metabolite inhibition. This pH-based feeding strategy resulted in very high cell count of about 2.4×10^{10} cfu/ml. In terms of the performance of the developed strategy, the fed-batch culture was found to be equivalent to approximately 20 conventional batch cultures as used by the industry.

10.4 Conclusion

Bio-inoculant formulations containing the culture broth of pseudomonads offer excellent combination of plant growth promotion and disease control. Most of the existing literature on bio-inoculants deals with genotype and phenotype characteristics of potential bio-inoculant microorganisms and enumeration of '*ai*' efficacy on host plants in glasshouse and field conditions. For large-scale applications of these bio-inoculants, they need to be cultivated in submerged culture for obtaining high cfu counts as well as the desired metabolites. This chapter has outlined the attempts that have been made at upstream (media development) and bioreactor engineering (mass cultivation through fed-batch

cultivation) components of bio-inoculant production supply chain based primarily on fluorescent pseudomonads. Although the focus microorganism was *Pseudomonas*, the challenges and rationale described here for carrying out such studies would help in designing submerged cultivation strategies for such extracellular PGPM in bio-inoculant development.

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Microbial Inoculants as Biofertilizers and Biopesticides

11

D.V. Pathak and Mukesh Kumar

Abstract

Bioinoculants are ecofriendly as they don't have any adverse effect on soil fauna and flora. These bioinoculants can also be used as biopesticides which do not have any residual effect on crop products. But the main problem with the bioinoculants is its quality, as the private agencies which supply various biofertilizers and biopesticides don't care for their quality parameters. The availability of good quality bioinoculants to the farmers is main hurdle in their success. There is lack of co-ordination between the extension workers and scientists. Due attention is needed regarding *Azotobacter*, *Azolla*, *Acetobacter*, *Trichoderma*, *Bacillus thuriengensis*, and *Azospirillum* and their application in various cereal and vegetable crops. These biofertilizers should be integrated with organic manures and chemical fertilizers to enhance the soil organic carbon and maintain sustainability in field and horticultural crops.

Keywords

Biofertilizers • *Azotobacter* • *Azospirillum* • Biopesticides • *Trichoderma* • *Bacillus*

11.1 Introduction

Agricultural productivity in Indian subcontinent has gained encouraging trends during last four decades. High-yielding variety seeds, availability of more water for irrigation, and enhanced use of chemical fertilizers have been the main factors for achieving high productivity. However, the

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pathway adopted by us has been dependent on nonrenewable energy resources, resulting in an exponential increase in the consumption of petroleum products. Urea is the main fertilizer being used across the globe in maximum quantities as compared to any other fertilizer. All the urea-manufacturing units depend upon petroleum products. According to an estimate, the manufacture, transportation, and application of one 1.0 kg urea involve an expenditure of 1.0 l petroleum products. Besides, an excessive use of urea to supplement nitrogen to the soil may render the groundwater polluted. Nitrate pollution in water may cause awful diseases like methemoglobinemia and hypertension among the infants, rendering them handicapped. In other words, excessive use of urea is not only expensive but also unsafe for human health and environment.

In view of sky rocketing population and growing grain demand, the necessity of intensive agriculture is likely to continue. Regular replenishment of plant nutrients to maintain the soil fertility is unavoidable. Consequently, any curtailment in the consumption of urea and other chemical fertilizers would not be feasible. In view of the necessity of intensive agriculture and keeping economy, health, and environment in mind, the need of the hour is to exploit all possible sources of plant nutrients so as to achieve the required productivity through intensive agriculture. Agriculturists suggest that the requirements of plant nutrients can be fulfilled only when the chemical fertilizers are judiciously used along with green manure, organic manure, and biofertilizers.

Biofertilizers are environment friendly, highly efficient, and low-cost agricultural inputs. The use of biofertilizers for various crops is, directly or indirectly, a true service to the soils of nation and the environment. Biofertilizers are mainly concerned with the nitrogen fixation in cereals and legume crops. Hence, to start with the biofertilizers, it is necessary to understand the mechanism of nitrogen fixation, so in the first part of the chapter, various aspects like biochemistry of nitrogen fixation, nodulation, and genetics of nodulation have been dealt with.

11.2 Biological Nitrogen Fixation

In the environment, nitrogen concentration is 78 % by volume; but the plant kingdom is unable to utilize it directly as the plant lacks the enzyme system required to convert N_2 into ammonia. Dinitrogen ($N \equiv N$) cannot be utilized as such because of the extremely stable triple-bonded structure of this gas and only certain prokaryotes have the utility to convert N_2 into ammonia with the help of nitrogenase system. Conversion of atmospheric elemental nitrogen into ammonia through a reductive process with the help of microbes is known as biological nitrogen fixation. These microbes include some eubacteria, blue-green algae, and actinomycetes (Table 11.1).

It was first discovered by Beijerinck in 1901 (Wagner 2012). In atmosphere the amount of free nitrogen present accounts to $4x10^{21}$gN out of which around 2.5×10^{11}kg NH_3 is fixed annually by biological means (Schlesinger 1991). In nature, 70 % of total nitrogen is fixed by biological means, the rest by chemical means and traces by physical means. Biological nitrogen fixation (BNF) is divided mainly in three groups: asymbiotic nitrogen fixation or free-living nitrogen fixers, associative nitrogen fixation, and symbiotic nitrogen fixation. The amount of nitrogen fixed by different modes has been shown in Table 11.2.

11.2.1 Asymbiotic Nitrogen Fixation

Free-living nitrogen fixers exist in the rhizosphere zone of plants. They take up carbon exudates from plants as nutrients and in return fix nitrogen under free-living state. *Azotobacter* is

Table 11.1 Nitrogen fixing microorganisms

Free living	Symbiotic
Azotobacter	*Rhizobium*
Azospirillum	*Azorhizobium*
Cyanobacteria	*Frankia*
Bacillus	*Acetobacter*
Clostridium	*Herbaspirillum*
Klebsiella	

Table 11.2 Amount of biological N_2 fixed by different inoculants

State	Aerobic/anaerobic	Bacteria	Amount of N_2 fixed Kg/ha/year
Free living	Anaerobic	*Clostridia*	2–5
	Aerobic	Azotobacter	10–20
	Facultative	*Klebsiella*	5–10
Associative	Legumes	*Rhizobia*	50–500
	Nonlegumes	*Azospirillum*	5–20
		Acetobacter	150
Blue green algae		*Anabaena*	20–25
		Azolla	70–100

the best example of this type which has potential to fix atmospheric nitrogen because it posses more than one type of nitrogenase enzyme. *A. chroococcum* possesses other properties like ammonia excretion (Narula et al. 1991), production of vitamins and growth substances (Shende et al. 1977; Martinez-Toledo et al. 1988), antifungal substances (Sharma et al. 1986), and siderospore production. All these properties favor its performance, increasing the biomass and grain yield of various crops (Lakshminarayana 1993; Goel et al. 1999). Other microorganisms involved in nitrogen fixation are *Clostridium*, *Rhodospirillum*, *Anabaena*, *Klebsiella*, and *Nostoc.*

11.2.2 Associative Nitrogen Fixation

Azospirillum, *Herbaspirillum*, and *Acetobacter diazotrophicus* are associated with the roots of Gramineae family. *Azospirillum* inoculation has sown marked effects on the seedlings of corn, wheat, sorghum, and other grasses. These bacteria can supply 20–25 % of total nitrogen requirements in rice and maize (Saikia and Jain 2007; Montanez et al. 2012). *Herbaspirillum* is beneficial to pearl millet. *Acetobacter diazotrophicus* is found to occur in the root and stem of sugarcane (Cavalcante and Dobereiner 1988; Gillis et al. 1989). It has high ability of nitrogen fixation which can fix up to 150 kg N/ha (Pathak et al. 1997).

11.2.3 Symbiotic Nitrogen Fixation

Rhizobium is the main contributor to the symbiotic nitrogen fixation in legume crops. Moore and Moore (1992) have divided it into four groups. They are fast-growing *Rhizobium*, six species; slow-growing *Bradyrhizobium*, a single species; *B. japonicum* and *Azorhizobium* (stem nodule forming), one species; and *Sinorhizobium*, two species (Table 11.3). On a global basis, of the total 17.2×10^7 tones of biologically fixed nitrogen, about 70–80 % is contributed by rhizobia in symbiosis (Ishizuka 1992). The details of nodulation, biochemistry, and genetics of nitrogen fixation also have been described in the chapter.

Azolla, a small, tree-floating aquatic fern, fixes nitrogen in association with nitrogen-fixing Cyanobacterium, *Anabaena azollae. Azolla* provides the suitable environment and nutrients to *Anabaena* in exchange of the fixed N and certain growth hormones. The heterocyst of symbiotic *Anabaena* is the site of nitrogen fixation. *Azolla* mainly contributes to rice crop by providing nitrogen and adding biomass to the soil.

Frankia, an actinomycete, is capable of forming nodules to actinorhizal plants, alders (*Alnus* sp.). The other genera which can be nodulated by *Frankia* include *Allocasuarina*, *Eleagnus*, *Myrica*, *Gymnostoma*, *Casuarina*, and *Coriaria*. All are monocots which have great future in agroforestry and land reclamation.

Table 11.3 Nodulation host range among legume strains

Group	*Rhizobium* spp.	Host
Rhizobium (fast growing)	*R. meliloti*	Alfalfa
	R. trifolii	Clover
	R. leguminosarum	Pea
	R. phaseoli	Bean
Bradyrhizobium (slow growing)	*B. japonicum*	Soyabean
	B. elkanii	Soyabean
Azorhizobium (fast growing)	*A. Caulinodans*	Sesbania (root and stem nodules)

11.2.4 Nodulation Process of *Rhizobium*

First of all, the legumes secrete root exudates in their vicinity to which host-specific rhizobia are attracted. This is followed by root hair curling and invasion of root hair by host-specific rhizobia. Indole acetic acid (IAA) and lectins are possibly concerned in this process. Following the microbial penetration into the root hair, a hyphae-like infection thread is formed. The bacteria are released into the cortical region of root system. Following the release, a period of rapid cell division takes place in the host cells. The cortical cells into the nodule region become tetraploid. Efficient nodules are pink in color due to the presence of leghemoglobin. The nodules are rounded, lobed, or club shaped depending upon the host. Infection thread branch and distribute themselves over the tetraploid cells. The root nodule results from tissue proliferation induced by the rhizobia via growth hormones. Once liberated from the infection thread, rhizobia assume a peculiar morphology, called bacteriods. These bacteroids proliferate rapidly and are irregularly shaped.

The root nodules formed by the bacteria on legumes fix atmospheric nitrogen and fulfill the nitrogen requirements of leguminous plants. Nodules are formed by an efficient strain of *Rhizobium* to meet the whole nitrogen requirement of the plant, and there is no need to supply nitrogen by other means. The legumes excrete excess amount of organic nitrogen into the soil to nourish the succeeding crop. In return to nitrogen fixation, the bacteria get protection and proper conditions for growth and photosynthate as source of energy. It may be mentioned here that neither the bacterium nor the plant can fix atmospheric nitrogen independently. Nitrogen fixation by different legume crops has been listed in Table 11.4.

Table 11.4 Nitrogen fixation by legumes

System	Nitrogen fixed (kg/ha/year)
Alfalfa	113–297
Red clover	75–171
Pea	72–132
Soybean	57–105
Cowpea	57–117
Vetch	79–140
Sesbania	80–100

11.2.5 Root Nodules in Nonleguminous Plants

Many higher plants, which are not members of leguminosae, also form root nodules with the ability to fix nitrogen. In most cases, these endosymbionts are actinomycetes belonging to genus *Frankia.* The host plant of such actinomycetes includes *Casuarina*, *Albus*, *Myrica*, *Dryas*, etc.

11.2.6 Biochemistry of Nitrogen Fixation

Nodules are generally pink in color because of the presence of an iron containing substance known as leghaemoglobin. Neither the plant nor the bacterium is individually capable of leghaemoglobin synthesis. The apoprotein globin is

encoded by a plant gene, and the synthesis of heme moiety is under the control of bacterial genes. Throughout the period during which the bacteriods persist, they actively fix atmospheric nitrogen. The reductant and ATP necessary for nitrogen reduction are derived from photosynthates provided by plants. The fixed nitrogen is excreted from the nodules to the plant vascular system as ammonia. About 15–20 mol of ATP are hydrolased per mole of ammonia fixed. It is provided by aerobic respiration within the bacteriods. Ammonia formed reacts with α-ketoglutarate to form glutamate which may be further converted into glutamine. Similarly, aspartate combines with ammonia to form asparagine. Various other products which are synthesized include glutamine, aspartate, and ureides like allantoin and allantoic acid, subsequently transported to plant tissues. Various steps involved in the nitrogen fixation have been mentioned in Fig. 11.1.

The most important plant bacterial interaction is that between legume plants and bacteria of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*. *Azorhizobium* forms stem nodules. In the nodules, precise oxygen levels are controlled by the oxygen-binding protein leghaemoglobin which functions as an oxygen buffer cycling between the oxidized ferric ions and reduced ferrous ions. These forms keep free oxygen levels within the nodule at a low but constant level. The ratio of free leghaemoglobin to bound form to oxygen in the root nodule is in the order of 10000:1.

Bacteriods are totally dependent on plants for supplying them energy sources for nitrogen fixation. The major organic compounds transported across the peri-bacterial membrane are citric acid cycle intermediates, in particular the C_4 acids succinate, malate, and fumarate. They are used as electron donors for ATP production and are converted into pyruvate. Ammonia is transported from bacteroid to plant cell and is assimilated to glutamine by glutamine synthetase enzyme by the plant and subsequently transported to plant tissue.

11.2.7 Genetics of Nodule Formation: *Nod* Genes

Nodulation in legumes by host-specific rhizobia is directed by a number of genes which are called *nod* genes. These are highly conserved and localized on large plasmid called *sym plasmid*. Cross-inoculation group specificity is controlled by *nod* genes. The *nod ABC* genes are common to all spe-

$$\text{Glutamate+ATP+NH}_3 \longrightarrow \text{glutamine+ADP+Pi}$$

Glutamine synthetase(GS)

$$\text{Glutamine+}\alpha\text{–ketoglutarate+NAD(P)H} \rightleftharpoons 2\ \text{glutamate+NAD(P)}^+$$

Glutamate Synthase (GOGAT)

$$\alpha\text{–ketoglutarate+NH}_3\text{+NAD(P)H+ATP}$$

$\downarrow$ GS/GOGAT or glutamate dehydrogenase

$$\text{Glutamate+NAD(P)}^+\text{+ADP+Pi}$$

$$\text{N}\equiv\text{N} \xrightarrow[2\text{H}]{} \text{HN=NH} \xrightarrow[2\text{H}]{} \text{H}_2\text{N-NH}_2 \xrightarrow[2\text{H}]{} 2\text{NH}_3$$

Overall reaction

$$8\text{H}^+ + 8\text{e}^- + \text{N}_2 \longrightarrow 2\text{NH}_3 + \text{H}_2$$

$$\text{18-24 ATP} \longrightarrow \text{18-24 (ADP+Pi)}$$

Fig. 11.1 Biochemistry of nitrogen fixation

cies of *Rhizobium* and are involved in the production of chitin-like molecules, called nod factors, which induce root hair curling and trigger cortical plant cell division. Nod factors consist of a backbone of N-acetyl-glucosamine to which various substituent are linked. *Nif* genes complex regulate the nitrogenase enzyme synthesis (Fig. 11.2).

11.2.8 Nitrogenase

In the fixation process, nitrogen is reduced to ammonia and ammonia is converted to organic form. The reduction process is catalyzed by the enzyme complex called nitrogenase, which consist of two separate proteins called dinitrogenase and dinitrogenase reductase. Dinitrogenase is the Mo-Fe protein, while dinitrogenase reductase is Fe protein. Some nitrogen-fixing bacteria can synthesize nitrogenase that lack molybdenum but contain vanadium.

11.3 What Are Biofertilizers?

All the microorganisms which add or make available different nutrients to the plants are called biofertilizers. These biofertilizers differ from the chemical fertilizers as the chemical fertilizers are manufactured in the factories and are direct source of nutrients, while the biofertilizers are the living or latent form of microorganisms which either mobilize different elements fixed in the soil or add nutrients from the environment to the soil. They also provide plant growth hormones and induce the plant protection mechanism and thus help them from plant pathogens. These biofertilizers improve the soil fertility by fixing atmospheric nitrogen, mineralization of various elements like phosphorus, sulfur, zinc, potash, and iron. These biofertilizers are also known as inoculants which are produced either on small scale under laboratory conditions or on large scale by batch fermentation (Hilda and Fraga 2000). The use of inoculants is ecofriendly and is not harmful to the environment (Rodríguez and Fraga 1999). Biofertilizers may be applied to the soil through seeds, roots, or directly to soil where microbes multiply and mobilize the inert nutrients. Commonly used biofertilizers which are made available to farmers by the government, semi-government, or private agencies have been mentioned in Table 11.5. The media used for commercialized production of bioinoculants is listed in Table 11.6.

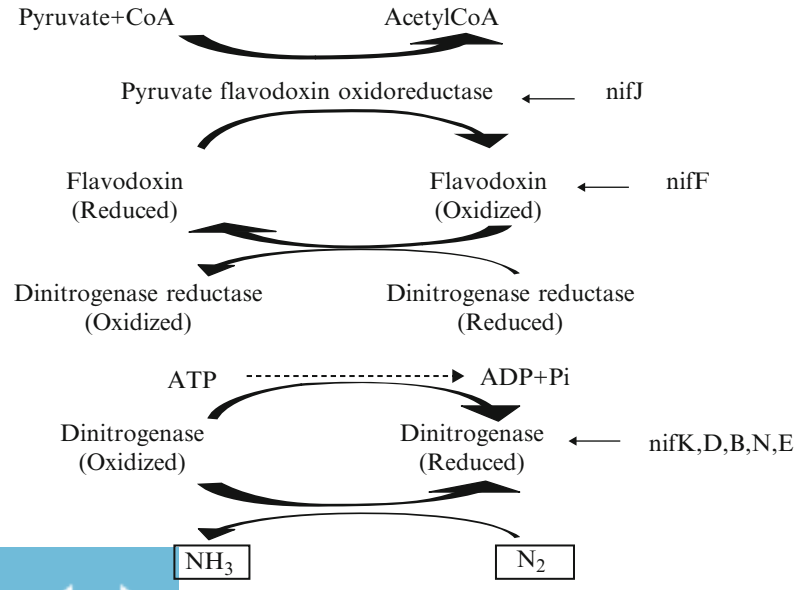

Fig. 11.2 Genetics of nitrogen fixation

Table 11.5 Types of biofertilizers commonly used

Sr No	Biofertilizers	Character specification requirement
a.	*Rhizobium*	Should show effective nodulation on all the species listed on the packet
b.	*Azotobacter*	The strain should be capable of fixing at least 10 mg of nitrogen per g of sucrose consumed
c.	*Azospirillum*	Formation of white pellicle in semisolid N-free bromothymol blue media
d.	PSB	The strain should have phosphate-solubilizing capacity in the range of minimum 30 %, when tested spectrophotometrically. In terms of zone formation, minimum 5 mm solubilization zone in prescribed media having at least 3 mm thickness

Table 11.6 Media for large-scale production

Bacteria	*Media*	*C- source*
Rhizobium	*YEMA*	*Mannitol or molasses, sugar, and glycerol*
Azotobacter	*Jenson*	*Sucrose or mannitol*
Azospirillum	*Malate or Okon's*	*Malate as C-source + yeast extract as vitamin source*
PSB	*Pikovaskaya's*	*Glucose as C-source + tricalcium phosphate*

11.3.1 Types of Biofertilizers/ Biopesticides

Biofertilizers/biopesticides can be generally categorized into four types

(a) Nitrogen supplementing
(b) Phosphate solubilizing
(c) Composting microorganisms
(d) Biopesticides/PGPRs

(a) *Nitrogen-supplementing microorganisms*

These microorganisms have the capability of fixing atmospheric nitrogen which is 78 % of the atmosphere. Most of the plants can utilize nitrogen only in the form of nitrate; hence, unless the nitrogen gas is converted to nitrate, it remains unavailable for plants. Certain microorganisms absorb nitrogen gas as their feed and convert it into ammonia through the activity of an enzyme called nitrogenase. Ammonia is converted into nitrate by nitrification or directly assimilated into the plant system.

11.3.1.1 *Rhizobium*

This bacterium fixes atmospheric nitrogen in the symbiotic association with the leguminous crops. *Rhizobium* enters the root system after germination of seeds and nodules are developed on the roots. These nodules inhabit rhizobia, which fix atmospheric nitrogen and keep supplying ammonia to the plant. Rhizobia are host specific as they form nodules and fix nitrogen on specific hosts. Hence, while procuring *Rhizobium* culture, it should be taken care of that name of the pulse crop should be mentioned on the culture for which it is used.

Benefited crops: Soybean, groundnut, berseem, sesbania, and all other pulse crops

Selection Criteria for *Rhizobium* and *Bradyrhizobium*

- Host specificity
- Nitrogen fixation potential
- Adaptation in different environments and soil conditions
- Competance with native *Rhizobium*
- Production of siderophores, auxins, vitamins, and other PGPS
- Production of bacteriocins and other secondary metabolites

11.3.1.2 *Azotobacter*

These bacteria fix atmospheric nitrogen in free-living conditions. They multiply in the vicinity of the root system and convert atmospheric nitrogen to ammonia. Plants assimilate the fixed nitrogen. The capability of fixing nitrogen in free-living conditions accredited to *Azotobacter* as a versa-

tile biofertilizer which can be successfully utilized against a broad range of crops belonging to different groups for supplementing chemical nitrogen. In addition to fixing nitrogen, they also produce plant growth-regulating substances in the vicinity of the plant.

Benefited crops: Wheat, maize, sorghum, pearl millet, mustard, sunflower, cotton, fruits, and flowers yielding crops, tea, coffee, vegetables, etc.

Selection Criteria for *Azotobacter* and *Azospirillum*

- Fix higher amount of N/g of C substrate in growth medium
- Excretion of ammonia
- Faster growth rates, survival, and competence in soil environment
- Tolerance of wider pH and temperature range
- Antibiosis and phosphate dissolving ability

11.3.1.3 *Acetobacter*

Similar to *Azotobacter*, this bacteria also multiply in the soil and fix nitrogen in aerial as well as underground parts of the plant. Most common sp. is *A. diazotrophicus* fixing nitrogen in sugarcane. Various field studies revealed that *Acetobacter* works more efficiently for sugar-yielding crops like sugarcane and sugar beet. It has been estimated that approximately one-fourth of total nitrogen requirement of sugar-yielding crops can be fulfilled by these bacteria. These bacteria are endosymbiont as they remain within the plant.

Benefited crops: Sugarcane, sugar beet, and pearl millet

(b) *Phosphate solubilizing microorganisms (PSM)*

Phosphate is the second most important plant nutrient. In general, chemical phosphatic fertilizers are used to supplement phosphates to the soil. Experiments have proved that 30–35 % of phosphatic fertilizers applied are actually utilized by the plants, while the remaining 65–70 % of chemical phosphatic fertilizer change to insoluble state and become unavailable to the plants. Certain microorganisms have the capability of resolubilizing this insoluble phosphate, making it available to the plants. PSM is a balanced blend of certain efficient phosphate-solubilizing microorganisms which work under diverse geographical conditions (Table 11.7). Since PSM has the capability of working in various types of soils under free-living conditions, this biofertilizer can be utilized against all the crops with equal efficiency. *Aspergillus* sp., soilborne fungi, is serving as an important phosphate solubilizer of the soil (Arcand and Schneider 2006). These fungi are capable of solubilizing both organic and rock phosphates; co-inoculation of these fungi will enhance the availability of phosphates to plants and in turn will reduce the requirement of synthetic fertilizers. *Aspergillus niger* also serves as phosphate-solubilizing fungi as it causes production of various organic acids like citric, gluconic, succinic, and oxalic acids and thus helps in pH drop (Nahas et al. 1990). Other than fungus, some bacteria are also involved in phosphate solubilization which are known as phosphate-solubilizing bacteria (PSB) or phosphotika, e.g., *Bacillus*, *Pseudomonas*.

Selection Criteria for Phosphate Solubilizers

- Ability to solubilize insoluble rock phosphate and tricalcium phosphate in liquid medium
- Production of organic acids, e.g., mono-, di-, tri-carbonic acids and gluconic acid

(c) *Composting microorganisms*

The use of compost and farm yard manure to replenish the nutrients in the soil is prevailing since ancient times. Dead leaves, plant parts, and other agricultural trash have got sufficient plant nutrients, but these are unavailable to crop plants

Table 11.7 Phosphate solubilizers

Bacteria and fungi	Mycorrhizal fungi	
Produces acidic metabolites	Endo	Ecto
Caused chelation of metal cation	*Mucor*	*Aminita*
Change the soil pH	*Glomus*	*Boletus*
Phosphate ion is released in soluble form		
Bacteria – *Bacillus, Pseudomonas*		
Fungi – *Aspergillus, Penicillin*		

unless their complex form is changed to simpler form through microbial decomposition. This process of decomposition is known as composting and involves specific microorganisms[1]. Composting microorganisms are available in the atmosphere and continue decomposing the dead organic matter. In case the population of efficient composing microorganisms is increased over the heap of agriculture waste, the process of composting becomes faster, and a good quality compost or organic manure is prepared in merely one-fourth time as compared to natural composting. The organic manure so obtained carries almost all the required plant nutrients in balanced quantities. The organic manure preparation can be fastened by the use of *Trichoderma*, *Penicillium*, and *Aspergillus*.

11.3.1.4 Urea-Coating Agents (UCA)

Nitrogen deficiency in soil is generally replenished by application of urea, but approximately 30 % is actually utilized by the plants, while the remaining 70 % either leaches down to groundwater or volatilized back to atmosphere. Immediately, after its application, urea tends to break into nitrates. This process is known as nitrification, which is much quicker than the nitrate assimilation by the plants. Consequently about 70 % of urea goes to waste and causes pollution. The mode of application of biofertilizers affects their quantity used as given in Tables 11.8 and 11.9.

(d) *Biopesticide/PGPRs*

We are aware of the losses due to certain fungal diseases in various crops. Generally, chemical fungicides are used to combat the fungal diseases. These poisonous chemicals persist in the environment for a long time and impose a slow but harmful effect on living beings and ultimately on human health. Biopesticides include bacteria, fungi, and plant viruses.

11.3.1.5 Bacteria

The bacteria which promote plant growth either by production of plant growth hormones or due to induction of plant protection mechanism have been designated as plant growth-promoting rhizobacteria (PGPR) by Kloepper et al. (1980). Various bacteria which have been identified as PGPRs in recent years include *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus*, and *Serratia* (Kloepper et al. 1989; Okon and Labandera-Gonzalez 1994; Glick 1995; Joseph et al. 2007). These bacteria have been commercialized by the production of their inoculants. They promote plant growth by different mechanisms that include suppression of plant disease (biopesticides), biofertilizers, or phytohormone production (biostimulants). The biopesticides protect the plant system by different mechanisms: induction of systemic (ISR), resistance synthesis of antibiotics, and production of siderophores. The microorganisms which produce siderophores chelate iron, thus making it unavailable to plant pathogen and thus suppress growth of plant pathogen. Induced systemic resistance is effective against a broad spectrum of plant pathogen (Pieterse et al. 2003).

Different strains of *Pseudomonas* serve as effective PGPRs due to their wide range of properties, viz., production of phytohormones (Timmusk et al. 1999; Verma et al. 2001; Bottini et al. 2004; Spaepen et al. 2008); phosphate solubilization (Vyas and Gulati 2009); sideropores production; production of antibiotics like 2,4-diacetylphoroglucinol (2,4-DAPG), phenazines, pyrrolnitrin, pyoluteorin, and surface-active antibiotics; and production of hydrogen cyanide (HCN) (Raaijmakers et al. 2002) and lytic enzymes like chitinases and proteoses (Haas and Defago 2005; Yadav et al. 2007). *Pseudomonas* also produces enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase which regulates ethylene level in plants helpful in protection from plant pathogens (Glick et al. 1998; Penrose and Glick 2003).

The soil bacterium, *Bacillus thuringiensis* (*Bt*), is currently being used worldwide, mainly for management of lepidopterous, coleopterous, and dipterous pests. The insecticidal activity of Bt is primarily due to the presence of proteinaceous crystals (delta endotoxins) pro-

[1] Composting culture –1 kg for 2–3 metric ton of agricultural waste.

Table 11.8 Doses of various biofertilizers for different crops

Target crops	Seed treatment (g/kg)		Soil application (kg/ha)	
	Trichoderma	*Acetobacter*	*Trichoderma*	*Acetobacter*
All pulses crops soybean, groundnut, mung, urd, lentil, pea, gram, etc.	4–5	–	2.5	–
A. Cereals, millets oilseed, wheat, jowar, bajra, mustard, and sunflower etc.	4–5	–	2.5	–
B. Cash crops (sugarcane, potato) vegetables and fruit crops	4–5	–	2.5	–
Sugarcane and sweet potato	–	2.5 kg	2.5 kg	2.5 kg

Table 11.9 Doses of various biofertilizers for different crops

Target crops	Seed treatment			Soil application		
	Rhizobium	*Azotobacter*	PSB	*Rhizobium*	*Azotobacter*	PSB
All pulses crops like soybean, groundnut mung, urd, lentil, pea gram, etc.	50 ml/10 kg seed	–	50 ml/10 kg seed	1.5 l	–	
A. Cereals, millets oilseed, wheat, jowar, bajra, mustard, and sunflower etc.	–	50 ml/10 kg seed For large seed crop and 50 ml per acre for small seed crop	Do	–	2 l liquid culture	2 l liquid culture
B. Cash crops {sugarcane, potato, vegetables, and fruit crops}	–	1.5 l	1.5 l liquid culture	–	2 l	3 l

Urea-coating agent (UCA)[b] is a balanced blend of certain herbs and minerals which inhibits the process of nitrification, resulting in slow release and more assimilation of urea by the plants. It is estimated that 40–50 % saving of urea can be achieved by coating the urea granules before application[b] UCA –1 kg/50 kg urea bag

duced during stationary and sporulating phases. In commercial production, the spores and crystals obtained from fermentation broth are concentrated and formulated variously. Upon ingestion of spores, crystals dissolve in the alkaline pH of the midgut larvae and protoxins of size 120–135 Kda are released which are further acted upon by the midgut proteolytic enzymes, and toxin fragments of size 60–70 Kda are released. These toxin fragments negotiate the receptors found in the columnar epithelial cells and cause pore formation, resulting in osmotic imbalance and eventually death of the insects.

11.3.1.6 Mode of Action of PGPRs

- Production of auxins
- Production of vitamins
- Production of siderophores
- Production of antibiotic substances
- Promoting plant defense mechanism by inducing flavonoids and phytoalexins

- *Acetobacter, Arthrobacter, Alcaligenes, Azospirillum, Bacillus, Pseudomonas, Flavobacterium*

11.3.1.7 Fungi

Fungi play an important role in the recycling of organic matter. These include nonpathogenic soil inhibiting saprophytes. They degrade cellulose, lignin, and hemicelluloses and thus mineralize the organic matter and help in soil aggregation. They also solubilize organic phosphates, e.g., *Alternaria*, *Aspergillus*, *Cladosporium*, *Dematium*, *Gliocladium*, *Helminthosporium*, *Humicola*, and *Metarhizium*. Some fungi promote plant growth by root colonization and are designated as plant growth-promoting fungi (PGPF). These include mycorrhiza (endomycorrhiza and ectomycorrhiza). Mycorrhiza increases the surface area of plant root system and thus helps in absorption of minerals, solubilization of phosphorus, and conversion of moisture. Due to abovementioned properties, it has been commercialized as inoculants.

Over 400 species of fungi infect insects and mites. *Deuteromycetes* and *Phycomycetes* contain most of the useful species for insect control. The entomopathogenic fungi have relatively broad host range and are amenable for mass production. The fungi penetrate through the insect cuticle and sporulate on the dried insects, which provide the way for epizootics. However, fungi are fairly fastidious with respect to humidity and temperature. In order to make effective use of a fungus, applying it at the right time and optimum amount is important for the successful management of insect pest on crops.

Trichoderma is a specific fungus having characteristic capability of inhibiting the growth of a broad range of pathogenic fungal species. Due to being biological, this bio-fungicide has got no adverse effect on the environment. Application of *Trichoderma* is known to prevent various diseases like stem and root rot, damping off, wilt, blight, and other diseases of leaves.

11.3.1.8 Viruses

Many of the commercial bioinsecticides are based on nuclear polyhedrosis viruses (NPVs) and to a lesser extent of granulosis viruses (GVS) and non-occluded viruses (NOVs). These viruses are highly host specific and safer to nontarget organisms including humans.

Upon ingestion of the viral particles, the polyhedron dissolves in the alkaline pH of the midgut, releasing virions. The virions enter the columnar epithelial cells through endocytosis and cause primary infection. Here the secondary infection takes place, ultimately causing death of the insect.

11.4 Constraints in Popularization of Biofertilizer Technology

- The quality of inoculants
- The lack of knowledge about the inoculation technology for the extension personnel and the farmers
- Ineffective inoculant delivery system
- Nonavailability of formulations to the farmers

11.5 Conclusion

The indiscriminate use of chemical fertilizers and pesticides has caused serious damage to the ecosystem; hence, it becomes imperative to turn to more ecofriendly methods of pest and nutrient management. Biofertilizers and biopesticides which are microbial in origin can become viable alternative to sustainable agriculture, although biofertilizers can't complement to chemical fertilizers but can become supplementary to them for maintaining soil health and crop productivity. Therefore, development of newer ecofriendly technology for pest and nutrient management is need of the hour. It is equally important to maintain the quality of biofertilizers and biopesticides. Timely delivery of these organic amendments and awareness to the farmers will help in the improvement of quality and quantity of food products. Biofertilizers and biopesticides are our tools to achieve the goals of not only higher yield but also a cleaner environment. Hence, an integrated approach of sci-

entists and extension workers should be followed for their success.

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Seed Bio-priming for Biotic and Abiotic Stress Management

12

S. Rajendra Prasad, Umesh R. Kamble, K.V. Sripathy, K. Udaya Bhaskar, and D.P. Singh

Abstract

In modern agriculture, advance technologies are being deployed for breaking yield barriers and enhancing crop productivity. Devising varied seed enhancement technologies is an important domain assuring uniform field emergence, better crop stand and realisation of higher yield in different crops. Integration of diverse plant extracts, microbial products and biotic agents through bio-priming for managing seed crop targeting against biotic and abiotic stresses has been considered as a unique approach, as it requires lesser amounts of chemicals, enhances efficacy of the seeds, reduces the cost of management and eliminates pollution hazards while causing minimum interference with biological equilibrium. Seed bio-priming is one of the vital seed enhancement tool in management of biotic as well as abiotic stresses and guarantees uniform stand establishment under stress conditions. Therefore, research programmes encompassing identification and genetic manipulations of novel biocontrol agents (fungal and bacterial strains) along with its commercial application needs to be devised.

Keywords

Bio-priming • Rhizosphere • Induced systemic resistance (ISR) • Systemic acquired resistance (SAR)

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12.1 Introduction

Seed is a growth driver of agriculture and efficacy of all other agricultural inputs, viz. irrigation, fertilisers and plant protectants, and human labour revolves around the use of quality seed. Seed is a tool for delivery of improved technolo-

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gies and is a mirror for portrayal of inherent genetic potential of a variety/hybrid. Seed offers to integrate production, protection and quality enhancement technologies through a single entity, in a cost-effective way. Seed can play a pivotal role in achieving higher productivity; the use of quality seeds alone could increase productivity by 15–20 % which highlights the important role of seed in agriculture.

In modern agriculture, advance technologies are being deployed for breaking yield barriers and enhancing crop productivity. Devising varied seed enhancement technologies is an important domain assuring uniform field emergence, better crop stand and realisation of higher yield in various crops. The quality of seed can be enhanced by different methods, viz. physical, mechanical, chemical and physiological seed treatments. Seed enhancements may be defined as "postharvest treatments that improve germination or seedling growth or facilitate the delivery of seeds and other materials required at the time of sowing". Seed priming is a technique of controlled hydration (soaking in water) and drying that result in more rapid germination when the seeds are reimbibed. There are different methods of priming like hydropriming, halopriming, thermopriming, bio-priming, etc. Numerous invigoration protocols as well as seed coating and pelleting technologies are used for enhancing planting value and storability of high value and poor storer seeds. Seed quality enhancement through second-generation drying, packing and quality enhancement technologies, viz. intelligent coating molecules, time and target-oriented seed additives, electron treatment, magnetic treatment, plasma coating and its commercial application holds the promise to deliver seeds with high vigour and better adaptability to biotic and abiotic stress. Use of third-generation seed quality augmentation strategies viz., nanotechnology for external as well as internal designing has unlocked new avenues in precision agriculture. Different types of seed enhancement technologies are being developed and deployed for seed invigoration and biotic as well as abiotic stress management.

12.2 Seed Enhancement Technologies

Any postharvest treatment that improves germination/seedling emergence or facilitates the development of more number of normal, rapid, uniform and healthy seedlings in the field condition is termed as seed enhancement. Various environmental factors can be circumvented by using seed enhancement techniques, viz. seed invigoration (priming), coating and pelleting.

12.2.1 Seed Invigoration or Priming

Seed invigoration or priming is a treatment, in which seeds are soaked in an osmotic solution/other solutions containing different active ingredients, that allows water imbibitions and permits early stages of germination but does not permit radical protrusion through the seed coat.

12.2.2 Osmopriming

Soaking the seed in osmotic solutions is osmopriming. Water is either made freely available to the seed (as in steeping or soaking) or restricted to a pre determined moisture contents, typically using water potential between – 0.5 Mpa and −2.0 Mpa. Several osmotica like inorganic salts such as potassium nitrate, potassium phosphate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, magnesium sulphate, magnesium chloride, calcium chloride, sodium chloride, sodium nitrate, sodium polypropionate, sodium sulphate, chemically inert compounds such as PEG 6000, PEG 8000 and mannitol are used. Details of different osmoticum used for priming in vegetable seeds are given in Table 12.1.

12.2.3 Solid Matrix Priming

Pre-sowing hydration in a solid-based medium is called solid-based matrix priming, and it is used for increasing the efficiency of fungicide/insecticide to control the seed-borne infection and soil

Table 12.1 Effect of osmotic seed priming in different crop species

Crops	Osmoticum	Results	References
Cabbage	PEG 305 g/kg seed 15 °C for 14 days	Accelerated emergence in heat-damaged seed	Ralph (1978)
Carrot	PEG 273 g/kg seed 15 °C for 14 days	Accelerated germination, field emergence and increased plant fresh weight	Broklehurst and Dearman (1983)

Table 12.2 Polymer film coating with reference to storage potential of seed

Crop	Finding	Reference
Turnip, carrot and cabbage	Coating seed with polyvinyl resin didn't decrease germination consistently after 18 months from storage	Sauve and Shiel (1980)
Tomato	Seed treated with Vitavax Power at 2 g + polymer coating at 20 ml per kg of seeds enhanced seed quality attributes and storability	Harish et al. (2014)

insects. In solid matrix priming seed slowly imbibes to reach an equilibrium hydration level, determined by the reduced matrix potential of the water adsorbed on the particle surfaces.

12.2.4 Seed Hardening

It is a process of soaking seeds in water for a precise period followed by drying, re-soaking and re-drying. This process of alternate hydration and dehydration cycles with water and later drying to original moisture is called seed hardening.

12.2.5 Seed Coating

Seed coating in broad sense includes seed film coating, seed colouring and seed pelleting. Details of the use of different chemicals for seed coating are given in Table 12.2.

12.2.6 Seed Pelleting

Seed pelleting is the mechanism of applying needed materials in such a way that they influence the seed or soil and the seed-soil interface. Pelleting is defined as the application of a layer of inert material that may obscure the original shape and size of the seed resulting in significant weight increase and improved palatability. These treatments are used to facilitate easy handling, precision placement and incorporation of beneficial microorganism. Seed pelleting is usually practised in seeds which are light in weight and irregular in shape. The largest commercial use of pelleting is for monger sugar beet, carrot, onion, lettuce, tomato and flower spp.

12.3 Bio-priming

Bio-priming is a process of biological seed treatment that refers to a combination of seed hydration and inoculation of the seeds with beneficial microorganisms. It improves seed viability, germination, vigour indices, plant growth and subsequent protection against diseases and finally enhances crop yield. In most of the cases microbial inoculants such as plant growth-promoting rhizo-microorganisms (bacteria or fungi) are used for the purpose of bio-priming of seeds. It is an environmentally sound ecological approach using selected microorganisms which enhance plant growth by producing plant growth-promoting substances or enhancing nutrient uptake or by protecting seedling/plants against soil-/seed-borne plant pathogenic organisms. In present-day agriculture, the biological seed treatment methods using microbial inoculants are providing an alternative to the chemical treatment methods (use of pesticides and/or plant growth-promoting nutrients), being eco-friendly and safer for future agriculture and gaining importance in the seed, plant and soil health improvement programmes.

Crop productivity in India suffers heavy loss due to diseases under field and storage conditions, and a majority of them are seed and soil borne in nature. Chemicals are being used so far to treat the seeds which are not effective under field conditions due to various soil and environmental factors. Moreover, chemicals used for seed treatment mostly act as contact fungicides which are unable to protect the plants from foliar pathogens during the later stages of crop growth. Seed bio-priming is a suitable alternative to seed treatment because the microbes multiply continuously, occupy the growing root surfaces, form a biofilm around the roots and protect the plants from soil-borne plant pathogens throughout the crop-growing stages. Other advantages using microbial bio-priming are the elicitation of systemic resistance in plants that can protect the plants from foliar pathogens during the later stages of their growth and development. This further strengthens the concept of popularising seed bio-priming technique among the farmers which will not only ensure seed and crop health but also help in ensuring ecological sustainability. Alternatively, seed bio-priming can also enhance seed's nutritional and physiological characteristics and result in better germination and adaptation under different soil conditions and when entwined with useful microbial agents associated with plant roots can augment plant productivity and immunity. However, recent work by several groups showed that such microorganisms also elicit so-called induced systemic tolerance (IST) against biotic and abiotic stresses.

Most of these microorganisms increase nutrient uptake from soils, thus reducing the need for fertilisers and preventing the accumulation of nitrates and phosphates in agricultural soils. A reduction in fertiliser use would lessen the effects of water contamination from fertiliser run-off and lead to savings for farmers in addition to impart drought tolerance capacity to plants. The investigators of the present project have been engaged with the isolation, trait characterisation and effective utilisation of several groups of microorganisms that are capable of promoting plant growth and suppressing various seed- and soil-borne diseases as well as foliar disease through induced systemic resistance mechanisms and can withstand high temperature, pH and salt concentrations.

Integration of plant extracts, microbial products and biotic agents along with bio-priming agents for managing plant growth and diseases has been considered as a novel approach as it requires low amounts of chemicals, enhances efficacy of the seeds, reduces the cost of control and eliminates pollution hazards while causing minimum interference with biological equilibrium. The use of bioagents, microbial metabolites or botanicals with priming agents has become an inevitable method of disease control, particularly in the absence of resistant cultivars.

12.3.1 Methodology

The method commonly recommended for bio-priming is to soak the seeds in water for 12 h. Selected formulated product of the microorganism is added to pre-soaked seeds at the rate of 10 g/kg of seed and mixed well. The treated seeds can be taken in polythene bags, heaped and covered with moist jute sack to maintain high humidity and maintained for 48 h at approximately 25–32 °C. During this period, the bioagent adhering to the seed grows on the seed surface to form a protective layer all around the seed coat. These bio-primed seeds can be sown in the nursery bed. Some studies have shown that bio-primed seeds can be safely stored up to 2 months. The microorganisms that have been commonly studied for this purpose include *Bacillus polymyxa*, *Pseudomonas fluorescens*, *Trichoderma harzianum*, *T. viride and Gliocladium* sp. These studies have clearly brought out that bio-primed seeds enhance *percent* germination, seed vigour, plant growth, yield and protection against seed- and soil-borne pathogens in crops like rice, sunflower, rape and several vegetable crops like carrot, radish, etc. Some studies revealed that bio-priming with more than one organism like *Trichoderma harzianum* with *Pseudomonas fluorescens* is more effective in enhancing plant growth compared to bio-priming with single organism. Similarly some workers have brought out that

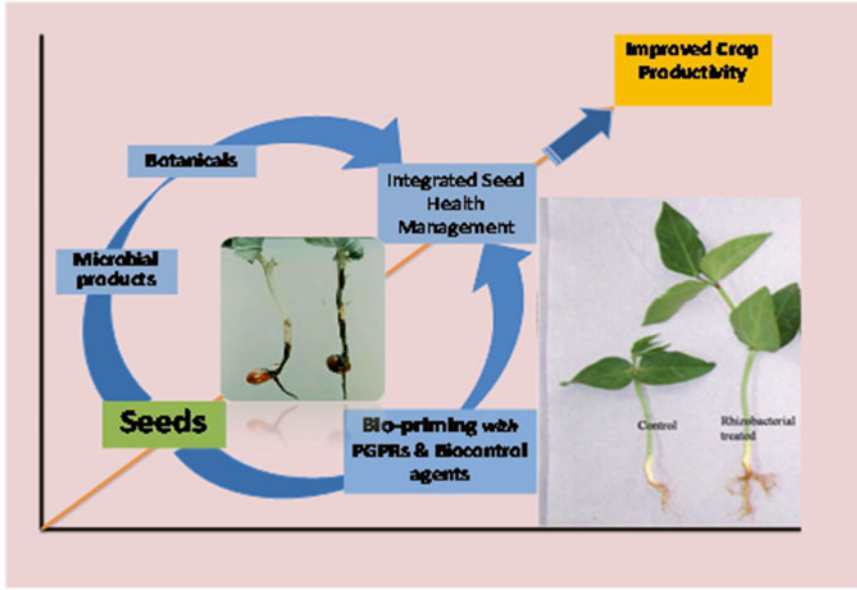

Fig. 12.1 Improving the crop productivity by the application of bio-priming agents

bio-priming along with osmopriming (with NaCl) is more effective in improving seed invigoration and seedling growth. Bio-priming process has potential advantages over simple seed coating with bioagents and results in more rapid and uniform seedling emergence even under adverse soil conditions (Fig. 12.1).

12.3.2 Signal Pathways of Induced Systemic Resistance (ISR) and Systemic Acquired Resistance (SAR)

Biocontrol agents, particularly rhizobacteria, have been shown to be effective in suppressing disease infection by inducing a resistance mechanism called "induced systemic resistance" (ISR) in varied crops (Van Loon et al. 1998). Induced resistance is defined as stimulation of plants with enhanced defensive ability of plants against different pathogens. Van Peer et al. (1991) showed that inoculation of *Pseudomonas fluorescens* strain WCS417r in the stem of carnation resulted in low infection of Fusarium wilt. This low level of Fusarium wilt was attributed to the induced resistance and deposition of phytoalexins in the stem region of carnation plant. Similarly, Wei et al. (1991) demonstrated that seed treatment with PGPR strains in cucumber resulted in reduction of anthracnose disease and further suggested that application of PGPR strains to seeds triggered induced systemic resistance, protecting leaves of cucumber plants against anthracnose disease caused by *Colletotrichum orbiculare*.

Beneduzi et al. (2012) stated that rhizobacteria-regulated induced systemic resistance and plant pathogen-induced systemic acquired resistance are almost regulated through same signal transduction pathway (Fig. 12.2). In case of ISR, jasmonic acid (JA) and ethylene (ET) responsive pathways are involved in defensive response of plants, whereas in case of SAR, salicylic acid pathway is vital to activate defence mechanism against pathogens. Both types of mechanisms are effective in ensuring protection to plants from various plant pathogens *viz*., fungus, bacteria, nematodes and insects.

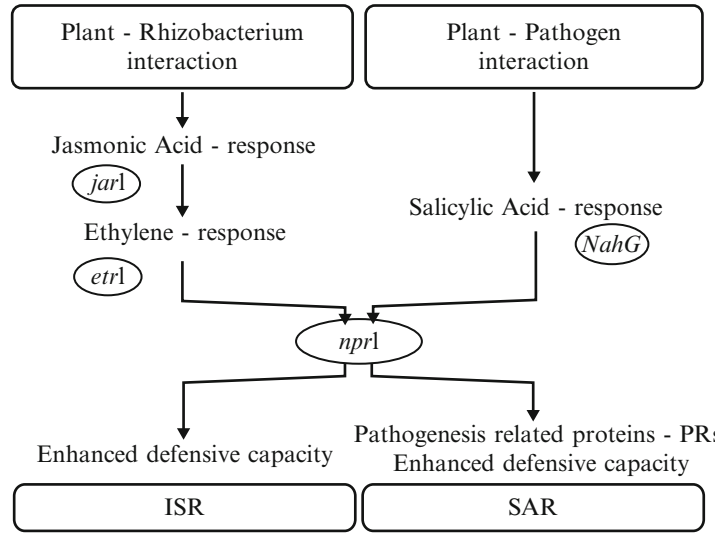

Fig. 12.2 Signal transduction pathways leading to pathogen-induced systemic acquired resistance (SAR) and rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis thaliana* (Source: Beneduzi et al. 2012)

12.4 Seed Invigoration Using Bio-priming

Seed germination, seedling emergence and crop establishment are important aspects of agricultural and horticultural production and are important components of seed/seedling vigour. Seedling vigour is critical when competition for light, nutrients, air and water becomes severe. Seedlings with a vigorous growth pattern can compete successfully under stress, influencing stand establishment and ultimately grain yield. The role of seed vigour comes in light when seeds are sown in adverse conditions, and the vigour of a seed becomes a deciding factor for the crop establishment and yield compared to normal conditions of plant growth.

Infestation by pathogens in seeds could adversely affect the ability of seed to germinate normally, resulting in loss of seed vigour (McDonald and Copeland 1997). Seed germination of Jasmine 85 rice affected by discoloration resulted in decreased number of filled grains/panicle and test weight (Phat et al. 2005). Hamman et al. (2002) concluded that high- and medium-vigour seed lots of soybean always showed higher final emergence (FE) and plant establishment. It was also observed that seedling with low vigour could not withstand stressful conditions, viz. deep planting and pathogen-infested soils during growth, and failed to attain final emergence.

Entesari et al. (2013) investigated efficacy of seed bio-priming treatment with fungal biocontrol agents, viz. *Trichoderma harzianum* (T. AS 19-2, T. bp4, T. BS1-1), *T. virens* (T.As19-1, T.As17-4, T.As10-5), *T. atroviride* (T.As18-5, T.cs5-1, T.Cs2-1) and a bacterium, *Pseudomonas fluorescens* (utpf5) on soybean seed. *Trichoderma harzianum* strain BS1(Th.4) showed positive correlation with soybean growth factors and resulted in enhanced shoot and root length, seedling dry weight and total chlorophyll content as compared to control.

12.4.1 Alleviation of Biotic Stress through Bio-priming

Different fungi isolated from the rhizosphere of various plants capable as biocontrol agents are given in Table 12.3; however, most of the research work is carried out to test efficacy of *Trichoderma* sp. in controlling plant pathogenic fungi. Major problem for commercial application of this biocontrol is its multiplication, formulation and suitable delivery method at end user. Bio-priming is an effective tool for delivery of biocontrol agents, and priming (bio-priming) is seen as an ideal delivery method for inducing resistance, which amplifies the efficiency of rhizobacteria-induced resistance in plants.

Table 12.3 Fungi isolated from the rhizosphere of various plants capable as biocontrol agents

Alternaria sp.	*Epicocuum* sp.	*Paecilomyces* sp.
Aspergillus sp.	*Fusarium* sp.	*Penicillium* sp.
Cephalosporium sp.	*Gliocladium* sp.	*Rhizopus* sp.
Chaetomium sp.	*Humicola* sp.	Sterile mycelia
Cladosporium sp.	*Mortierella* sp.	*Talaromyces* sp.
Coniothyrium sp.	*Mucor* sp.	*Trichoderma* sp.
Curvularia sp.	Mycorrhizal fungi	*Verticillium* sp.
Cylindrocarpon sp.	*Myrothecium* sp.	

Maize is one of the important cereal crops grown in India, and Fusarium ear rot is one of the most devastating diseases inflicting both pre- and postharvest losses in maize. Further *Fusarium verticillioides* is capable of producing varied mycotoxins, viz. fumonisin, moniliformin, zearalenone and trichothecene, damaging approximately 20 % of grains in storage. Chandra Nayaka et al. (2008) studied the effect of bio-priming with potential *Trichoderma harzianum* on maize to control Fusarium ear rot disease and fumonisin accumulation in different maize cultivars grown in India. They concluded that the pure culture of *T. harzianum* was more effective in reducing the *F. verticillioides* and fumonisin incidence followed by talc formulation than the carbendazim. Formulations of *T. harzianum* (1X 108 spore/ml and 10 g/kg of seed) were effective at reducing the *F. verticillioides* and fumonisin infection and also increasing the seed germination, vigour index, field emergence, yield and thousand-seed weight in comparison with the control.

In maize plants, sh2 genes are responsible for high sugar content leading to increase in occurrence of damping off disease caused by *Pythium ultimum*. Callan et al. (1990) used an isolate of *Pseudomonas fluorescens* (AB254) with at least 1×10^7 cfu/seed and allowed to imbibe moisture up to 35–40 % under warm conditions and offered better protection than chemical seed treatments.

Niranjan Raj et al. (2004) studied the effect of bio-priming on pearl millet seeds with different isolates of *Pseudomonas fluorescens* and concluded that among different isolates, UOMSAR14 and UOM SAR 80 showed enhanced germination and seedling vigour in pearl millet plants. Further, bacterial isolate UOM SAR 14 elicited resistance against downy mildew disease under greenhouse as well as field conditions.

Seed bio-priming provides numerous advantages over other delivery methods, and it is reported to alleviate physiological and pathological stresses in plants. Bio-priming of corn seeds with root-colonising *Pseudomonas fluorescens* AB254 resulted in better plant establishment in *Pythium ultimum*-infested soil and was almost equivalent with fungicide seed treatment of metalaxyl (Mathre et al. 1999). Jensen et al. (2004) demonstrated that bio-priming of carrot seeds with fungal isolates of *Clonostachys rosea* (IK726) assured better protection against seedborne pathogens *Alternaria dauci* and *Alternaria radicina* without any antagonistic effects on plant establishment in carrot (Fig. 12.3).

Mapping of disease-free seed production zones and combinations of integrated disease and pest management practices ensures quality seed production in various crops. Modifying seed and soil interface with addition of beneficial microbes may be proved as an important tool in quality seed production of vegetable crops. Pill et al. (2009) concluded that slurry coating of osmotically primed or non-prime seeds with a combination of *Trichoderma harzianum* and *Trichoderma virens* is at least as effective as mefenoxam coating reducing damping off caused by *Pythium aphanidermatum*-infested seedbed of cucumber. Further, seeds coated with Th, Tv or ThTv can be stored for 4 weeks with the *Trichoderma* viability remaining fairly stable at 4 °C and increasing from 3 to 4 weeks at 21 °C.

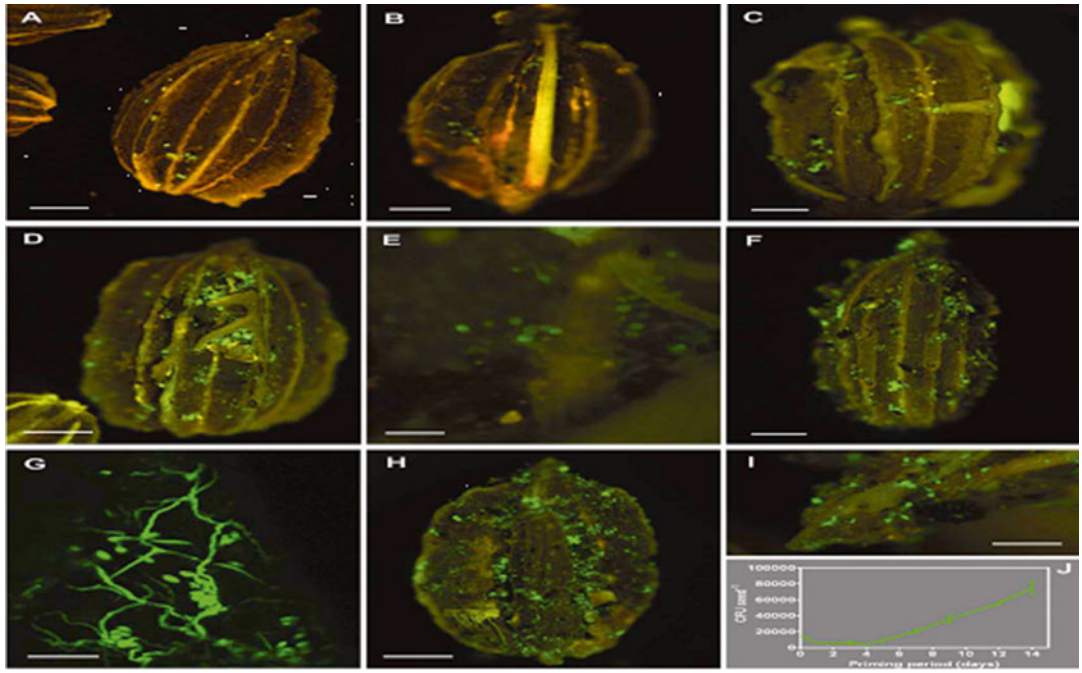

Fig. 12.3 Growth of isolate IK726d11 was visualised with SFM on seeds after a bio-priming period of A, 1 day; B, 2 days; C, 3 days; D, 5 days; F, 7 days; and H, 14 days. E, Sporulation with verticillate conidiophores was observed after 4–5 days. I, Penicillium conidiophores were observed after 6 days. G, With CLSM, a fine web of hyphae was seen on the pericarp at day 7. J, The development in density of *C. rosea* IK726d11 on seeds (CFU/seed) was observed during bio-priming (Source: Jensen et al. 2004)

Today biopesticides with their commercial application are available in India, but biological seed protectant market share is negligible as compared to chemical sales. Progress in biological control with respect to formulation and reliability must be top priority. Commercial formulations available in the market are given in Table 12.4. Key goals of such type of product development invariably should be long shelf life (1–2 years), high density of viable propagules, stability under unfavourable conditions, ease in application and low production cost (Lewis et al. 1991).

12.4.2 Alleviation of Abiotic Stress through Bio-priming

Plants usually face several abiotic stresses that can affect seed quality and yield; these abiotic stresses can decrease germination, vigour and plant stand ultimately affecting the seed yield. In order to optimise the seed crop husbandry, apart from conventional approaches, integration of microbial inoculants in such production systems is gaining importance these days, which is highly efficient and cost-effective. Understanding the complexity of microbial adaptations into stressed rhizosphere and effect of these microorganisms on biological, chemical and physical properties of rhizosphere and plant remains a significant challenge (Yang et al. 2009). At present, significant interest resides in the development and integration of trait-specific microbial inoculants to seed through bio-priming for its enhanced performance in abiotic stress conditions (Nadeem et al. 2007; Yang et al. 2009; Neelam and Meenu 2010). There are satisfactory evidences suggesting that the use of beneficial microbes can enhance plant's resistance to adverse environmental stresses, viz. drought, salt, nutrient deficiency, heavy metal contamination and climate change-induced stresses (Glick et al. 2007).

Table 12.4 Commercial formulations of biocontrol agents available in India

Product	Bioagent	Use
Antagaon-TV	*T. viride*	As seed and soil treatment for control of *Rhizoctonia solani* and *M. phaseolina* in pulses and vegetables
Biocon	*T. viride*	Available in broth and dust used for control of root and stem disease in tea
Bioderma	*T. viride* + *T. harzianum*	Seed treatment against the fungal pathogens in vegetables and pulses
BioGuard	*T. viride*	As seed and soil treatment of seed-borne diseases in vegetables and pulses
Bioshield	*Pseudomonas fluorescens*	As seed, soil and seedling dip against fungal pathogens of cereals and pulses
Biotak	*Bacillus subtilis*	Available in broth formulation and used for the control of black rot disease of tea
Defence-SF	*T. viride*	As seed and soil treatment for control for different diseases in crops

Source: Bhattacharjee and Dey (2014)

12.4.3 Manifestation of Abiotic Stresses in Seed Crop

Stress manifests itself in reduced plant-microbe interaction, water balance and nutrient availability and increased disease incidence and heavy metal toxicity in plant system (Mayak et al. 2004; Egamberdieva and Kucharova 2009). Among the microorganisms many fungal and bacterial strains augmented to seed through bio-priming were found with immense ability to alleviate abiotic stresses by means of various mechanisms thereby enhancing plant growth (Paul and Nair. 2008). Abiotic stresses lead to a series of morphological, physiological, biochemical and molecular changes adversely affecting plant growth and yield (Wang et al. 2001). Various abiotic stress factors like drought, salinity, extreme temperatures and oxidative changes are well connected resulting in cell damage (Wang et al. 2003). High temperature stress results in extensive protein denaturation and aggregation leading to cell death, and low temperature stress weakens metabolic processes by altering the membrane system (Heino and Palva 2003). Heavy metals like Pb, Cu, Hg, etc., were taken up by plant cell and subsequently target enzymes vis-à-vis Cu/Zn-SOD and ethylene receptors and further reduce molecular oxygen leading to formation of reactive oxygen species (ROS) causing extensive cellular damage (Polle and Schützendübel 2003).

12.4.4 Microbial Inoculants for Bio-priming for Alleviation of Abiotic Stresses

The application of beneficial microbes in agricultural production systems started about 60 years ago (Kloepper et al. 1980), and the effect of these microbes was amply addressed in a variety of crops especially in cereals, legumes and oilseeds. Integration of beneficial microorganism into seed crop husbandry through bio-priming for management of biotic and abiotic stresses is gaining enormous importance.

12.4.5 Potential Fungal Bio-inoculants for Bio-priming

Wide range of fungal bioagents through its novel interactions with plant has made it beneficial for alleviating biotic and abiotic stresses. *Trichoderma harzianum* is most widely used for bio-priming for its vast range of antagonism against plant pathogens, mainly fungi and nematode (Singh et al. 2004); increased plant growth especially roots

particularly under stress (Harman 2000; Shoresh et al. 2010); systemic resistance to abiotic plant stresses including drought, salt and temperature (Mansouri et al. 2010; Shoresh et al. 2010); decomposition of organic matter thereby increasing humic acid in soil; solubilisation and mobilisation of phosphorus; and increased nitrogen use efficiency and nutrient availability per se (Singh et al. 2004). Symbiotic fungi, vesicular-arbuscular mycorrhiza (VAM), viz. *Acaulospora* sp., *Ambispora* sp., *Gigaspora* sp., *Glomus* sp., *Pacispora* sp. and *Paraglomus* sp., have shown significant influence on plant nutrient uptake, growth and colossal capacity to resist abiotic stress, especially drought situations (Oliveira et al. 2006); however, the success of establishing symbiotic interaction was limited through bio-priming, but recent reports suggest that inclusion of some *biostimulants* has made it successful by increasing the occurrence of viable colonies and *percent* infection at early seedling growth stages. Seeds of tomato treated with *T. harzianum* Rifai strain T-22 alleviated abiotic stress factors like osmosis, salinity, chilling and high temperature (Mansouri et al. 2010). Further, many endophytic fungi confer abiotic stress tolerance as detailed in Table 12.5.

Table 12.5 Fungal endophytes inducing abiotic stress tolerance

Fungal strains	Host plant	Responses	Reference
Drought/water stress			
Neotyphodium sp.	*Festuca pratensis*	Induce resistance by osmoregulation and stomatal regulation	Malinowski et al. (1997)
	F. arizonica		
Acremonium sp.	Tall fescue	Osmotic protection through secondary metabolites	White et al. (1992)
Phialophora sp.	*F. pratensis*	Osmotic adjustments	Malinowski et al. (1997)
Colletotrichum magna	*Lens esculentum* and *Capsicum annuum*	Osmotic protection and increased water use efficiency	Redman et al. (2001)
C. orbiculare			
C. musae			
Fusarium culmorum	*Oryza sativa* and *L. esculentum*	Osmotic adjustments and expression of genes	Rodriguez et al. (2008)
Piriformospora indica	*Brassica campestris* and *Arabidopsis* sp.	Involved in expression of diverse stress-related genes	Sun et al. (2010) and Sherameti et al. (2008) respectively
Trichoderma hamatum	*Theobroma cacao*	Induced systemic resistance	Bae et al. (2009)
Salinity stress			
Piriformospora indica	*Hordeum vulgare*	Symbiotic interaction with enhanced nitrate reductase synthesis	Waller et al. (2005)
Fusarium culmorum	*Leymus mollis*, *L. esculentum* and *O. sativa*	Confers salt tolerance symbiotically in coastal habitats through osmotic adjustments	Rodriguez et al. (2008)
Trichoderma harzianum	*Allium cepa*	Osmoregulation through physiological response	Hanci et al. (2014)
Heat stress			
Curvularia sp.	*L. esculentum* and *Dichanthelium lanuginosum*	Symbiotic association found in geothermal soils of Yellow Stone National Park.	Rodriguez and Redman, (2008)
Fusarium sp. and *Alternaria* sp.	*L. esculentum*	Interaction leads to upregulation of stress-related genes	Rodriguez and Redman (2008)

Source: Singh et al. (2011)

The bipartite and tripartite beneficial interactions among various fungi, bacteria and even viruses within the fungi or bacterial cell against abiotic stresses were well demonstrated. Tripartite interaction among *Paenibacillus lentimorbus*, *Piriformospora indica* and *Cicer arietinum* (chickpea) enhanced root nodulations and plant growth which is evident by enhanced N, P, K and S uptake by plants (Nautiyal et al. 2010). Hence, these fungal bio-inoculants when integrated with seed through bio-priming have potential to alleviate the ill effects of abiotic stresses in different crops.

12.4.6 Alleviation Mechanism of Abiotic Stresses in Fungal Bio-inoculants

A variety of mechanism has been projected for microbial stimulated abiotic stress tolerance in plants. Stress tolerance conferred to plants symbiotically involves two mechanisms: (1) activation of host stress response systems soon after exposure to stress, allowing the plants to avoid or mitigate the impacts of the stress (Schulz et al. 1999; Redman et al. 1999), and (2) biosynthesis of antistress biochemicals by endophytes (Miller et al. 2002; Schulz et al. 2002). The manifestation of biosynthesis of antistress compounds results in various mechanisms like *osmotic adjustment* conferring tolerance to abiotic stresses. Osmotic adjustments through enhanced production of osmolytes result in increased retention of water in cells, thereby increasing water use efficiency of plant. Increased osmolyte concentration in plant cell results in increased cell wall elasticity and turgid weight to dry weight ratio (TW/DW) (White et al. 1992). Endophytes are involved in the synthesis of alkaloids like lolines conferring *osmotic protection* by reducing stomatal conductance and alleviating drought stress (Morse et al. 2002); these alkaloids protect macromolecules from denaturation and/or reactive oxygen species (ROS) associated with drought stress (Schardl et al. 2004). Apart from these other potential osmoregulators and protectants are soluble sugars and sugar alcohols, produced by the endophyte, plant or both (Richardson et al. 1992). Symbiotic endophytes increased biomass levels but decreased water consumption and improved recovery after drought period conferring enhanced water use efficiency allowing plants for alleviating drought/heat stress conditions (Rodriguez et al. 2008). Development of mutualistic association of plants and endophytes also confers some systemic properties that enable plant to scavenge ROS burst initiated in plant system as abiotic stress response and thereby reducing the cellular damage (Rodriguez et al. 2008). It is a common acceptance that antioxidant enzymes play an important role in fungal symbiosis conferring abiotic stress tolerance. Further, Rouhier and Jacquot 2008; Rouhier et al. 2008 reported that ROS scavenging compounds include low molecular weight glutathione, ascorbate and tocopherol and enzymes, viz. superoxide dismutases, catalases, ascorbate- or thiol-dependent peroxidases, glutathione reductases, dehydroascorbate reductases and monodehydroascorbate reductases.

12.4.7 Potential Bacterial Bio-inoculants for Bio-priming

Bacteria are the most abundant soil microbes and integral part in nutrient cycling for maintaining soil fertility. Beneficial bacteria in rhizosphere are of two types: (a) bacteria forming symbiotic relationship through specialised structures and (b) free-living bacteria present in the vicinity of plant domain which are often known as plant growth-promoting rhizobacteria (PGPR). PGPR include a wide range of bacteria belonging to genera *Azotobacter*, *Arthrobacter*, *Agrobacterium*, *Azospirillum*, *Enterobacter*, *Streptomyces*, *Bacillus*, *Burkholderia*, *Klebsiella*, *Pseudomonas* and *Serratia* (Gray and Smith 2005; Vessey 2003). Co-application of PGPR to seed via bio-priming improves plant performance under stress environments and consequently enhances yield both directly and indirectly (Dimkpa et al. 2009). Some PGPR may exert a direct stimulation on plant growth and development by providing plants with fixed nutrients and phytohormones that have been sequestered by

bacterial siderophores (Hayat et al. 2010; Rodrìguez and Fraga 1999). Strains of *Rhizobium leguminosarum* bv. *viciae* confer tolerance to abiotic stress factors like drought and salinity by maintaining its capacity to nodulate and fix nitrogen in faba bean (Belal et al. 2013). Some of the PGPR capable of alleviating abiotic stresses in different crops are presented in Table 12.6.

Co-inoculation of PGPR through seed bio-priming shows synergistic effects, where one acts as a helper for enhanced performance of other inoculant. In the rhizosphere the synergism between various bacterial genera such as *Bacillus, Pseudomonas* and *Rhizobium* are well demonstrated to promote plant growth and development. Compared to single inoculation, co-inoculation improved the absorption of nitrogen, phosphorus and other mineral nutrients by seed crop (Figueiredo et al. 2011; Yadegari et al. 2010). Presently bio-priming of seeds for alleviating abiotic stress is achieved through only few PGPR; enormous scope exists for inclusion of underutilised biological agents with varied capacity to confer tolerance for various abiotic stresses.

12.4.8 Alleviation Mechanism of Abiotic Stresses in PGPR Bio-inoculants

Bacteria in association with plant are endowed with certain specialised traits to encounter the ill effects of abiotic stress. Under stress conditions, the endogenous ethylene production in plant system is well documented (Jackson 1997), which adversely affects the root growth and consequently the growth of the plant as a whole. Production of enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which cleaves ACC, the precursor molecule of ethylene, is well documented in bacteria (Wang et al. 2001; Saleem et al. 2007). Some PGPR are endowed with certain unique abiotic stress alleviation traits by inducing physical and chemical changes in plants known as *induced systemic tolerance* (IST) to alleviate abiotic stresses (Yang et al. 2009). PGPR induce the expression of drought response-related gene in the plant system through production of some specific inducer and also enhance the level of ROS scavenging enzymatic antioxidants by upregulating the gene involved in its synthesis (Kohler et al. 2008). PGPR are involved in the synthesis of some specialised compounds like *exopolysaccharides* (EPS) which are involved in soil aggregation and help in the maintenance of soil structure in the vicinity of root system even in water stress conditions (Konnova et al. 2001). Plant roots along with fungal hyphae fit in the pores between microaggregates and thus stabilise macroaggregates, thereby increasing the root-adhering soil/root tissue (RAS/RT) ratio (Oades and Waters 1991). Further, PGPR enhance the nutrients uptake of plants in soil conditions where limited nutrient is freely available for plant uptake due to fixation (Munns and Tester 2008). In most cases salinity decreased availability of phosphorus, potassium, iron, zinc and copper to plant (Hayat et al. 2010; Rodrìguez and Fraga 1999). These PGPR convert insoluble form of macro- and micronutrients into available form (Richardson et al. 2009; Khan et al. 2009; Rodrìguez and Fraga 1999). PGPR were also involved in the synthesis of phytohormones, viz. indoleacetic acid (IAA) and gibberellins, which enhance root and shoot development in plant, thereby increasing the plant biomass for better alleviation of abiotic stress conditions (Patten and Glick 2002).

12.5 Conclusion

At present, bio-priming of seeds, development of suitable microbial bio-priming agents and their commercial application to facilitate penetration among farming community are very essential. Therefore, microbial identification and characterisation of potential strains for the development of bio-priming agents, development of formulation and microscale production of bio-priming agents and their suitable delivery mode, mass-scale multilocational field trials and generation of bioefficacy data on different crops, popularisation of technologies among the farmers and registration and commercial production by the agro-industries are needs of the hour. Further

Table 12.6 Bacterial strains inducing abiotic stress tolerance

Bacterial strains	Host plant	Responses	References
Drought/water stress			
Achromobacter piechaudii ARV8	*Lycopersicon esculentum* and *Capsicum annuum*	Synthesis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase which reduces ethylene production	Mayak et al. (2004)
Ensifer meliloti bv. *mediterranense*	*Phaseolus vulgaris*	Synthesis of ACC deaminase	Mnasri et al. (2007)
Variovorax paradoxus	*Pisum sativum*	Synthesis of ACC deaminase which reduces ethylene production	Dodd et al. (2005)
Pseudomonas putida, *Pseudomonas* sp. and *Bacillus megaterium*	Undescribed plant	Production of phytohormones	Marulanada et al. (2009)
Pseudomonas sp.	*Helianthus annuus*	Increase in biomass and (root-adhering soil/root tissue) RAS/RT of seedlings	Sandhya et al. (2009)
Bacillus sp.	*Lactuca sativa*	Enhanced AM fungi association in roots and incremental photosynthesis	Vivas et al. (2003)
Pseudomonas fluorescens	*Catharanthus roseus*	Improved plant growth	Jaleel et al. (2007)
Paenibacillus polymyxa and *Rhizobium tropici*	Common bean	Altered phytohormone balance and stomatal conductance	Figueiredo et al. (2008)
Pseudomonas mendocina	*Lactuca sativa*	Increased phosphatase activity in roots and proline accumulation in leaves	Kohler et al. (2008)
Salinity stress			
Pseudomonas putida	Canola	Accumulation of proteins and increased availability of nutrients	Cheng et al. (2007)
Pseudomonas fluorescens	*Arachis hypogaea*	Enhanced ACC deaminase activity	Saravanakumar and Samiyappan (2007)
Rhizobium and *Pseudomonas*	*Zea mays*	Decreased electrolyte leakage and increase in proline production in leaves	Bano and Fatima (2009)
Pseudomonas putida	*Gossypium* sp.	Increase the absorption of useful cations and decrease uptake of deleterious Na^{2+}	Yao et al. (2010)
Bacillus subtilis	*Arabidopsis thaliana*	Decreased electrolyte leakage and increase in proline production in leaves	Zhang et al. (2010)
Azospirillum sp.	*Lactuca sativa*	Increase in N metabolism and synthesis of high molecular weight proteins	Hamdia et al. (2004)
Heat and cold temperature stress			
Pseudomonas putida NBR1097	*Cicer arietinum*	Overexpression of stress sigma factor and biofilm formation	Srivastava et al. (2008)
Pseudomonas AKM-P6	*Sorghum bicolor*	Biosynthesis of HSPs and accumulation of proline in leaves	Ali et al. (2009)

(continued)

Table 12.6 (continued)

Bacterial strains	Host plant	Responses	References
Burkholderia phytofirmans PsJN	*Vitis vinifera*	Increase in root and plant biomass and accumulation of starch, proline and phenolics in leaves	Barka et al. (2006)
P. putida UM4	Canola	ACC deaminase synthesis	Cheng et al. (2007)
Burkholderia phytofirmans	*Solanum tuberosum*	Accumulation of proline, antioxidants and phenolics in leaves	Bensalim et al. (1998)
Pseudomonas fluorescens, Pantoea agglomerans, Mycobacterium sp.	*Triticum aestivum*	Upregulation of stress-related genes	Egamberdiyeva and Hoflich (2003)
Waterlogging stress			
Pseudomonas and *Enterobacter*	*Lycopersicon esculentum*	Synthesis of ACC deaminase which reduces ethylene production	Grichko and Glick (2001)
Heavy metal stress			
Kluyvera ascorbata	*Lycopersicon esculentum*	Toxic effects of Ni^{2+}, Pb^{2+} and Zn^{2+} not pronounced on plant	Burd et al. (2000)
Methylobacterium oryzae and *Burkholderia* sp.	*Lycopersicon esculentum*	Reduced uptake and translocation of nickel and cadmium	Madhaiyan et al. (2007)
Pseudomonas brassicacearum Am3, *P. marginalis* Dp1 and *Rhodococcus* sp. Fp2	*Pisum sativum*	Stimulation of root growth and enhanced nutrient uptake	Safronova et al. (2006)
Rhizobium sp.	*Pisum sativum*	Enhanced plant growth	Wani et al. (2008)
Nutrient deficiency stresses			
Pseudomonas fluorescens and *Bacillus megaterium*	*Lycopersicon esculentum*	Enhanced availability of phosphorus and calcium	Lee et al. (2010)
Azospirillum sp. and *Azotobacter* sp.	*Oryza sativa*	Fixation of atmospheric nitrogen	Wada et al. (1978)
Paenibacillus glucanolyticus	*Piper nigrum*	Solubilisation of fixed potassium	Sangeeth et al. (2012)
Frateuria aurantia	Field and vegetable crops	Solubilisation of fixed potassium	Commercial product
Pseudomonas sp. P29	*Zea mays*	Solubilisation of zinc	Goteti et al. (2013)

research on the viability of the introduced microorganisms and its fate, existence and mode of work on bio-primed seeds is one important area which needs immediate attention.

While *Trichoderma* and *Pseudomonas* have been studied by many researchers extensively, hardly any attention has been paid to identification of crop-specific novel strains of various beneficial microbes. Therefore, future research programme needs to be devised for identification and genetic manipulations of novel biocontrol agents with compatibility studies on seed surface and commercial viability. Further, using bio-primed seeds along with bio-priming of nursery beds with obligate symbionts like AM fungi, particularly in marginal soils, should be given special attention. Similarly, a huge number of fungal and PGPR representing diverse genera have been identified and characterised for their capability to augment the alleviation strategies of plant as a response to biotic/abiotic stress factors in agro-ecosystem. Application of these bio-inoculants for enhancing performance of seed under limiting environmental conditions through bio-

priming has proved beyond doubt, but still a large number of microorganisms remain underutilised for this purpose with the capacity to alleviate varied biotic/abiotic stresses. These bio-inoculants in association with plant have much better stimulatory effect on managing pest/diseases, plant growth and nutrient uptake in stressful environmental conditions. Thus integrating these bio-inoculants to seed through bio-priming can successfully alleviate biotic as well as abiotic stress conditions in agricultural system thereby improving the seed quality and yield in limiting environments.

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Azotobacter: PGPR Activities with Special Reference to Effect of Pesticides and Biodegradation

13

Chennappa Gurikar, M.K. Naik, and M.Y. Sreenivasa

Abstract

Among all the microorganisms, plant growth-promoting rhizobacteria (PGPR) have significant influence on soil physiological and structural properties. PGPR help to replace chemical fertilizer for the sustainable agriculture production by fixing the atmospheric nitrogen and producing growth-promoting substances. Among the PGPR group, *Azotobacter* are ubiquitous, aerobic, free-living, and N_2-fixing bacteria commonly living in soil, water, and sediments. Being the major group of soilborne bacteria, *Azotobacter* plays different beneficial roles and is known to produce varieties of vitamins, amino acids, plant growth hormones, antifungal substances, hydrogen cyanide, and siderophores. The growth-promoting substances such as indoleacetic acid, gibberellic acid, arginine, etc., produced by *Azotobacter* have direct influence on shoot and root length as well as seed germination of several agricultural crops. *Azotobacter* species are efficient in fixation of highest amount of nitrogen (29.21 μg $NmL^{-1}day^{-1}$), production of indoleacetic acid (24.50μgmL^{-1}) and gibberellic acid (15.2 μg 25 mL^{-1}), and formation of larger phosphate solubilizing zone (13.4 mm). Many species of *Pseudomonas*, *Bacillus*, and *Azotobacter* can grow and survive at extreme environmental conditions, viz., tolerant to higher salt concentration, pH values, and even at dry soils with maximum temperature. Different factors affect *Azotobacter* population in soil such as pH, phosphorus content, soil aeration and moisture contents, etc. *A. chroococcum* found tolerant to a maximum NaCl concentration of 6 % with a temperature of 45 ° C and also up to pH of 8. *Azotobacter* species such as *A. vinelandii*, *A. chroococcum*, *A. salinestris*, *A. tropicalis*, and *A. nigricans*

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are able to produce antimicrobial compounds which inhibit the growth of common plant pathogens, viz., *Fusarium*, *Aspergillus*, *Alternaria*, *Curvularia*, and *Rhizoctonia* species. Pesticides used to control pests, insects, and phytopathogens are known to cause direct effect on soil microbiological aspects, environmental pollution, and health hazards in all living beings of the soil ecosystem. The species of *Azotobacter* are known to tolerate up to 5 % pesticide concentration and also to degrade heavy metals and pesticides. *A. chroococcum* and *A. vinelandii* proved their biodegradation efficiency of many commonly used pesticides, viz., endosulfan, chlorpyrifos, pendimethalin, phorate, glyphosate, and carbendazim. From these results, it is clear that the *Azotobacter* strains not only produce plant growth-promoting substances (PGPS) but are also tolerant to abiotic stress under different physiological conditions.

Keywords
Azotobacter • PGPR • Abiotic stress • Antifungal activity • Pesticide tolerance • Biodegradation

13.1 Introduction

Plant growth-promoting rhizobacteria (PGPR) are a group of bacteria that actively colonize rhizosphere around the roots, the rhizoplane, i.e., in the root surface and often within the roots as well, and increase plant growth and yield. Root-colonizing bacteria that exert beneficial effects on plant development via direct or indirect mechanisms have been defined as rhizobacteria by Kloepper and Schroth (1980). PGPR have gained worldwide importance and acceptance and appear to be the trend for the future. PGPR can influence plant growth directly either by providing specific compounds that help plant growth or by facilitating uptake of nutrients from the soil and indirectly by suppressing the phytopathogenic organisms in the rhizosphere (Mishra et al. 2005). The PGPR have been found to produce plant growth regulators which are produced by the different species of *Azotobacter*, *Bacillus*, *Rhizobium*, *Pseudomonas*, and *Klebsiella*.

Among them, free living *Azotobacter* is one of the important soil borne bacteria and are capable of producing secondary metabolites in different environmental conditions which have wide range of application. Plant-interacting microorganisms can establish either mutualistic or pathogenic associations. Although the outcome is completely different, common molecular mechanisms that mediate communication between the interacting partners can be involved. Specifically, nitrogen-fixing bacterial symbionts of legume plants, collectively termed rhizobia and phytopathogenic bacteria, have adopted similar strategies and genetic traits to colonize, invade, and establish a chronic infection in the plant host. Several types of pesticides are known to cause direct effect on soil microbiological aspects, environmental pollution, and health hazards in all living beings. Different species are known to tolerate and biodegrade chemical compounds which adversely affect the population of soil microorganisms, viz., *Azotobacter*, *Pseudomonas*, etc., which include pesticides that are being used worldwide for the management of many agricultural crops (Bagyaraj and Patil 1975; Ramaswami et al. 1977).

13.2 *Azotobacter* Diversity

The genus *Azotobacter* was first described in 1901 by Martinus Beijerinck (1851–1931), a Dutch microbiologist and one of the founders of

Environmental Microbiology. *Azotobacter* belongs to the kingdom Bacteria, phylum Proteobacteria, class Gammaproteobacteria, order Pseudomonadales, family Azotobacteraceae, and genus *Azotobacter*. Beijerinck was the first person who isolated and cultured *Azotobacter chroococcum* and *A. agilis*. Later several other species of *Azotobacter* have been isolated and described as *Azotobacter vinelandii*, *A. beijerinckii*, *A. insignis*, *A. macrocytogenes*, *A. paspali*, *A. salinestris*, *A. armeniacus*, *A. brasilense*, *A. tropicalis*, and *A. nigricans* (Mulder and Brontonegoro 1974; Page and Shivprasad 1991; Aquilanti et al. 2004; Kizilkaya 2009).

The family *Azotobacteraceae* is composed of free nitrogen fixing bacteria commonly living in soil, water, and sediments (Aquilanti et al. 2004). *Azotobacter* requires neutral to slightly alkaline pH for the growth, and the pH range is between 5.5 and 8.5 but the optimum pH is 7.0–7.5 (Channal et al. 1989; Akhter et al. 2012). The optimum temperature is 28–32 ° C and the maximum temperature is around 38 ° C and the minimum is 22 ° C. The diversity and morphological characters of *Azotobacter* species, viz., *A. chroococcum*, *A. vinelandii*, *A. paspali*, *A. beijerinckii*, *A. salinestris*, *A. armeniacus*, *A. brasilense*, *A. insignis*, *A. agilis*, *A. tropicalis*, and *A. nigricans*, are well studied since the last two decades because of its plant growth-promoting activity for sustainable agriculture (Chennappa et al. 2013; Jimenez et al. 2011; Aquilanti et al. 2004). Among them, *A. chroococcum* and *A. vinelandii* are found almost in all the rhizosphere soils.

Azotobacter species exist in dry, hot steppes, deserts, sands, rocky terrains, and valleys and on mountain summits. In Indian soils, the population of *Azotobacter* is not more than 10 thousand to 1 lakh/g of soil (Subbarao 1988). The population of *Azotobacter* is mostly influenced by other microorganisms present in soil. The occurrence and dominance of *Azotobacter* in the rhizosphere of various agricultural crops such as ragi, sorghum, green gram and soybean, sugarcane, rice, and cereals were reported by Bagyaraj and Patil (1975). *Azotobacter* population was found more in black soil than in red soil, and the number may decrease with depth but the decrease was more drastic in black soils (Ramaswami et al. 1977). *Azotobacter* population variation was also observed in desert soils of India with respect to organic matter content (Rao and Venkateswarlu 1982).

The species of *Azotobacter* are ubiquitous in nature, and the occurrence of *Azotobacter* in soil is influenced by many factors, viz., soil pH, organic matter, and calcium, phosphorus, and potassium content (Rangaswamy and Sadasivan 1964). Genus *Azotobacter* is one of the major diazotrophic bacteria capable of atmospheric nitrogen fixation, living at an optimum temperature range between 20 and 30 ° C in all soil conditions and at varied pH (5.5–9.5) values. *Azotobacter* is commonly found in neutral to alkaline soils but not in acidic soils (Kaushik and Sethi 2005). They are also found in the Arctic and Antarctic soils, cold climates, and aquatic habitat (Garg et al. 2001), but the population may vary from soils of different geographical regions.

Different nitrogen-free media are used for the isolation, cultivation, and maintenance of *Azotobacter* with different carbon sources such as sucrose, glucose, and mannitol. Other commonly used nitrogen-free media for the isolation of *Azotobacter* are Waksman No. 77 (Waksman 1952) and Burk's (Burk 1932). The ability of the *Azotobacteraceae* family to grow in a medium free from nitrogen helps in their selective isolation. An organic carbon source and phosphate are usually the minimum nutrients required for the development of *Azotobacter* under natural conditions (Becking 1981).

Azotobacter is a gram-negative bacterium and is pleomorphic, i.e., can have different shapes from bacillary to spherical (Page and Shivprasad 1991), and their size ranges from 1.0 to 3.8 μm. *Azotobacter* are relatively large and usually oval, can be alone or in pairs, form irregular clusters or occasionally chains of varying lengths, and produce cysts. The genus *Azotobacter* is ubiquitous in all soil conditions and is a common diazotroph and free-living and nitrogen-fixing bacterium found in agricultural soils playing different beneficial roles (Tejera et al. 2005). The cells move through the multiple flagella and, in later stages,

lose their motility (Page and Shivprasad 1991; Chennappa et al. 2013), become almost an inactive form, and produce a thick layer of mucus, which forms the capsule cells.

Mobility is seen in *A. chroococcum*, *A. armeniacus*, and *A. paspali* by means of peritrichous flagella, and in fresh cultures, the cells move through the multiple flagella. *Azotobacter* in old culture produces melanin which gives blackish color to the culture, and it has been used for the production of biofertilizer, food additives, and biopolymers. Nonsymbiotic nitrogen fixers morphologically differ from one another because of their pigment production and the appearance of the colonies. The pigmentation that is produced by *Azotobacter* in aged culture is melanin which is due to oxidation of tyrosine by tyrosinase enzyme which has copper. The color can be seen in liquid medium. *A. chroococcum* produces brown-black; *A. beijerinckii* produces yellow-light brown; *A. vinelandii*, *A. paspali*, and *A. agilis* produce green fluorescent; *A. macrocytogenes* produces pink; and *A. insignis* produces grayish-blue pigmentations (Moreno et al. 1986; Akhter et al. 2012; Rubio et al. 2000).

During growth, *Azotobacter* species will produce water-soluble pigments, causing cultures to appear in shades of yellow, green, red, and brown color. In media containing sugars, some of the *Azotobacter* species will produce copious amounts of an extracellular polysaccharide (Barrera and Soto 2010). The highest population was recorded in soils having maximum amount of organic carbon (2.5 %), and the lowest was found in acid soils with less organic carbon (0.94 %). This indicates that increase in organic carbon in soil favored increased population of *Azotobacter*, and lack of organic carbon in soil has a limiting effect on proliferation of *Azotobacter* species. This species can be grown in any of the N-free media, and the genus *Azotobacter* are chemoorganotrophs, capable of using sugars, alcohols, and salts of organic acids for growth (Tejera et al. 2005).

The *Azotobacter* can survive in brown soil for 18 months, and similarly, the *Azotobacter* species remained viable for more than 24 years in dry soil. The cysts survived in dried agar medium for 10 years under laboratory conditions in the same way as endospores of gram-positive bacteria (Moreno et al. 1986; Kirokasyan et al. 1955). Aquilanti et al. 2004 isolated *Azotobacter* and screened for growth, colony morphology, pigment production, and acidification activity on N-free LG medium containing sucrose as sole carbon source and bromothymol blue as pH indicator. Khanafari (2007) proved more significant growth of *Azotobacter* in whey agar than in mannitol agar medium and also showed two strains of *A. chroococcum* in whey agar media producing colonies that are mucoid, ropy, and capsule positive with yellow pigment in 24 h at 30 ° C.

Azotobacter do not produce endospores, but they form thick-walled cysts (Subhani et al. 2000) as part of their life cycle and germinate under favorable conditions to give vegetative cells (Reinhardt et al. 2008). Cyst representatives of the genus *Azotobacter* are visible central body with vacuoles, and multilayered shell and cysts are more resistant to adverse factors of the environment than the vegetative cells. The ability to form cysts is an important diagnostic character and is determined by examining old cultures of *Azotobacter* grown on nitrogen-free agar. Cysts are twice more resistant to UV radiation than vegetative cells and resistant to drying, gamma radiation, solar irradiation, and effects of ultrasound (Parker and Socolofsky 1966). The formation of cysts is induced by changing the concentration of nutrients in the nutrient medium and the addition of some organic substances (ethanol, n-butanol).

13.3 Applications of *Azotobacter*

Among all, PGPR have more influence on the growth and yield of the crops. PGPR can help to replace nitrogen from chemical fertilizer for the sustainable cultivation by fixing the atmospheric N_2 and producing PGPS (Ahmad et al. 2005). *Azotobacter* is known to produce secondary metabolites such as vitamins (riboflavin), amino acids (thiamine), plant growth hormones (nico-

tine, indoleacetic acid, and gibberellins), antifungal compounds, and siderophores, and importantly they can fix atmospheric-free nitrogen (Myresiotis et al. 2012). These growth-promoting substances have direct influence on shoot and root length as well as seed germination of several agricultural crops (Ahmad et al. 2005).

These secondary metabolites influence plant growth promotion by excreting vitamins, amino acids, and auxins. Siderophores can provide iron to plants and polyhydroxybutyrate (PHB) which can be used in large-scale production of alginic acid. Antifungal compounds, HCN, can inhibit the pathogenic organisms in plant rhizosphere. The ability to synthesize phytohormones is widely distributed among plant-associated bacteria, and 80 % of the bacteria isolated from plant rhizosphere are able to produce plant growth-promoting substances. *Azotobacter* species improves seed germination and plant growth. *Azotobacter* is the heaviest breathing organism and requires a large amount of organic carbon for its growth. *Azotobacter* is less effective in soils with poor organic matter content (Bhosale et al. 2013; Barrera and Soto 2010).

13.3.1 Vitamins

A. vinelandii strain ATCC 12837 and *A. chroococcum* strain H23 (CECT 4435) produced the B-group vitamins which are niacin, pantothenic acid, riboflavin, and biotin. Vitamins are essential compounds for the physiological functions of the living beings which are produced by several groups of bacteria. They are used to maintain metabolic processes of living beings, but the production of vitamins is controlled by several factors such as growth conditions, pH, incubation temperatures, availability of nitrogen and carbon, nitrogen sources, and their concentrations (Revillas et al. 2000).

Riboflavin or vitamin B2 is required for a wide variety of cellular processes, and it plays a key role in metabolism of fats, ketone bodies, carbohydrates, and proteins. Genetically engineered *Bacillus subtilis* and *Corynebacterium ammoniagenes* are used for mass production of riboflavin by which they overexpress genes of the enzymes involved in riboflavin biosynthesis (Almon 1958; Revillas et al. 2000).

13.3.2 Amino Acids

Amino acids and proteins are the building blocks of life, and when proteins are digested or broken down, amino acids are released. Amino acids are biologically important organic compounds that combine to form proteins composed of amine ($-NH_2$) and carboxylic acid ($-COOH$) functional groups along with a side chain specific to each amino acid. Carbon, hydrogen, oxygen, and nitrogen are the key elements of amino acids; however, some of the other elements are also found inside chains of certain amino acids. *A. vinelandii* and *A. chroococcum* produced amino acids supplemented with phenolic compounds as sole carbon source under diazotrophic conditions (Revillas et al. 2000). Production of amino acids (methionine, lysine, arginine, tryptophan, and glutamic acid) has been recorded (Lopez et al. 1981) by using *Azotobacter* spp. during growth in culture media amended with glucose as sole carbon source under diazotrophic conditions.

Production of thiamine and pantothenic acid by *A. vinelandii* ATCC 12837 in Burk's N-free media amended with glucose (0.5 ± 2 %) has been reported and increased after the addition of 0.3 % NH_4Cl. Revillas et al. (2000) reported that the *A. vinelandii* strain ATCC 12837 and *A. chroococcum* strain H23 (CECT4435) were able to grow in different chemically defined media supplemented with protocatechuic acid or sodium p-hydroxybenzoate as sole source of carbon. The same two strains produced different types of amino acids such as glutamic acid, threonine, alanine, cysteine, tyrosine, valine, arginine, serine, proline, methionine, lysine, glycine, isoleucine, histidine, leucine, aspartic acid, and phenylalanine in chemically defined media supplemented with phenolic compounds or sodium succinate as a C source along with 0.1 % NH_4Cl.

13.3.3 Plant Growth Hormones (Nicotine, IAA, GA)

Indoleacetic acid (IAA) is the main plant auxin produced by different groups of bacteria commonly living in soils (Barazani and Friedman 1999). Saline soil is a rich source of IAA-producing bacteria, whereas 75 % of the bacterial isolates are active in IAA production. Many *Azotobacter* species are found to produce IAA in the range of 2.09–33.28 µg/mL (Chennappa et al. 2013). Most commonly, IAA-producing PGPR strains are known to increase root growth and length resulting in greater root surface area which enables plants to access more nutrients from soil. The IAA is responsible for the division, expansion, and differentiation of plant cells and tissues and stimulates root elongation.

Many rhizobacteria and some of the pathogenic, symbiotic, and free-living bacterial species have been reported to produce IAA in different conditions. Rhizobacteria use different pathways to produce IAA from tryptophan, although they can produce the IAA by using tryptophan-independent pathways. However, the production of IAA by pathogenic bacteria mainly through indoleacetamide pathway can induce tumor formation in plants. In contrast, the indolepyruvic pathway appears to be the main pathway present in plant growth-promoting beneficial bacteria (Patten and Glick 2002). The rhizobacteria have also been reported to produce phytohormones such as auxin (Spaepen et al. 2007).

Among PGPR species, *Azospirillum* is one of the best-studied IAA producers, and other bacteria belonging to genera *Aeromonas*, *Burkholderia*, *Azotobacter* (Ahmad et al. 2008; Chennappa et al. 2013), *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Rhizobium* (Ghosh et al. 2010) species have been isolated from different rhizosphere soils. IAA-producing bacteria have been used to stimulate seed germination, to accelerate root growth or modify the architecture of the root system, and to increase the root biomass.

13.3.4 Gibberellic Acid (GA)

Another important activity of *Azotobacter* is the production of gibberellins. Gibberellic acids (GA) include a wide range of chemicals that are produced naturally within plant rhizosphere by bacteria and fungi (Chennappa et al. 2014a). GA was first discovered by Japanese scientist Eiichi Kurosawa from a fungus called *Gibberella fujikuroi* under abnormal growth stage in rice plants. Gibberellins are important in seed germination and enzyme production that mobilizes growth of new cells. In grain (rice, wheat, corn, etc.) seeds, a layer of cells called the aleurone layer wraps around the endosperm tissue, and absorption of water by the seed causes production of GA. GA promote flowering, cellular division, and seed growth after germination. Gibberellins also reverse the inhibition of shoot growth and dormancy induced by abscisic acid.

13.3.5 P Solubilization

Phosphorus (P) is a chemical element, and as a mineral, it is always present in its oxidized state as an inorganic phosphate rock, but due to its high reactivity, phosphorus is never found as a free element on earth. Phosphorus is an important limiting nutrient and is one of the least soluble mineral nutrients in soil. The phosphorus content of soils may be up to 19 g/Kg, but usually less than 5 % of this is available to the plants and microorganisms in soluble form, and 95 % is unavailable in the form of insoluble inorganic phosphate and organic phosphorus complexes. Microbes play a significant role in the transformation of phosphorus and are referred to as phosphobacteria. Phosphate-solubilizing bacteria such as *A. chroococcum*, *Bacillus subtilis*, *B. cereus*, *B. megaterium*, *Arthrobacter ilicis*, *Escherichia coli*, *P. aeruginosa*, *Enterobacter aerogenes*, and *Micrococcus luteus* are identified. Phosphorus compounds are used in explosives, nerve agents, friction matches, fireworks, pesticides, toothpaste, and detergents (Kumar et al. 2000).

Phosphorus is a major component of nucleic acids, ATP, and also phospholipids involved in the formulation of all cell membranes. It is also used as a major ingredient in many of the chemically synthesized pesticide and chemical fertilizers to replace the phosphorus that plants remove from the soil. Phosphate-solubilizing bacteria are a group of beneficial bacteria capable of hydrolyzing organic and inorganic phosphorus from insoluble compounds. P-solubilization ability of the microorganisms is considered to be one of the most important traits associated with plant phosphate nutrition. Currently, different species of bacteria have been identified as biofertilizer, and three new species, *Pantoea agglomerans*, *Microbacterium laevaniformans*, and *Pseudomonas putida*, have been recently identified as the highly efficient phosphate solubilizers (Garg et al. 2001; Upadhyay et al. 2009).

13.3.6 Antifungal Compounds

Antibiotics constitute a wide and heterogeneous group of low molecular weight organic compounds that are produced by a wide variety of microorganisms. Antibiotics are active upon many microorganisms but not all, or only to a very limited extent. This group includes most of the substances that have found extensive application as chemotherapeutic agents, notably penicillin, streptomycin, chloramphenicol, Aureomycin, Terramycin, and neomycin. This group of substances appears to be the most significant from the point of view of their utilization in the treatment of fungal diseases. It is sufficient to mention actidione, antimycin, fradicin, and fungicidin, and these substances vary greatly in their chemical nature, antifungal spectra, and toxicity to animals.

The production of antibiotics is considered one of the most studied biocontrol mechanisms for combating phytopathogens. Under laboratory conditions, different types of antibiotics produced by PGPR have shown to be effective against phytopathogenic agents (Bowen and Rovira 1999). *Azotobacter* can provide protection against drought and produces antifungal antibiotic substance which inhibits the growth of soilborne fungi such as *Aspergillus*, *Fusarium*, *Curvularia*, *Alternaria*, and *Helminthosporium* (Khan et al. 2008; Mali and Bodhankar 2009; Agarwal and Singh 2002). The species of *Azotobacter* produce different types of antibiotics which include 2,3-dihydroxybenzoic acid, aminochelin, azotochelin, protochelin, and azotobactin (Kraepiel et al. 2009).

Azotobacter species act as biocontrol agents for many plant pathogens: *A. chroococcum* inhibit the growth of *Aspergillus*, *Alternaria*, *F. oxysporum*, and *R. solani*, and they are known to produce antimicrobial agents such as 2,3-dihydroxybenzoic acid, aminochelin, azotochelin, protochelin, and azotobactin. The species of *Azotobacter* and *Arthrobacter* inhibit root colonization of *F. verticillioides* and suppress fumonisin B-1 production by *A. armeniacus*. Antifungal activity of *A. vinelandii* against *F. oxysporum* showed maximum zone of inhibition (40 mm) which was known to cause diseases of agricultural crops, viz., chili and pigeon pea (Chennappa et al. 2014a; Cavaglieri et al. 2005; Bhosale et al. 2013).

The antibiotics produced by PGPR include 2-acetamidophenol, phenazine-l-carboxamide (PCN), hydrogen cyanide (HCN), resin, butyrolactones, eumycin, pyocyanin, hernipyocyanin, tyrothricin, zwittermicin A, kanosamine, oligomycin A, oomycin A, phenazine-I-carboxylic acid, pyoluteorin, pyrrolnitrin, viscosinamide, xanthobaccin, and 2,4-diacetylphloroglucinol (2,4-DAPG) (Whipps 2001). 2,4-DAPG is one of the most efficient antibiotics in the control of plant pathogens and is produced by various strains of *Pseudomonas*. The 2,4-DAPG has a wide spectrum of properties such as antifungal, antibacterial, and antihelminthic (Naik et al. 2013).

13.3.7 HCN

Apart from the production of antibiotics, some of the rhizobacteria are capable of producing HCN (hydrogen cyanide). HCN is a volatile, secondary metabolite that suppresses the development of microorganisms and that also influences the growth and development of plants. HCN is one of the significant inhibitors of cytochrome C oxidases and other metalloenzymes which are involved in the metabolic activities of phytopathogens. HCN is mainly synthesized by an enzyme HCN synthetase, which is known to be associated with plasma membrane of certain rhizobacteria. Presently many bacterial species have shown to be capable of producing HCN, including species of *Azotobacter*, *Alcaligenes*, *Aeromonas*, *Bacillus*, *Pseudomonas*, and *Rhizobium* (Naik et al. 2013; Ahmad et al. 2008).

Rhizobacterial groups are known to produce HCN, among which *Pseudomonas* is one of the common producers. Some studies showed that about 50 % of the *Pseudomonas* isolates obtained and characterized from wheat and potato rhizospheric soil are able to produce HCN. Various studies reported that HCN can inhibit the growth of phytopathogenic nematodes such as *Meloidogyne javanica* and *Thielaviopsis basicola* which are known to cause root knot and black rot of tomato and tobacco, respectively, in the rhizospheric soil. The subterranean termite *Odontotermes obesus*, an important pest in agricultural and forestry crops in India, is also controlled by HCN produced by rhizobacteria (Sakthivel and Karthikeyan 2012; Kannapiran and Sriramkumar 2011).

13.3.8 Siderophores

Siderophores are iron-chelating agents which are produced and utilized by a number of bacteria and fungi. These low molecular weight compounds are produced in soil rhizosphere under neutral to alkaline pH condition where there is an iron deficiency due to low iron solubility at elevated pH. Iron is one of the essential components for the cellular growth and metabolic activities of the bacteria. The bacteria utilize iron through siderophore production, which plays an essential role in determining the ability of bacteria to colonize plant roots and also to compete for iron with other microbes (Naik et al. 2013; Johri et al. 2003).

A. vinelandii produces siderophores under limited iron conditions. Recently, it has been found that *A. vinelandii* produces at least five different siderophores which are antibiotic in nature such as 2,3-dihydroxybenzoic acid, aminochelin (monocatechols), azotochelin (bis-catechol), protochelin (tris-catechol), and the yellow-green fluorescent pyoverdine-like azotobactin (Kraepiel et al. 2009; Barrera and Soto 2010; Page and Von Tigerstrom 1988). The main biotechnological applications of siderophores are as drug delivery agents (Mollmann et al. 2009), antimicrobial agents, and soil remediation. The PGPR with the siderophore-producing ability can prevent the root infection by phytopathogens in the rhizospheric region. In certain conditions, plants can also use microbial siderophores as iron sources where there is a lack of sufficient iron concentration in soil (Naik et al. 2013).

13.3.9 Polyhydroxybutyrate

Azotobacter cells have the ability to biosynthesize molecules that are most important in the field of biotechnological and biomedical applications. Under determined nutritional and favorable environmental conditions, *A. vinelandii* produces the intracellular polyester, i.e., poly-β-hydroxybutyrate (PHB), extracellular polysaccharide alginate, and catechol compounds (Barrera and Soto 2010).

13.3.10 Enzymes

Polyphenol oxidases (PPOs) and phenol oxidases (POs) are produced by the group of multi-copper protein family bacteria. Most phenol oxidases have been characterized from filamentous fungi, insects, and several plants. The distribution, occurrence, structural organization, and

localization of prokaryotic phenol oxidases seemed to be restricted to some species or to those at distinctive morphological or physiological stages of cell differentiation. The presence of phenol oxidases in members of the family *Azotobacteraceae* is highly presumed.

The strain *Azotobacter* sp. SBUG 1484 isolated from soil was confirmed for production of phenol oxidases at extracellular and intracellular environment with regard to a variety of cultural conditions. *Pseudomonas* species are also known to produce phenol oxidases and polyphenol oxidases which are also having similar functions. The interest in exploitation of *Azotobacter* in industrial applications such as pulp delignification, textile dye bleaching, biopolymer synthesis, etc. is emerging significantly. Significant interest in the application of phenol oxidases has also been generated in scientific fields concerning the detoxification and degradation of environmental pollutants and also concerning the production of fine chemicals and antibiotics (Herter et al. 2011).

13.3.11 Biofertilizer

The commercial history of biofertilizer (rhizobia) began with the launch of "Nitrogin" by Nobbe and Hiltner in 1895, followed by the discovery of *Azotobacter* and then the cyanobacteria. *Azotobacter* is used as a biofertilizer for the cultivation of most agricultural crops because of its high nutritional and plant growth-promoting rhizobacterial activities. Nitrogen-fixing bacteria are able to fix atmospheric nitrogen under different conditions independently, or in close association with other organisms or in strict symbiosis with them, such as *Rhizobium* legume plant symbiosis.

The *Rhizobium* is the most efficient type of association between diazotrophic microorganisms and plants and is one of the major components for agricultural practices in soybean crop. Different kinds of formulations have been developed from carrier materials such as talc, lignite, and vermicompost base which are being readily used all over the world. *A . chroococcum* has been used as a biofertilizer for many agricultural crops such as cereals, pulses by direct application, seed treatment, and by seedling dip methods.

Azotobacter species directly or indirectly increases germination of seeds; seeds having less germinating ability can increase germination by 20–30 %; this is because of the production of the PGPS by the bacteria, which reduce chemical nitrogen and phosphorus by 25 %, stimulating the plant growth. Plant growth-promoting rhizobacteria (PGPR) have been introduced by Kloepper and Schroth (1980) to define free-living, beneficial, root-colonizing bacteria. The term PGPR refers to all bacteria that are inhabitants of rhizospheric region of the plants which directly help the plant to uptake nutrients from soil by various mechanisms and also indirectly help the plant by suppressing the growth and activities of phytopathogens (Glick 1995).

The direct promotion of plant growth by PGPR may include the production and release of secondary metabolites such as plant growth regulators or facilitating the uptake of certain nutrients from the root environment. Application of PGPR also increases the rate of seed germination, root and shoot length, weights, chlorophyll content, tolerance to drought, salt stress, delayed leaf senescence, and yield of the crop (Polyanskaya et al. 2002).

The strains of *Azotobacter* showed their ability to invade the endo-rhizosphere of wheat and higher production of cellulase and pectinase. *A. chroococcum* are beneficial for plantation as they enhance growth and induce IAA production and phosphorus solubilization compared to agrochemicals and biofertilizer on agriculture crops (Sachin 2009). The higher the concentration of agrochemical application, the lower the plant growth (Martin et al. 2011). Different kinds of formulations have been developed from carrier materials such as talc, lignite, and vermicompost which are being readily used all over the world. Among different carrier materials used, vermicompost was the best carrier material for the survival of *A. chroococcum* and their cells have the most significant effect on improving the growth and yield parameters of summer rice cv. IR-36 (Roy et al. 2010).

13.3.12 Nitrogen Fixation

Nitrogen fixation is the biological reaction where nitrogen gas is converted into ammonia (NH_3). Ammonia is a form of nitrogen that can be easily utilized for biosynthetic pathways; nitrogen fixation is a critical process in the completion of the nitrogen cycle (Murcia et al. 1997; Barrera and Soto 2010). The nitrogen gas occupies about 79–80 % of the total atmospheric gases, and earth's atmosphere contains about 386 × 1016 Kg of nitrogen. Biological fixation of the atmospheric nitrogen can be estimated at about 175 million metric tons per year or about 70 % of all nitrogen fixed on the earth per year, the remaining of which is fixed by some microorganisms, autotrophs, or heterotrophs as free fixers. Living organisms that are present in the soil have profound effect on transformation, which provide food and fiber for an expanding world population. Although nitrogen is very abundant in nature, it often limits plant productivity because atmospheric nitrogen is only available to a wide range of organisms symbiotically associated with higher plants (Khan et al. 2008; Barrera and Soto 2010).

PGPR are root-colonizing bacteria which are able to fix atmospheric nitrogen through symbiotic and nonsymbiotic nitrogen fixation process. Some of the important nitrogen-fixing PGPR bacteria are *Azospirillum*, *Azotobacter*, *Pseudomonas*, *Azomonas*, *Alcaligenes*, *Bacillus*, *Rhizobium*, *Frankia*, cyanobacteria, etc.

Among all the *Azotobacter* species, *A. chroococcum* and *A. vinelandii* are the most studied diazotrophic, nonsymbiotic nitrogen-fixing bacterial spp. which are aerobic soil bacteria with a wide variety of metabolic capabilities (Mirzakhani et al. 2009; Khan et al. 2008). These bacteria are capable of synthesizing various plant growth-promoting substances, which may augment their performance as efficient inoculants for crop plants.

The species of *Azotobacter* are known to fix on an average 10 mg of N/g of carbohydrate under in vitro conditions. *A. chroococcum* happens to be the dominant inhabitant in arable soils capable of fixing N_2 (2–15 mg N_2 fixed/g of carbon source) in culture medium. The most efficient strains of *Azotobacter* would need to oxidize about 1000 Kg of organic matter for fixing 30 Kg of N/ha. Besides, soil is inhabited by a large variety of other microbes, all of which compete for the active carbon. Plant needs nitrogen for its growth and *Azotobacter* fixes atmospheric nitrogen non-symbiotically and plants get benefited especially cereals, vegetables, fruits, etc., and are known to get additional nitrogen requirements from *Azotobacter* (Tilak et al. 2005; Khan et al. 2008; Mirzakhani et al. 2009; Tejera et al. 2005).

13.4 Effect of Pesticides on *Azotobacter*

The production and utilization of food crops increased with the population from the past two decades to meet the demand of national food security. However, still there is need to increase food production to sustain self-sufficiency within the available land. Among all agricultural crops, rice is one of the major food crops in the Asian countries, and cultivation is found in all irrigated lands. Major production of rice comes from Asian countries such as China, Korea, India, Bangladesh, Japan, Vietnam, Pakistan, Indonesia, Thailand, Myanmar, and the Philippines. Even today, Asian farmers account for 92 % of the world's total rice production.

Farmers used to follow traditional cultivation practices for the production of rice prior to the introduction of advanced cultivation practices such as machineries, pesticides, chemical fertilizers, and high-yielding varieties. Among them, different pesticides such as insecticides, herbicides, fungicides, etc., are extensively used for the control of pests and diseases that resulted in better crop yield.

Due to unscientific methods and their extensive use, pesticides are largely distributed and contaminate soil, ground water, and sediments (Castillo et al. 2011). On the other hand, the demand for agricultural crops is increasing day by day due to the rapidly growing industrialization along with increasing population. There is need to increase the quantity of agricultural produce as well as improvement in the quality.

Pesticides and chemical fertilizers reaching the soil in significant quantities have direct effect on soil microbiological aspects, environmental pollution, and health hazards (Martin et al. 2011) leading to alterations in ecological balance of the soil microflora (Naik et al. 2007) and causing everlasting changes in the soil microflora (Aleem et al. 2003). They adversely affect soil fertility and crop productivity, inhibit N_2-fixing bacterial activity, suppress nitrifying bacteria, alter nitrogen balance of the soil (Sachin 2009), interfere with ammonification, and hamper mycorrhizal symbiosis in plants and nodulation in legumes (Reinhardt et al. 2008). This alters the soil living ecosystem, soil microbe interaction, plant growth and soil structure, soil fertility and crop productivity, organic matter decomposition, and biogeochemical cycling of elements and inhibits the soil microorganisms as biocontrol agents and biological N_2 fixation processes.

On the other hand, there are some chemical compounds which adversely affect the population of *Azotobacter* in soil such as pesticides, insecticides, fungicides, herbicides, and nematicides which are being used worldwide for the management of many agricultural crops. The population of *Azotobacter* was also affected by several factors in soil. In the context of soil, pests are fungi, bacteria, insects, worms, nematodes, etc., that can cause damage to field crops. Thus, in broad sense, pesticides are insecticides, fungicides, bactericides, herbicides, and nematicides that are used to control or inhibit plant diseases and control weeds and insect pests.

Due to increase in the world population, demand for increased in the food production was felt, to cater to the needs of national food security. In view of this, in the last two decades, the production and utilization of food crops were also increased. However, still there is a need to increase the production of food significantly to sustain self-sufficiency within the available land. Among all the crops, rice is one of the staple food and main agricultural crops in many countries including India. For cultivation of paddy, several kinds of pesticides and chemical fertilizers are being applied extensively since 1992 in major paddy-growing areas of the Asiatic region (www.fao.orgldocrepI003/x6905e/x6905e.htm) in order to control pests and diseases to improve rice yield. Rice is a staple food of states in southern and eastern India, and cultivation is found almost all over India. Plant protection has been an integral part of agricultural crop production. Average yield losses in India are estimated to be 10–30 % which are caused by insects, diseases (bacterial and fungal), and weeds. Nearly 43.5 % of total pesticides are used to protect cotton and 38.6 % for protection of rice crop (Kadam and Gangawane 2005).

Although wide-scale application of pesticides is an essential part of augmenting crop yields, an ideal pesticide should have the ability to destroy target pest and should be able to undergo degradation into nontoxic substances as quickly as possible. The commonly used organochlorine pesticides for the control of pests in paddy are dieldrin, aldrin, heptachlor, toxaphene, hexachlorocyclohexane (HCH), endosulfan, carbofuran, monocrotophos, phorate, diazinon, sodium pentachlorophenate, fenthion, phosphamidon, methyl parathion, and azinphos-methyl. Excessive use of these chemicals leads to microbial imbalance, environmental pollution, and health hazards. Finally they enter the human and animal food chain causing neurotoxicological disorders.

Herbicides used for agriculture are harmful to *Azotobacter* spp. Glyphosate herbicides have shown to not only inhibit the nitrogen fixation process in *Azotobacter chroococcum* but also reduce the bacterium's respiration rate by 40–60 % and hence preclude its positive effects (Chennappa et al. 2014b). Simazine is an herbicide widely used in agriculture to control broad-leaved annual and perennial weeds and is applied at concentrations in the range from 1.0 to 4.0 Kg/ha. It has been previously reported that simazine affects nitrogenase activity and ATP content of *Azotobacter*. Niewiadomska (2004) reported the effect of carbendazim, thiram, and imazetapir on nitrogenase activity in soil.

Nitrogen-fixing activity of *Azotobacter* strains was not significantly affected by 5 % phorate concentration, while 5 % glyphosate concentration showed negative impact on nitrogen-fixing ability of *Azotobacter* strains. On the other hand,

5 % concentration of pendimethalin was lethal to bacterial respiration and reduced nitrogen fixation (Chennappa et al. 2013).

13.5 Biodegradation of Pesticides

Azotobacter species is known to biodegrade chlorine-containing aromatic compounds, such as insecticide, fungicide, and herbicide, which are found lethal to human and animal health. Several physical, chemical, and biological forces act up on the pesticides when they reach the soil. However, biological forces particularly microbes play a significant role in degrading the pesticides than the physical and chemical forces.

Several biodegradation studies have been carried out throughout the world in order to minimize the pesticide residues in food and food chain. For degradation of these hazardous compounds, a number of microorganisms are used, and some of the soilborne bacterial species such as *Arthrobacter* spp., *Burkholderia* spp., *Bacillus* spp., *Azotobacter* spp., *Flavobacteria* spp., *Pseudomonas* spp., and *Rhodococcus* spp. are widely used in the majority of biodegradation and bioremediation studies (Castillo et al. 2011).

Many soil microorganisms have the ability to act upon pesticides and convert them into simpler nontoxic compounds. For example, bacterial genera like *Pseudomonas*, *Azotobacter*, *Clostridium*, *Bacillus*, *Thiobacillus*, *Achromobacter*, etc., and fungal genera like *Trichoderma*, *Penicillium*, *Aspergillus*, *Rhizopus*, *and Fusarium* play an important role in the degradation of the toxic chemicals or pesticides in soil. Natural hydrocarbons in soil like waxes, paraffins, oils, etc., are degraded by fungi, bacteria, and actinomycetes (Castillo et al. 2011; Latifi et al. 2012; Chennappa et al. 2013). For example, ethane (C_2H_6), a paraffin hydrocarbon, is metabolized and degraded by *Mycobacteria*, *Nocardia*, *Streptomyces*, *Pseudomonas*, *Flavobacterium*, and several fungi. *Azotobacter* facilitate the mobility of heavy metals in the soil and thus enhance bioremediation of soil from heavy metals, such as cadmium, mercury, and lead, which are used in plant protection.

The highest amount of IAA (34.40 µg/mL) was produced by the different isolates of *Azotobacter* species in the media supplemented with 5 % pendimethalin, chlorpyrifos, glyphosate, and phorate. This shows the tolerance of *Azotobacter* toward pesticides (Chennappa et al. 2013; Shafiani and Malik 2003). Hydrocarbon-degrading potential of marine nitrogen-fixing bacterium *A. chroococcum* was isolated from Tuticorin harbor which revealed the possibility of using marine nitrogen-fixing hydrocarbon-degrading bacteria and their biosurfactants in the abatement of marine oil pollution. Maximum degradation of lindane was recorded at 10 ppm concentration by *A. chroococcum* on the 8th week of incubation (Anupama and Paul 2009).

Degradation is often considered to be synonymous with mineralization, and the degradation pathways of catechol, protocatechuic acid, gentisic acid, ferulic acid, resorcinol, and 2,4-dichlorophenoxyacetic acid are described (Mrkovacki et al. 2002).

Insecticides influence soil microflora and their biochemical activities associated with soil fertility and can also trigger the growth of *Azotobacter* (Das and Mukharjee 1998). Relatively, a study was conducted to establish a relation between pendimethalin herbicide and *A. chroococcum* which showed a transformational process of the herbicide degradation into nontoxic products, thus exhibiting the importance of the bacterium not only in agriculture but also for the environment (Chennappa et al. 2014b; Kole et al. 1994).

Azotobacter also biodegrade chlorine-containing aromatic compounds, such as 2,4,6-trichlorophenol which was previously used as an insecticide, fungicide, and herbicide for many field crops but later found to have mutagenic and carcinogenic effects. *Azotobacter* dissimilates many aromatic substances including simple phenols, substituted phenolics, and pesticides. Moneke et al. (2010) studied the biodegradation of glyphosate herbicide in vitro using *Azotobacter* isolates from rice fields. Kadam and Gangawane (2005) reported the degradation of phorate by *Azotobacter* spp. under in vitro conditions with effective concentration which has been used as a pesticide against cotton crop. Thiram

(fungicide) is degraded by a strain of *Pseudomonas*, and the degradation products are dimethylamine, proteins, sulpholipids, etc. Similarly, *Azotobacter* growth was observed in the media supplied with 1 and 2 mg/L endosulfan, which could be due to the tolerance of bacterium to these pesticide concentrations but bacterial growth was reduced.

The accumulation of endosulfan in the cysts of *Azotobacter* will stimulate the faster degradation of endosulfan isomer resulting in the formation of metabolites. An oxidation reaction occurs in the metabolic pathway, producing endosulfate, followed by double hydration producing endodiol and finally a dehydration process generating endosulfan ether. In accordance with the IAA production by *Azotobacter* spp., no significant effect was determined with 2–10 mg/L endosulfan under in vitro conditions (Castillo et al. 2011).

Hence, biodegradation of pesticides and chemical fertilizers plays an important role in agricultural practice to serve safe food to the world and clean environment for the future generation. *Azotobacter* species are natural inhabitants of rhizosphere and have the ability to utilize the abovementioned herbicides and insecticides as sole carbon source for their energy. Hence, usage of *Azotobacter* will help in organic farming to improve soil nutrients and fertility and also to remove environmentally hazardous chemicals from food chain, thereby ensuring the supply of healthy food for mankind. It is clear from the above discussion that *Azotobacter* can play an important role in agriculture under diverse environmental conditions by raising crop yield, protecting plants, and increasing soil enzyme activities directed against pollutants.

Among the hundreds of species of beneficial soil bacteria, there are groups that will pull nitrogen out from the air and put it into a liquid form available to feed plants. When there are sufficient nitrogen-fixing bacteria in a soil, the need for fertilizer goes way down. Other bacteria will decompose organic matter and even degrade pesticide residues if they are in soil. Soilborne bacteria will actually reduce soil compaction by improving soil structure, creating microscopic spaces in the soil to hold air or water. Some soil bacteria will suppress soil pathogens that could cause disease in plants, reducing the need to use any fungicides. There are a number of products in the market that will help to restore many of the beneficial soil microbes lacking in the soil. These products are available in the market in powder or liquid form and are applied as a seed treatment and foliar spray or drenched directly into soil around the crops.

13.6 Conclusion

According to the guidelines for the approval of pesticides, effects of pesticides on soil microorganisms and soil fertility should be determined. The fertility of soil depends not only on the texture of soil but also on the biological ability within it. The microbial diversity may have been changed following pesticide use and such changes may affect soil fertility. Soil microorganisms therefore play an important role in soil fertility. The use of pesticides to protect crops may alter the soil biological ability either by direct or indirect mode, but the knowledge of soil microbial ability to degrade pesticides and the influence of pesticides on microbial diversity in soil are still limited. Understanding the effect of pesticides on soil microflora and their beneficial activities is an important part of the pesticide's risk assessment. Certain pesticides stimulate the growth of microorganisms, but other pesticides have depressive effects or no effects on microorganisms when applied at normal rates. In view of this, the diversity of *Azotobacter* species has the ability to tolerate and degrade pesticides efficiently under *in vitro* and *in vivo* conditions.

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Beneficial Effects and Molecular Diversity of Endophytic Bacteria in Legume and Nonlegumes

14

Surjit Singh Dudeja

Abstract

Endophytes colonize the plant inner tissues, commonly coming from the soil. Endophytes could be considered of two types, one having plant growth-promoting activity, while another type having the ability to fix nitrogen. Endophytic bacteria can stimulate plant growth directly through production of phytohormones and volatiles, enhance nutrient acquisition, and suppress stress-induced ethylene synthesis. Bacterial endophytes protect against disease, against abiotic stresses of salinity and heavy metals. Pathogenic, symbiotic nitrogen fixers and mycorrhiza coordinate sequential expression of plant or microbial genes. But in both types of endophytic association, partial interactions, signaling pathways, coordination, and gene expression of host and bacteria are known. To obtain nitrogen-fixing cereals, now emphasis has been shifted from rhizobia to actinorhizal symbiosis based on the recent studies on model legumes. Endophytic bacteria have been found in almost every plant studied. All plants may harbor one or more number of bacteria of genera mainly *Bacillus*, *Paracoccus*, *Sphingomonas*, *Inquilinus*, *Pseudomonas*, *Serratia*, *Mycobacterium*, *Nocardia*, *Brevibacillus*, *Staphylococcus*, *Lysinibacillus*, *Bosea*, *Rhodopseudomonas*, *Phyllobacterium*, *Ochrobactrum*, *Starkeya*, *Agromyces*, *Ornithinicoccus*, *Actinobacterium*, *Paenibacillus*, *Methylobacterium*, *Pedobacter*, *Aerococcus*, *Stenotro-phomonas*, *Streptomyces*, *Dyella*, and others. Most of the endophytic isolates upon inoculation in different agricultural crops significantly increased plant

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growth under greenhouse conditions. This plant growth promotion is the result of many different factors that can act directly or indirectly. Efficacy of two endophytic bacterial strains *Bacillus subtilis* and *B. licheniformis* under field conditions showed that an increase up to 22.5 % in grain yield of chickpea was observed with *Bacillus subtilis* inoculation. However, inoculation with all the recommended biofertilizers – *Mesorhizobium*, PSB and PGPR – could increase up to 14.4 % grain yield in chickpea.

Keywords
Endophytes • Plant growth promotion • N_2 fixation • Microbial inoculants • Molecular diversity

14.1 Introduction

In recent years, interest in endophytic microorganisms has increased, as they play a key role in sustainable agriculture and environment. Endophytes colonize the plant inner tissues, commonly coming from the soil and entering the plants by intercellular spaces or little root cracks. Endophytic bacteria influence the microbial balance in the plant host and participate in the plant development. Communication between plants and microorganisms is important for plant colonization by pathogenic or nonpathogenic bacteria (Hardoim et al. 2008). To attract microorganisms, plants exude flavonoids, ethanol (Williams and Yavitt 2009), and methanol (Sy et al. 2007; Jourand et al. 2005). Bacterial communication during the interactions with plant is coordinated and creates favorable environment for the changes in microbial population (Soto et al. 2006; Sanches-Contreras et al. 2007).

Bacterial endophytes inhabit the below- and above-ground tissues of all terrestrial plants examined and can affect plant physiology and growth under normal and stressed conditions. Endophytic bacteria can stimulate plant growth directly through production of phytohormones and volatiles (Hardoim et al. 2008), enhance nutrient acquisition (Hurek et al. 2002; Boddey et al. 2003), and suppress stress-induced ethylene synthesis (Glick 2004). Bacterial endophytes protect against disease (Conn et al. 2008) and against abiotic stress of salinity and heavy metals (Idris et al. 2004; Mayak et al. 2004; Han et al. 2015; Babu et al. 2015; Kolbas et al. 2015).

One of the goals of research on pathogenic, symbiotic nitrogen fixers, mycorrhizal interactions with legumes and nonlegumes is to identify and assign a function to all genes acting in the pathways leading from microbial recognition to the development of pathogenic or symbiotic structures. Legumes encode and synchronize all functions necessary for nodule development. Both symbionts coordinate sequential expression of plant or rhizobial genes. Up to some extent, rhizobial, pathogenic, and mycorrhizal interactions are similar (Carole et al. 2013). Though majority of the signals and expression of genes in plants and microorganisms are known (Dudeja et al. 2012a), still a lot is to be unrevealed. Similarly there are many plant-associated endophytic bacteria known, which are living within plants without triggering persistent and apparent defense responses or visibly do not harm the plant. In some cases, even a stimulation of plant growth due to the presence of these microbes within the plant system has been reported (Dudeja et al. 2012b; Dudeja and Nidhi 2014; Dudeja and Giri 2014; Turner et al. 2013). It is now generally accepted that understanding these interactions is possible if both partners are considered simultaneously (Zilber-Rosenberg and Rosenberg 2008). It is still mostly unknown which particular plant genetic loci are controlling the interactions with the plant-microbe endophytic system and which signals are responsible for these interactions.

Endophytic microbes are able to overcome plant defense responses and are successful in colonization of the host (Zamioudis and Pieterse 2012; Alqueres et al. 2013).

Finely tuned interactions and molecular communication between endophytes and plants seem to be similar to mycorrhiza or rhizobia and are also systemically regulated processes. The induction of systemic immunity responses like induced systemic resistance or the systemic-acquired resistance response by pathogens is the result of multiple responses. A detail of the entire interactive response is still not fully understood. Secondary metabolites, like the surfactin lipopeptide, are produced by certain biocontrol *Bacilli* (Garcia-Gutiérrez et al. 2013) or volatile compounds of plant-associated microbes. The biocontrol activity of microbial inoculants is due to multiple effects.

14.2 Beneficial Effects of Endophytes

Plant-beneficial endophytic properties are continually benefiting the plants in natural ecosystems. A better understanding of the bacteria that inhabit legume and nonlegume plants will help in better exploitation of these for different ecosystem processes. Nitrogen-fixing epiphytic or rhizospheric bacteria have been identified in several genera of α- and β-proteobacteria including *Acetobacter, Azoarcus, Azospirillum, Azotobacter, Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Glucenobacter*, and *Pseudomonas* (Schmid and Hartmann 2007; Cocking 2009; Richardson et al. 2009). Few of these bacteria also exist endophytic. Particularly *Azoarcus* spp., *Herbaspirillum seropedicae*, and *Glucenobacter* can form endophytic association with maize, rice, and wheat and contribute to improved plant growth. These endophytes in the roots don't induce any specialized structure-like legume nodules but invade plant tissues and reside intracellularly in the living plant cells. Endophytic microbes able to fix nitrogen may have an advantage over epiphytic or associative nitrogen fixers, as they colonize the interior of plant roots and can establish themselves in niches that provide more appropriate conditions for effective nitrogen fixation and subsequent transfer of the fixed nitrogen to the host plant (Reinhold-Hurek and Hurek 1998, 2011).

Endophytic bacteria can stimulate plant growth directly through the production of phytohormones and volatiles, enhance nutrient acquisition, and suppress stress-induced ethylene synthesis; bacterial endophytes have also been found to protect against disease (Conn et al. 2008) and against abiotic stress such as salinity and heavy metals (Idris et al. 2004; Mayak et al. 2004). The production of the auxin IAA together with cytokinin has been reported in numerous endophytic bacterial strains. These two hormones play a central role in regulating plant development (Kramer and Bennett 2006; Angulo et al. 2014). Phytohormones produced by bacteria thus enhance root growth, which in turn favor the uptake of soil water and minerals and has a positive effect on plant growth (Steenhoudt and Vanderleyden 2000). IAA has been suggested as an important signaling molecule involved in the plant–endophytic communication process. Gibberellin and ethylene are also involved in enhancing plant growth. Majority of the endophytes promoted the growth of chickpea roots in root growth promotion assay in agar plates; however, chickpea nodule endophytic bacteria were better root growth promoters as compared to others (Saini et al. 2015a). Similarly in field pea root growth promotion assay, 63.3 % of nodule endophytic bacteria out of 60 isolates were root growth promoters (Narula et al. 2013a).

Other beneficial effects of PGP activity of endophytes include the production of siderophores, vitamins, and solubilization of phosphorous. Iron is essential for plant growth and acts as a global regulator and, in bacteria, is a key element for nitrogen fixation activity. Microorganisms produce siderophores for iron acquisition. Microbial siderophores may stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots or by inhibiting pathogen growth. However, 11 endophytic bacterial isolates from legumes and nonlegumes also produced siderophores but showed low biocontrol activity against plant pathogens (Giri and Dudeja 2015). Vitamins, thiamine, biotin,

riboflavin, and niacin have been shown to be produced by strains of *Azospirillum* and *Azotobacter* (Richardson et al. 2009).

Insoluble phosphorous is converted into soluble form through acidification, secretion of organic acids or protons, and chelation by endophytic bacteria, thereby helping to improve phosphate nutrition in the associated plants (Sturz and Nowak 2000; Richardson et al. 2009; Kumar et al. 2013). A study showed that a total of 56 % of nodule endophytic bacteria from field pea and chickpea, 57 % of isolates from roots of legumes, and 36 % from nonlegume roots were solubilizing phosphate (Narula et al. 2013a; Saini et al. 2015b).

For some plant growth-promoting (PGP) positive effects of endophytes on plant growth are indirect and result from mechanisms involving antagonism toward phytopathogens and the induction of systemic resistance pathways in the plant (Raaijmakers et al. 2009; Bhattacharya and Jha 2012). These beneficial bacteria can help to suppress a broad spectrum of viral, bacterial, and fungal pathogens (Saharan and Nehra 2011). Interestingly endophytes can also confer tolerance to a number of abiotic stresses and can stimulate plant growth even in areas affected by drought (Creus et al. 1996), salt (Creus et al. 1997; Bacilio et al. 2004), and in soils polluted by heavy metals (Belimov and Dietz 2000). All factors act in combination to achieve a significant increase in plant biomass in the field (Bhattacharjee et al. 2008).

Despite of the difficulty to understand the complexity of mechanisms involved in plant-microbe interactions, some bacterial genes responsible for metabolism, stress defense, and pathogenicity that present an important role on plant-bacterial interactions has partially been characterized. Little is known about the role of plant exudates and N-acyl-homoserine lactones as signaling molecules on the expression of bacterial genes involved in endophyte-plant interactions (Camilli and Bassler 2006; Sanches-Contreras et al. 2007; White and Winans 2007). Biofilm formation on the plant may be the first step toward endophytic colonization by *Methylobacterium* (Rossetto et al. 2011). Genes coding PAT protein in maize are reported to be involved in association of endophytic microbial communities (da Silva et al. 2014).

Expression of methanol dehydrogenase enzyme provides competitive advantage on the plant surface (Williams and Yavitt 2009). Further genes for phosphate regulation, stress, antibiotic production (Li and Zhang 2007; Gristwood et al. 2009), and virulence gene expression (Cheng et al. 2009) are also involved in the initial interactions. Gene responsible for the transport of solutes with sodium solute symporter is reported to be associated with the stress response and to plant metabolism (Hardoim et al. 2008). Lycopene synthesis protects the cell against oxidative damages, different types of radiations, and dissections. Aminocyclopropane deaminase that degrades ACC is known to be involved in the initial interactions. Production of enzymes for the hydrolysis of membrane phospholipids results in membrane damage by the pathogenic bacteria which help in effective host colonization. Further α-glucosidase from *Cladosporium* endophyte is involved as biocontrol agent (Singh et al. 2015). Endophytic *Bacillus* produces antifungal lipopeptides and induces host defense gene expression in maize (Gond et al. 2014).

Root colonization involves migration toward the plant roots, adsorption, and attachment onto the root system, microbial proliferation, and the formation of biofilm structures at the surface of roots (Compant et al. 2010; Reinhold-Hurek and Hurek 2011; Dourado et al. 2013; Hartmann et al. 2014). However, endophytic bacteria fixing nitrogen in root tissues probably employ different mechanisms, either alone or in combination, to successfully colonize plant roots and have edge over other soil microorganisms. This includes chemotaxis toward roots, twitching motility, and surface attachment. In *Azoarcus*, the *pil*A, *pil*B, and *pil*T genes were essential for root-surface colonization and for infection of plant tissues in rice (Krause et al. 2006; Böhm et al. 2007). Involvement of surface polysaccharides like exopolysaccharides and lipopolysaccharides in the colonization of roots is also advocated (Downie 2010). Mutant of *Azospirillum brasilense* having modified lipopolysaccharide composition resulted in impaired attachment of the mutant to maize roots and reduced root colonization (Jofré et al. 2004). The ability to attach to the surface and colonize the internal tissue of maize roots

was 100-fold lower than that of the wild type in two mutants of *H. seropedicae* in the genes *rfb*B and *rfb*C involved in the biosynthetic pathway of rhamnose (Balsanelli et al. 2010). A purified outer membrane protein from *A. brasilense* was shown to bind to roots of wheat, corn, and sorghum seedlings (Burdman et al. 2001). In endophytes, undifferentiated tissues above the root tips and the points of emergence of lateral roots are the sites for primary colonization and entry into the plant (Reinhold-Hurek and Hurek 1998; Giri and Dudeja 2013a, b), and cellulolytic and pectinolytic enzymes contribute to the infection process by degrading plant cell walls (Adriano-Anaya et al. 2005). Rice plant differentially regulates the genes of nitrogen-fixing bacteria *Burkholderia kururiensis* for endophytic colonization (Coutinho et al. 2015). Inoculated *Azoarcus* bacteria were observed inside the root after 3 weeks of inoculation, while the number of colonized cells considerably decreased in case of a mutant defective in endogluconase activity. However, since genes encoding cell wall-degrading enzymes have not been found in all endophytic PGP bacteria, some of them may passively enter the root system using lateral roots. After penetration, some endophytes may then colonize nutrient-rich intercellular spaces of the root cortex, move toward the xylem, and spread into stems and leaves (Olivares et al. 1996).

To ascertain the role of different traits responsible for entry into the root, based on presence or absence of different characteristics, different combinations of co-inoculants were used in addition to their individual inoculation. A total of four combinations of co-inoculants were used, (1) cellulase$^+$ and cellulase$^-$, (2) root entry$^+$ and root entry$^-$, (3) P solubilization$^+$ and P solubilization$^-$, and (4) cellulase$^+$, root entry$^+$, P solubilization$^+$, auxin$^+$, and siderophore$^+$, to observe the entry in legume (chickpea, field pea) and nonlegume (wheat and oats) roots. All the co-inoculants were now able to enter the roots of chickpea, field pea (Table 14.1), wheat, and oat (Table 14.2) being grown in MS medium tubes irrespective of their individual behavior. There was a statistically significant difference in colonization pattern as well as fresh root growth as compared to control in all the crops, and better results were obtained as compared to their single inoculation. Co-inoculation of LRE7, CNE215, ORE27, PNE17, and PNE92 in chickpea, field pea, wheat, and oat resulted in highest growth promotion, number of root epiphytes, and fresh weight of roots. The root exudates of different crops positively promoted the growth of endophytic bacterial isolates irrespective of their host or tissue from which these were isolated. Following rhizosphere colonization, bacteria were also found to attach to the rhizoplane, i.e., the root surface. Further with an increase in age of the plant roots, increase in endophytic detection was observed in roots of all the four crops, i.e., chickpea, field pea, wheat, and oat. Possibly with the increase in age of plant roots, more cracks in roots occur, and this ultimately resulted in endophytic colonization. As such presence of cellulases or other enzymes in endophytes is unable to make an entry in the roots. The co-inoculation of cellulase-negative endophytes with cellulase-positive endophytes leads to entry of both endophytes in roots. Even endophytic bacteria with other traits were also able to enter roots of chickpea, field pea, wheat, or oat after co-inoculation, meaning that both endophytic bacteria complement or compensate the lacking trait and the presence of cell wall-degrading enzymes is not mandatory.

Rhizobia–legume, rhizobia–*Parasponia*, and actinorhizal and mycorrhizal symbioses have led to renewed interest in exploiting the possibility of transferring nitrogen-fixing ability to nonlegume crops (Charpentier and Oldroyd 2010; Beatty and Good 2011; Geurts et al. 2012). It has been known for several years that several components of the legume symbiotic signaling pathway play a role in both nodulation and mycorrhizal symbiosis. The common system is also required for actinorhizal nodulation (Gherbi et al. 2008a, b; Markmann et al. 2008). Nod factor signal transduction pathway is similar in root nodules in legumes and actinorhizal plants (Hocher et al. 2011). Series of well-characterized symbiotic genes in legumes exhibit similar expression patterns as in actinorhizal symbiosis. Signaling mechanisms involved in mycorrhizal symbioses were evolved first. The majority of land plants, including cereals, can form mycorrhizal association with fungi belonging to the phylum

Table 14.1 Root colonization in legumes co-inoculated with bacterial endophytes

Endophytic bacterial isolates	Log CFU at 21 days			Presence of root endophytes		Fresh root weight g plant^{-1}	
	Growth promotion (per mL)	Root epiphytes (per plant roots)	Root endophytes (per plant roots)	14 days	21 days	14 days	21 days
Chickpea							
Control	–	–	–	–	–	0.96	1.10
LRE7 + ORE35	7.21	3.12	2.78	–	Both +	2.00	2.81
PNE 92+ ORE35	6.99	3.08	2.67	–	Both +	1.91	2.21
CNE 215 + WRE4	6.91	3.83	2.13	–	Both +	2.12	2.83
LRE7 + CNE215+ ORE27 + PNE17+ PNE92	7.38	4.06	2.56	–	All +	2.23	2.90
SE(m)	0.058	0.119	0.203	–	–	0.05	0.01
CD at 5 %	0.184	0.380	0.647	–	–	0.17	0.04
Field pea							
Control	–			–		0.91	1.11
LRE7 + ORE35	7.39	3.42	2.06	–	Both +	1.95	2.30
PNE 92+ ORE35	7.09	3.67	2.45	–	Both +	1.91	2.21
CNE 215 + WRE4	6.86	3.36	2.06	–	Both +	1.97	2.80
LRE7 + CNE215+ ORE27 + PNE17+ PNE92	7.96	3.96	2.98	–	All +	2.03	2.91
SE(m)	0.075	0.119	0.198	–	–	0.01	0.06
CD at 5 %	0.252	0.354	0.544	–	–	0.04	0.19

Cellulase$^+$ + cellulase$^-$ = LRE7 + ORE35; root entry$^+$ + root entry$^-$ = PNE 92+ ORE35; P solubilization$^+$ + P solubilization$^-$ = CNE 215 + WRE4; cellulase$^+$ + root entry$^+$ + P solubilization$^+$ + Auxin$^+$ + Sid$^+$ = LRE7 + CNE215 + ORE27 + PNE17 + PNE92

Glomeromycota. Most of the genes closely related to those involved in the signaling pathways leading to nodulation or mycorrhizal symbiosis have been identified in the rice genome. Now it is well established that rice genes are able to restore mycorrhizal symbiosis and nodulation in a *Medicago* mutant and a rice mutant fails to develop mycorrhizal symbiosis (Chen et al. 2007). Though nodules mostly did not contain rhizobia or bacteria which were not released from infection threads (Godfroy et al. 2006). Indicating that rice can trigger an appropriate signaling pathway, which would be helpful to engineer nitrogen-fixing symbiosis in cereals.

Further the nonlegume actinorhizal and Parasponia symbioses could be more suitable models based on the recent studies on model legumes to obtain nitrogen-fixing cereals. Actually Frankia strains can interact with nonlegumes and could be a better choice than rhizobia to infect cereals. In contrast to extensive molecular knowledge of rhizobia–legumes interactions, there are still only limited data available on the molecular aspects and signaling interactions leading to associative and/or endophytic associations. Particularly the interactions with PGP endophytes appear to be less complex as compared to nitrogen-fixing endophytes. This will provide additional knowledge leading to a broad view of the plant and microbial gene interactions and expressions that could be manipulated to engineer new nitrogen-fixing plants.

14.3 Molecular Diversity of Endophytes in Legumes and Nonlegumes

Our knowledge of the role, diversity, and transmission of bacterial endophytes colonizing native

Table 14.2 Root colonization in nonlegumes co-inoculated with bacterial endophytes

Endophytic bacterial isolates	Log CFU at 21 days			Presence of root endophytes		Fresh root weight g plant^{-1}	
	Growth promotion (per mL)	Root epiphytes (per plant roots)	Root endophytes (per plant roots)	14 days	21 days	14 days	21 days
Wheat							
Control	–			–		0.11	0.17
LRE7 + ORE35	6.98	3.45	2.36	–	Both +	0.36	0.45
PNE 92+ ORE35	6.79	3.34	2.77	–	Both +	0.38	0.42
CNE 215 + WRE4	7.01	3.37	2.83	–	Both +	0.32	0.49
LRE7 + CNE215+ ORE27 + PNE17+ PNE92	7.18	4.08	2.64	–	All +	0.39	0.51
SE(m)	0.069	0.121	0.205	–	–	0.01	0.01
CD at 5 %	0.204	0.389	0.649	–	–	0.04	0.04
Oats							
Control	–			–		0.12	0.17
LRE7 + ORE35	6.86	3.32	2.44	–	Both +	0.40	0.46
PNE 92+ ORE35	6.79	3.28	2.36	–	Both +	0.32	0.40
CNE 215 + WRE4	6.65	3.94	2.57	–	Both +	0.40	0.48
LRE7 + CNE215+ ORE27 + PNE17+ PNE92	7.04	4.97	2.96	–	All +	0.41	0.57
SE(m)	0.12	0.10	0.12	–	–	0.04	0.07
CD at 5 %	0.35	0.30	0.35	–	–	0.01	0.02

Cellulase$^+$ + cellulase$^-$ = LRE7 + ORE35; root entry$^+$ + root entry$^-$ = PNE 92+ ORE35; P solubilization$^+$ + P solubilization$^-$ = CNE 215 + WRE4; cellulase$^+$ + root entry$^+$ + P solubilization$^+$ + Auxin$^+$ + Sid$^+$ = LRE7 + CNE215 + ORE27 + PNE17 + PNE92

plants is still limited. Both culturable and unculturable endophytic bacteria belonging to large number of different classes and genera of bacteria have been reported from different parts of world. However, the most commonly observed endophytic bacteria belongs to *Actinobacterium*, *Aerococcus*, *Agromyces*, *Arthrobacter*, *Azoarcus*, *Bacillus*, *Bosea*, *Bordetella avium*, *Bradyrhizobium*, *Brevibacillus*, *Burkholderia*, *Clavibacter*, *Curtobacterium*, *Devosia*, *Dyella*, *Ensifer*, *Enterobacter*, *Escherichia*, *Gluconobacter*, *Inquilinus*, *Klebsiella*, *Lysinibacillus*, *Mesorhizobium*, *Methylobacterium*, *Microbacterium*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Ochrobactrum*, *Ornithinicoccus*, *Paenibacillus*, *Pantoea*, *Paracoccus*, *Pedobacter*, *Phyllobacterium*, *Pseudomonas*, *Pseudomonas chlororaphis*, *Rhizobium*, *Rhodopseudomonas*, *Serratia*, *Sphingomonas*, *Staphylococcus*, *Starkeya*, *Stenotrophomonas*, *Streptomyces*, and *Streptomyces oryzae* sp. nov. (Sessitsch et al. 2012; Dudeja et al. 2012b; Zhao et al. 2013; Kumar et al. 2013; Dudeja and Giri 2014; Saini et al. 2015a, b).

Recently one of the endophytes isolated from root nodules of legume *Sophora alopecuroides* was closely related to *Pseudomonas chlororaphis* (Zhao et al. 2013). Tagging with *gfp* gene indicated that *P. chlororaphis* colonize in root or root nodules. Co-inoculation with *Mesorhizobium* sp. resulted in increased siderophore production, phosphate solubilization, organic acid production, IAA production, antifungal activity, and growth index in growth assays under greenhouse conditions. Endophytic bacteria of wild soybean root belonged to six bacterial groups, and Proteobacteria and Firmicutes were the dominant endophytes in wild soybean with 46.8 % and 13.6 % of total clones. Actinobacteria, Bacteroidetes,

Acidobacteria, Deincoccus-Thermus, and Archaea were less represented. 18.8 % of clone sequences were similar to those of uncultured bacteria in the environment (Wu et al. 2014).

A large number of determinants like bacterial species, host genotypes, host developmental stage, and environmental and soil condition determine the microbial population density in a particular host. Most commonly, endophytes has been isolated from *Acacia*, *Acacia salicina*, *A. stenophylla*, alfalfa, *Argyrolobium*, banana, bean, carrot, chickpea, chilly, citrus berry, clover, coffee, *Conzattia*, cowpea, fenugreek, field pea, *Hedysarum*, *Kennedia*, *Leucaena*, *Lotus*, maize, *Medicago*, *Melilotus*, *Mimosa*, mung bean, oats, O*nobrychis*, orchids, *Ornithopus*, *Oxytropis*, *Panax notoginseng*, peanut, potato, *Psoralea*, rice, *Scorpiurus*, *Sesbania*, *Sophora alopecuroides*, soya bean, *Stemona earthnet*, sugarcane, *Tetragonolobus*, Thai jasmine rice, *Typha angustifolia*, *Vicia*, and wheat (Rosenblueth and Martínez-Romero 2006; Muresu et al. 2008; Palaniappan et al. 2010; Hoque et al. 2011; Wei and Wu 2012; Dudeja et al. 2012b; Rungin et al. 2012; Kumar et al. 2013; Etesami et al. 2013; Ma et al. 2013; Narula et al. 2013b; Wang et al. 2013; Dudeja and Nidhi 2014; Dudeja and Giri 2014; Wu et al. 2014; Jia et al. 2014; Mingma et al. 2015; Saini et al. 2015a).

14.4 Beneficial Effects of Endophytic Bacteria

In most of the studies, inoculation of endophytic isolates significantly increased plant growth and productivity in different agricultural crops (Carvalho et al. 2014). Strains of *Paenibacillus macerans* due to the presence of multiple traits promoted plant growth under greenhouse conditions during seedling acclimatization in orchid species of *Cymbidium eburneum* (Faria et al. 2013). Using these two bacterial strains as inoculants, enhancement in crop productivity in all the tested crops was observed. Sturz et al. (1997) showed that root bacterization of bacterial endophytes like *Bacillus megaterium*, *Bordetella avium*, and *Curtobacterium luteum* consistently promoted growth of red clover. Gyaneshwar et al. (2001) isolated six closely related N_2-fixing bacterial strains identified as *Serratia marcescens* from different rice varieties. Inoculation of the strain resulted in a significant increase in root length and root dry weight of rice. The inoculated plants confirmed the strain was endophytically established within the roots, stems, and leaves using light and transmission electron microscopy combined with immunogold labeling. Li et al. (2010) reported some of endophytic bacteria from narrow leaf cattail (*Typha angustifolia* L.) roots capable of fixing nitrogen and can therefore improve plant growth. Similarly Shi et al. (2009) showed that inoculation of endophytic bacterial isolates resulted in significant increase in plant height, fresh and dry weights, and number of leaves per plant. Presence in roots was also confirmed. Two endophytic bacterial isolates in combination increased the germination, shoot length, and root length of chili plants (Muthukumar et al. 2010). Saini et al. (2015a) tested 79 endophytic bacterial inoculations in chickpea under pot culture conditions and showed enhanced plant growth, nodulation, and nitrogen-fixing parameters. The most efficient isolates were identified as *Bacillus subtilis* and *Bacillus amyloliquefaciens*. Similarly 41 bacterial endophytes were inoculated in field pea, and these enhanced nodulation, root growth, plant growth, and nitrogen content in shoot of field pea (Narula et al. 2013a).

The efficacy of two endophytic bacterial strains *Bacillus subtilis* strain CNE215 (isolated from chickpea nodules) and *B. licheniformis* strain CRE1 (isolated from chickpea roots) was evaluated under field conditions in comparison to uninoculated and inoculated controls (Saini et al. 2015b). Standard microbial inoculants recommended for chickpea crop *Mesorhizobium* sp. strain 1233; phosphate-solubilizing bacterial (PSB) strain PS 36 and plant growth-promoting rhizobacterial (PGPR) strain LK 884 were used as inoculated control. In all the treatments, there were more nodules and nodule biomass as compared to uninoculated control. Inoculation with nodule endophytic bacteria *Bacillus subtilis* strain CNE215 along with *Mesorhizobium* was better as compared to inoculation with all

the recommended biofertilizers – *Mesorhizobium*, PSB and PGPR. Therefore, in legumes instead of using multiple inoculants of rhizobia, PSB and PGPR, endophytic bacteria with rhizobial inoculation have more potential for enhancing crop productivity. Such studies under field conditions with other crops need to be conducted.

14.5 Conclusion

A few endophytic bacteria fix N_2 and have plant growth promoting mechanisms, while other endophytic bacteria possess only plant growth-promoting mechanisms. Endophytic bacteria in a single plant host are not restricted to a single species but comprise several genera and species. It seems that the bacteria best adapted for living inside plants are naturally selected. Signaling for becoming endophytic are still not fully understood, but it seems N_2-fixing endophytes follow different pathway as compared to non-N_2-fixing endophytes. Like rhizobia–legume, host specificity is not a major issue in plant endophytic interactions. Better understanding of these interactions will pave the way for the development of N_2-fixing system in cereals and nonlegumes. Development of efficient endophytic inoculants able to fix N_2 and having PGP activity will enhance crop productivity with sustainable agriculture and environment.

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Pseudomonas fluorescens: A Promising Biocontrol Agent and PGPR for Sustainable Agriculture

15

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Abstract

Indiscriminate use of chemicals as fertilizers and pesticide caused incredible harm to the environment and ecosystem including animals and humans. To replace such type of hazardous agrochemicals, biological solution is provided by nature in the form of microorganisms having capacity to promote the plant growth without substantially harming the environment. One of the biological approaches for the control of different phytopathogenic agents is the use of biocontrol plant growth-promoting rhizobacteria (PGPR), which is capable of suppressing or preventing the phytopathogen damage. The best characterized biocontrol PGPR belong to the bacteria genus *Pseudomonas*. Fluorescent pseudomonads are suitable for application as biological control agents due to their abundant population in natural soils and plant root system and their capability to utilize many plant exudates as nutrient. Fluorescent pseudomonads are known to have important traits in bacterial fitness such as the ability to adhere to soil particles and to the rhizoplane, motility and prototrophy, synthesis of antibiotics, and production of hydrolytic enzymes. Moreover, *Pseudomonas* also possesses plant growth-promoting traits such as nitrogen fixation, phosphate solubilization, iron chelation, and phytohormone production. Such multidimensional utility of fluorescent *Pseudomonas* makes them a bioagent of choice to be exploited in the field of agriculture.

Keywords

Biocontrol • Rhizobacteria • Phytopathogens • Fluorescent • Pseudomonas

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15.1 Need of Biocontrol Agents

Across the world, plant diseases are major cause of yield loss. The global market for phytosanitary products is dominated by synthetic pesticides (Thakore 2006). There are many disadvantages of using such chemical pesticides which include accumulation of toxic residues in environment and adaptation of pathogens to such chemicals which in turn reduce its efficiency and led to undesirable effect on nontarget organisms prevailing in the same niche. Moreover, nowadays, consumers are becoming more and more concerned about pesticide-free safer foods which results in emergence of eco-friendly strategies for plant disease management, i.e., biocontrol agents.

15.2 What Are Biocontrol Agents ?

Biocontrol agents can be defined as living organisms or natural products derived from living organisms (genetically modified crops, insects, nematodes, and microorganisms; Fig. 15.1) that are used to suppress plant pathogen pest populations.

Among these biocontrol agents, microorganism-based products (bacteria, fungi, virus, and yeasts) represent 30 % of total sales (Thakore 2006). Microbial biocontrol agents are having different modes of action for dealing with pathogens. The application of biocontrol agents and disease suppressing chemicals can reduce the possibility of resistance development among pathogen representing an integrated pest management strategy with the goal of minimizing the use of chemicals. Most of the bacterial strains exploited as biocontrol agents belong to the genera *Agrobacterium, Bacillus*, and *Pseudomonas* (Fravel 2005).

15.3 *Pseudomonas* as Biocontrol Agent

Research carried out at the University of California, Berkeley, during the late 1970s (Weller 1988) has awakened the global interest in the *Pseudomonas* sp. as biocontrol agents. Species of fluorescent *Pseudomonas* are capable of utilizing wide range of organic and inorganic compounds which imparts them capacity to live in varied environmental conditions. Members of this genus are found in large numbers in all the major natural environments, viz., terrestrial, freshwater, and marine, and they also form intimate associations with plants and animals. This widespread dispersal suggests a significant amount of physiological and genetic flexibility (Nowak-Thompson et al. 1997). The bacteria belonging to genus *Pseudomonas* are functionally diverse and ecologically noteworthy microorganisms because of their multiple utility as plant growth-promoting agents and bioremediators. Pseudomonads are gram-negative, chemoheterotrophic, and motile rods with polar flagella as defined by Palleroni (1984). *Pseudomonas* has been recognized as a complex collection of a large number of described species (Gardener et al. 2005). The functional and metabolic heterogeneity of *Pseudomonas* has been well documented from comprehensive studies dating to more than 45 years ago. Species of the genus *Pseudomonas* embodies an attractive biocontrol agent because of their catabolic adaptability, their outstanding root-colonizing abilities, and their capacity to produce a wide range of antifungal metabolites. Among various *Pseudomonas* spp., fluorescent pseudomonads have received particular attention as biocontrol agent of choice. *Pseudomonas* exerts its biocontrol activity through direct antagonism of phytopathogens and induction of disease resistance in the host plant (Cartieaux et al. 2003). Fluorescent *Pseudomonas* is a widely studied group among common inhabitants of the rhizosphere. They can be visually distinguished from the other *Pseudomonas* species of soil by their ability to produce water-soluble yellow-green pigments. They comprise of *P. aeruginosa*, the type species of the genus, *P. aureofaciens*, *P. chlororaphis*, *P. fluorescens*, *P. putida*, and the plant pathogenic species *P. cichorii* and *P. syringae* (Landa et al. 2003; De La-Funte et al. 2006). *Pseudomonas* spp. are well adapted for inhabiting in the rhizosphere. Pseudomonads possess many traits that make them well suited as biocontrol and growth-promoting agents (Weller 1988). These include their ability to (1) grow faster which makes them

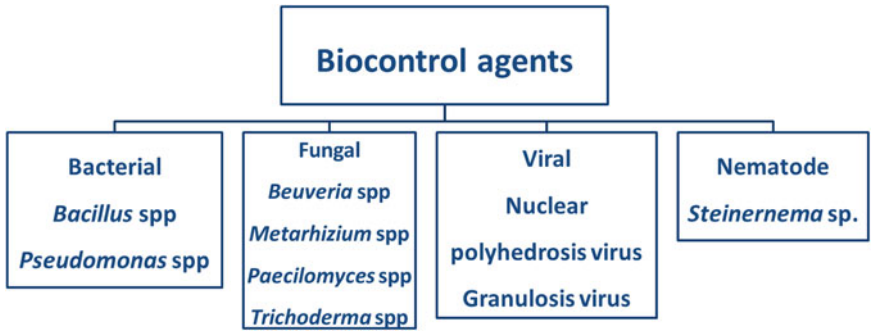

Fig. 15.1 Classification of biocontrol agents

easy to be mass produced in the laboratory, (2) readily consume seed and root exudates, (3) colonize and multiply in the rhizosphere and spermosphere environments and in the interior of the plant, (4) produce a wide spectrum of bioactive metabolites (i.e., antibiotics, siderophores, volatiles, and growth-promoting substances), (5) compete aggressively with other microorganisms, (6) adapt to environmental stresses, and (7) easily colonize plants upon subsequent reinoculation in soil by seed bacterization. The presence of pseudomonads in soil provides natural suppressiveness to the soil against some soil-borne pathogens (Weller et al. 2002).

Several strains live in commensal relationship with plants, protecting them from infection by pathogens that would otherwise cause disease. Control of root diseases by beneficial bacteria involves a blend of possible mechanisms that may complement each other. The primary mechanism of biocontrol includes production of antibiotics or inactivation of virulence trait of pathogens (Diby et al. 2005). Another important mechanism is the indirect inhibition of the pathogen by bacterial stimulation of defense responses in the plant host. Many of the plant-associated strains belong to fluorescent *Pseudomonas* group, which currently includes more than 50 named species (Yamamoto et al. 2000; Mulet et al. 2010).

Pseudomonas plays key role in better growth and development of plant through its capacity to protect plants against pathogens during various developmental stages. The above said benefit of pseudomonads depends on their ability to efficiently consume root exudates and resist predation by soil predators such as nematodes and protozoa (De Mesel et al. 2004; Abuzar and Haseeb 2010). Bacteria have evolved an array of antipredatory mechanisms, such as toxicity. Extracellular metabolites of *Pseudomonas* sp. drive complex interactions with predators, affecting their physiology and behavior. Secondary metabolite works specifically on predators, acting as repellents, stressors, or toxics. Production of such secondary metabolites by biocontrol bacteria serves multiple functions, and metabolites protecting plants against pathogens improve bacterial resistance (Gadoury et al. 1989).

Pseudomonas sp. can utilize variety of organic compounds as energy sources and produce an array of secondary metabolites foremost as 2, 4-diacetylphloroglucinol (DAPG, Phl), lipopeptides, phenazines, pyrrolnitrine, pyochelin, and hydrogen cyanide (Keel et al. 1992; Haas and Defago 2005). Biocontrol strains of *Pseudomonas* sp. with a proven effect in plant bioassays produce one or several antibiotic compounds. In vitro, these antibiotics have been proven as inhibitory compounds, and they are also showing active response for the plant health management in field conditions. Strains that produce the antifungal compound DAPG play an important role in the suppression of some root diseases when introduced into the rhizosphere via seed or soil treatments (Reddy et al. 2009). *Pseudomonas* sp. plays a key role in suppression of plant diseases and commercially exploited for plant disease management in agriculture sector. Biological control of plant diseases through antagonistic bacteria is less popular among the farming com-

munity in comparison to other disease control measures, but it has potential to transform plant disease management strategies.

15.4 Concept of Disease Suppressive Soil

Suppressive soils are soils in which phytopathogens are unable to persist or are present but fail to induce severe disease symptoms on susceptible crops. Plants are protected from diseases generally caused by soil-borne phytopathogens such as bacteria, fungi, and even nematodes in suppressive soils. Suppressiveness in soil is mainly attributed to the presence of high number of antagonistic bacteria having disease suppressive properties. Here the plant roots harbor plant-beneficial microbial communities which are having general beneficial effect on plant health and thereby also known as plant probiotics. Pasteurization of soil results into loss of disease suppressiveness which proves that microorganisms play an important role in disease suppressiveness of soil. Most of the soil pathogens such as fungi, bacteria, and plant-deleterious nematodes get suppressed in such soils. Dominant microfloras of suppressiveness in soil are *Trichoderma*, *Pseudomonas*, and *Bacillus* species. How these bacteria achieve this and what they have, to protect plant from pathogenic fungi, have been analyzed in biocontrol strains of fluorescent *Pseudomonas. Pseudomonas* competitively colonizes plant roots and stimulates plant growth and/or reduces the incidence of plant disease. *Pseudomonas* acts by production of antibiotics or by induction of systemic resistance within the plants during its colonization. It also has reported that growth regulatory compounds and beneficial enzymes are present in them (Haas and Defago 2005). *Pseudomonas* owes their fluorescence due to extracellular diffusible pigments such as pyoverdin (Pvd), pyochelin, and ferripyoverdin (Pvd Fe^{3+} complex) (Paez et al. 2005). The phenomenon of natural suppressive soils has been described for *Gaeumannomyces graminis* var. *tritici* (take-all of wheat), *Fusarium oxysporum* (wilt), *Phytophthora cinnamon* (root rot), *Pythium* spp. and *Rhizoctonia solani* (damping-off of seedling), *Thielaviopsis basicola* (black root rot), *Streptomyces scabies* (bacterial scab), *Ralstonia solanacearum* (bacterial wilt), and *Meloidogyne incognita* (root swelling and root-knot galls) (Haas and Defago 2005).

15.5 Mechanism of Biocontrol by *Pseudomonas*

Over the last few years, a great diversity of rhizosphere microorganisms has been described, characterized, and, in many cases, tested for activity as biocontrol agents against soil-borne plant pathogens. Such microorganisms can produce substances that may limit the damage caused by phytopathogens, e.g., by producing antibiotics, siderophores, and a variety of enzymes or by induction of systemic resistance in host plants. These microorganisms can also function as competitors of pathogens for colonization sites and nutrients. The major mechanisms by which *Pseudomonas* exerts its biocontrol effect are:

1. Competition for niche and nutrient acquisition
2. Antibiotic production
3. Induced systemic resistance

15.5.1 Competition for Niche and Nutrient Acquisition

The high microbial diversity, density, metabolic activity, and competition occurring in the rhizosphere environment represent a challenging "biological buffering" (Keel et al. 1996) that generally limits the establishment of exogenous, foreign microorganisms into the rhizosphere. Thereby, it is essential to evaluate the ability of introduced pseudomonads to colonize roots and provide protection against major and minor soil-borne pathogens. Several definitions of root colonization by rhizobacteria were proposed (Lemanceau et al. 1995; Van Loon et al. 1998), and that defines microbial colonization of plant as movement of the rhizobacteria from an inoculum source to the roots, multiplication, and persistence in the presence of native soil microflora. Weller et al. (2002) defined root colonization as the process

whereby rhizobacteria introduced into the seeds, vegetative propagated plant parts, or soil become distributed along roots growing in raw soil, multiply, and then survive for several weeks in the presence of indigenous soil microflora. Root colonization included colonization of the rhizosphere, rhizoplane, and/or inside the root. Rhizosphere competence describes the relative root-colonizing ability of a rhizobacterium. Bacterial inoculants become more powerful when they multiply on the root and colonize it. So the establishment of inoculant is an important factor for the disease suppression by bio-inoculant. Root colonization not only results in high population densities on the root system, it also functions as the delivery system of antifungal metabolites along the whole root. The extent of colonization ability of applied strain may also be dependent on the mechanism by which a biocontrol agent performs its action. The biocontrol of plant disease can be achieved by antibiosis wherein optimum colonization is needed for delivery of antifungal compounds to entire root system, whereas for ISR colonization of plants by limited number of bacteria is sufficient to induce ISR response in plant. The speed and degree of colonization by biocontrol is supposed to be an important trait. Most of the *Pseudomonas* strains are having short generation time. Microcolonies of *P. fluorescens* WCS365 appeared on the tomato root (Chin-A-Woeng et al. 1997; Bloemberg et al. 2000) 1 day after seed inoculation. Bacterial antagonist generally colonizes intracellular junction between root epidermal cells as they are nutritionally rich which represent small surface area of total root surface area (Chin-A-Woeng et al. 1997). Dhingani et al. (2013) studied colonization of fluorescent *Pseudomonas* isolates as a plant growth-promoting attribute. They isolated 30 isolates of fluorescent *Pseudomonas* from six different locations of Junagadh district, Gujarat, India, and confirmed various PGPR traits present in the fluorescent *Pseudomonas* which may help in the improved plant growth promotion during colonization with suppressive rhizospheric soils. Many of the biocontrol systems are dependent on positive relationship between colonization and pathogen suppression. During the last 40 years, the process of root colonization, the biotic and abiotic factors affecting colonization, and the bacterial genes and traits that contribute to rhizosphere competence has been clearly elucidated from the experimental systems using *Pseudomonas* sp.

Soil area around the root and influenced by root is known as rhizosphere (Hiltner 1904) which is richer in microbes than bulk soil. The rhizospheric microflora is mainly affected by root exudates that contain organic acids, sugars, and amino acids. Biocontrol agents applied to the soil have to race with injurious microorganisms and pathogens for limited available nutrients in root exudates and suitable colonization niches and finally outnumber them. After inoculation, the biocontrol agent can cause inhibition of soil pathogen only for a short period of time. Soil microorganisms have to become highly dependent upon nutrients present in the rhizosphere or root exudates. So, we can assume that there must be strong competition for nutrients between the biocontrol agent and the indigenous microflora in the rhizosphere of the host plant. Native microbial strains or aggressively colonizing biocontrol bacteria can therefore prevent the establishment and consequent deleterious effects of a pathogen. The ability of pseudomonads to establish in niche and rapidly compete for nutrient acquisition is thought to be a general mechanism for antagonistic activity dispersed by biocontrol strains of pseudomonads and thereby acting as plant probiotic. Fungal pathogens can be eliminated from the soil by increasing competition for nutrients such as carbon, nitrogen, or iron which in turn reduce the ability of fungal pathogens to proliferate in the soil (Leong 1986; Loper and Buyer 1991). The generation time of pseudomonads is 3–6 h in rhizosphere which is slower than that in nutrient-rich laboratory media as microorganism in the rhizosphere live under nutrient limiting (Lugtenberg and Kamilova 2009; Haas and Defago 2005). Populations of *Pseudomonas* established on the plant roots could act as a sink for the accessible nutrients and limit the nutrient availability for pathogen and its successive root colonization. This mechanism is generally used by fluorescent pseudomonads because of their nutritional versatility and high growth rates in the rhizosphere

(Walsh et al. 2001). Moreover, the pseudomonads compete with indigenous microbial populations for nutrition in the rhizosphere for successful removal of the pathogens. Siderophores are organic compounds produced by pseudomonads which sequester most of the available Fe^{3+} in the rhizosphere and starve the pathogens for their iron requirement and thereby play a main role in defeating pathogens in the same ecological niche (O'Sullivan and O'Gara 1992). Fluorescent siderophores have high affinity for ferric iron, which forms ferric-siderophore complex that becomes unavailable to other organisms, but the producing strain can utilize this complex via a very specific receptor in its outer cell membrane (Koster et al. 1993, 1995; Buyer and Leong 1986). In this way, fluorescent *Pseudomonas* strains may restrict the growth of deleterious bacteria and fungi on the plant root (Loper and Buyer 1991).

Failure of a pathogen to compete effectively with the biocontrol strain and use the available nutrient sources in same ecological niche will restrict the pathogen's spread. A classical example of niche exclusion is the control of leaf frost injury caused by *P. syringae*, which has an ice nucleation protein on its cell surface (Lindow 1983a, b; Lindow et al. 1983). Well-known example of competition for nutrients is limitation of iron as iron – an essential cofactor for growth in all organisms. The availability of Fe^{3+} in soils is lower at neutral and alkaline pH, which in turn leads to Fe^{3+} limitation. Fluorescent *Pseudomonas* species utilize Fe^{3+} by production of siderophores which are high-affinity iron chelating compounds. The capacity of iron scavenging under iron limitation gives the biocontrol organism a selective advantage over phytopathogens that possess less efficient iron binding and uptake systems. As compared to wild-type parental strains, siderophore-deficient mutants were found to be less effective against pathogens (Bakker et al. 1986).

15.5.2 Antibiotic Production

Antibiotic-producing bacterial biocontrol agents occur frequently and are efficient agents for plant disease management as they can be easily isolated from soil. Many factors affect the production of antibiotics such as temperature, pH, and the levels of various metal ions, particularly of Zn^{2+} (Duffy and Defago 1997). Among the variety of *Pseudomonas* species inhabiting the rhizosphere, certain strains of fluorescent pseudomonads have received particular attention because of their potential to control seed- and soil-borne pathogenic fungi and oomycetes (Keel et al. 1992, 1996). Plant-beneficial microorganisms help in exclusion of plant pathogens from rhizosphere through secretion of antimicrobial metabolites which in turn improves plant health (Haas and Keel 2003; Handelsman and Stabb 1996; Raaijmakers et al. 2002; Thomashow and Weller 1996). A triangular interaction occurs among plants, pathogens, and bacteria for regulation of antifungal traits of *Pseudomonas* (Jain et al. 2011). Due to this reason, efficient colonization is required for antibiosis (Chin-A-Woeng et al. 2003), and that's why it is not unexpected that some strains, which show antifungal activity under laboratory conditions, do not act as biocontrol agents in vivo. The identification and quantification of the antibiotics which are produced during biocontrol in situ are a challenge and have been shown only for a few cases (Thomashow and Weller 1996). The slow growth rate of bacteria in the rhizosphere favors the production of secondary metabolites (Haas and Defago 2005). Most of the identified *Pseudomonas* biocontrol strains produce antifungal metabolites, of which DAPG, phenazines, pyrrolnitrin, pyoluteorin, and volatile hydrogen cyanide are the most frequently detected classes. However, novel antifungal metabolites viscosinamide (Nielsen et al. 1999) and tensin (Nielsen et al. 2001) have been discovered and play a role in protection of plants against phytopathogens. Fluorescent pseudomonads producing antibiotic DAPG are an important group of biocontrol agents for suppressing diseases of roots and young seedlings of various crops, e.g., suppression of black root rot of tobacco by *P. fluorescens* CHA0 (Stutz et al. 1986), take-all of wheat (Keel et al. 1992), and *Fusarium* wilt, crown, and root rot of tomato (Duffy and Defago 1997; Tamietti et al. 1993). Moreover, *Pseudomonas* sp. F113 is found to suppress damping-off of sugar beet (Fenton et al. 1992; Shanahan et al. 1992), and *P. fluorescens* Q2-87 (Harrison et al. 1993;

Pierson and Weller 1994) and Q8r1-96 (Raaijmakers and Weller 1998) suppress take-all of wheat. DAPG-producing strains of *P. fluorescens* are also having a key role in the natural biocontrol of take-all disease (Raaijmakers and Weller 1998; Raaijmakers et al. 1997). The exact mechanism of action of DAPG on pathogens is yet to be discovered. The importance of DAPG as biocontrol molecule has been demonstrated by genetic approaches (Thomashow 1996) as well as direct isolation of disease suppressive strains producing DAPG from rhizosphere of crop plants (Bonsall et al. 1997; Duffy and Defago 1997; Raaijmakers and Weller 1998).

Development of resistance among the human and animal pathogens against the antibiotics used for treatment is believed to be the main risk of using an antibiotic-producing biocontrol agent. Moreover, there is also possibility of transfer of genes encoding the antibiotic production to related strains (Zhang et al. 2003), which seems to be realistic as some conjugative transfers require quorum sensing that are dependent on a high density of microbes. This type of cross transfer of genes is possible in root where pseudomonads form microcolonies under a mucoid layer (Chin-A-Woeng et al. 1997). The genetic material is exchanged at a high frequency in the rhizosphere. These are the reasons for slow process of registration of biocontrol products based on antibiotic-producing microbes.

15.5.3 Induced Systemic Resistance (ISR)

In simple words, ISR can be defined as a broad spectrum plant immune response activated by plant-beneficial bacteria that live in association with plant roots. Few strains of pseudomonads such as *P. fluorescens* (van Loon and Bakker 2006; van Wees et al. 1997; Kamilova et al. 2005) trigger ISR response to combat against a broad spectrum of plant pathogens. Such immunized plants express defense responses faster and stronger after pathogen attack, which results in enhanced level of protection (Van Peer et al. 1991). Such beneficial microbes induce resistance in distant parts of the plants such as leaves, and that's why it is known as ISR response. ISR response induced by beneficial microbes is effective against broad range of pathogens, viz., bacteria, fungi, and viruses (van Loon et al. 1998; van Loon 2007), but the response is believed to be random (Verhagen et al. 2003). There exists the host specificity among the ISR-inducing microbial strains as the ISR induction was found to be dependent on the plant species and cultivar (van Loon and Bakker 2006; van Wees et al. 1997). Generally the plant hormones, viz., jasmonate and ethylene, are believed to be key regulators of ISR response (van Wees et al. 2000). ISR response was observed in many plant-pathogen systems wherein the bacterium and the challenging pathogen remained spatially separated. Many effective biocontrol pseudomonads provoke ISR (Ongena et al. 2004; Ton et al. 2002; Zehnder et al. 2001). ISR does not require complete root colonization. In addition to live microbes, such as *Bacillus*, *Pseudomonas*, and *Trichoderma*, dead microbial cells and some of the products of bacterial metabolites, viz., siderophores, lipopolysachharides, salicylic acid, pyocyanin, and pyochelin as well as organelles such as flagella, are the main inducers of ISR response in plants (Audenaert et al. 2002). Moreover, the volatile 2,3-butanediol (Ryu et al. 2003), the signal molecule AHL (Schuhegger et al. 2006), the antibiotic phloroglucinol (Iavicoli et al. 2003), and some c-LPs (Ongena et al. 2002; Pérez-García et al. 2011) are also believed to be important triggering molecules of ISR response.

15.6 Role of *Pseudomonas* for Plant Growth Promotion

Pseudomonads possess many traits that make them well suited as biocontrol and growth-promoting agents (Weller 2007). There are several ways in which different plant growth-promoting *Pseudomonas* have been reported to directly facilitate the proliferation of their plant hosts. The direct promotion of plant growth by PGPR generally entails providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of nutrients from the environment. Direct mechanisms of plant growth

promotion are (1) phytohormone production, (2) nitrogen fixation, (3) siderophore production, and (4) phosphate solubilization.

15.6.1 Phytohormone Production

15.6.1.1 Indole 3 Acetic Acid

Many rhizospheric strains of *Pseudomonas* produce indole acetic acid (IAA) which helps in stimulating plant growth (Loper and Schroth 1986). The phytohormone indole-3-acetic acid (IAA) is known to be involved in root initiation, cell division, and cell enlargement. IAA production by microorganisms increases root length and surface area which in turn enables plants to increase absorption of water and nutrients from their ecosystem (Salisbury 1994). Increase in root length as well as the number of secondary roots in young seedlings through IAA production by microorganisms increases the chances of survival of seedlings due to enhanced capacity to anchor to the soil and absorb water and nutrients from the surroundings (Patten and Glick 2002). In IAA-producing bacteria, L-tryptophan-dependent auxin production was observed and reported to increase the grain yield and the number of branches (Asghar et al. 2002, 2004). Patten and Glick (2002) reported the role of IAA-producing *P. putida* in the development of the host plant root system.

15.6.1.2 Cytokinins

Cytokinins promote cell divisions, cell enlargement, and tissue expansion and are believed to be the signals for mediation of environmental stress from roots to shoots. *P. fluorescens* can produce cytokinins as reported by Garcia et al. (2001).

15.6.1.3 1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase

The stress hormone ethylene is the only gaseous phytohormone and produced upon physical or chemical to the plants which causes inhibition of plant root growth. Glick et al. (1998) reported that some of the PGRP strains can produce a stress-relieving enzyme named as ACC deaminase that breaks down ACC, which is the precursor for biosynthesis of ethylene in plants. Production of ACC deaminase enzyme by microorganisms can decrease the concentration of ethylene in the plant roots and thereby elongates plant roots (Glick et al. 1994). Shah et al. (1998) reported that insertion of ACC deaminase gene within *Pseudomonas* spp. aided bacteria with capacity to produce ACC deaminase enzyme and thereby release stress which in turn elongates seedling roots. Pseudomonas strains having capacity to produce ACC deaminase enzyme were reported to promote plant growth under stressful condition such as flood (Grichko and Glick 2001) or heavy metal contamination (Burd et al. 1998).

15.6.2 Nitrogen Fixation

The first evidence for nitrogen fixation by *Pseudomonas* like microorganisms has been reported by Anderson in 1955. Nitrogen-fixing ability of members of the genus *Pseudomonas* is poorly understood. The mechanism of nitrogen fixation and the protection of nitrogenase against oxygen deactivation were also not revealed (Young 1992). However, recently several workers demonstrated among the strains of pseudomonads (Desnoues et al. 2003; Krotzky and Werner 1987). The optimum conditions for the nitrogen fixation and structure of genes encoding nitrogenase enzyme in *Pseudomonas* sp. were studied in detail using *P. stutzeri* A15 (A1501), isolated from rice paddies in China (Desnoues et al. 2003). So, one can classify the *Pseudomonas* spp. as nitrogen fixers based on their physiological properties, nitrogenase assays, phylogenetic studies, and detection of *nifH* DNA by hybridization or PCR amplification (Chan et al. 1994; Vermeiren et al. 1999). After detection presence of nitrogen-fixing traits among the species of *Pseudomonas* genus, nitrogen-fixing strains of *Pseudomonas* spp. were reassigned genera in α- and β-proteobacteria (Chan et al. 1994). Krotzky and Werner in 1987 isolated two nitrogen-fixing *Pseudomonas* strains, viz., *P. stutzeri*. and *P. stutzeri* CMT.9.A, from the roots of sorghum, and You et al. (1991) isolated *P. stutzeri* strain A15 from rice paddies from China (You et al. 1991).

15.6.3 Solubilization of Phosphorus

The second important macronutrient required for plant growth is phosphorous. Phosphorous is present in insoluble forms such as iron and aluminum phosphates in acidic soils and calcium phosphates in alkaline soils. In phosphorous-rich soil, only a small proportion of phosphate (~0.1 %) is available to plants (Stevenson and Cole 1999). Phosphate-solubilizing bacteria (PSB) secrete organic acids and phosphatase enzymes to convert the insoluble phosphates into soluble forms. This process is known as phosphate solubilization which leads to an increase in the content of available phosphate for plants (Gyaneshwar et al. 2002). Almost all the soil types contain phosphate-solubilizing bacteria (Gyaneshwar et al. 2002), among which *Bacillus*, *Enterobacter, Erwinia*, and *Pseudomonas* spp. are most prevalent. Generally rhizospheric region of plant is colonized by phosphate-solubilizing bacteria where they bring about solubilization of insoluble inorganic phosphatic compounds. Most commonly the phosphate-solubilizing ability of PGPR strains is dependent on the availability of other macronutrients such as carbon and nitrogen as well as metal ions (Kim et al. 1998). Generally, phosphate-solubilizing bacteria produce various types of organic acids, among which the most abundant is β-ketogluconic acid, a secondary oxidation product of glucose metabolism. The oxidation of glucose is catalyzed by an enzyme glucose dehydrogenase (GDH) present in cytoplasmic membrane of bacteria, and as a result of the enzyme activity, gluconic acid and β-ketogluconic acid are produced which bring about phosphate solubilization.

15.6.4 Sequestering Iron by Siderophores

Iron is essential for life for all living organisms and is required as a component of proteins involved in important processes such as respiration, photosynthesis, and nitrogen fixation.

Despite the abundance of this element on the earth's surface, soil organisms such as plants and microbes have difficulty in obtaining enough iron to support their growth because iron in soil is largely present as insoluble, ferric hydroxides, which cannot be readily transported into cells. Microorganisms and some plants can secrete low molecular weight, organic, iron binding molecules known as siderophores which help in iron scavenging from soil. Each functional group presents two atoms of oxygen or less commonly nitrogen that bind to iron. In general, catecholate-type siderophores are typical to bacteria. It is known that many bacteria, including *Pseudomonas* spp., react to limiting Fe^{3+} concentrations by inducing a high-affinity iron uptake system (Braun 1985; Neilands 1982) consisting of siderophores, Fe^{3+} chelating molecules, and outer membrane receptor proteins with a high affinity for the matching Fe^{3+} siderophore complex (De Weger et al. 1986). Production of siderophores by plant growth-promoting *Pseudomonas* spp. during iron starvation is considered as the one of the mechanism in inhibition of phytopathogens. But whenever the concentration of iron in the medium is sufficient, such antagonism will not be observed (Geels and Schippers 1983). The following scenario was proposed to account for the enhancement of plant growth by the *Pseudomonas* spp. (Kloepper et al. 1980). After the inoculation of seeds, the *Pseudomonas* bacteria rapidly colonize the roots of the developing plant. The limiting Fe^{3+} concentration in the soil induces the high-affinity iron uptake system. The siderophores bind Fe^{3+}, and as an uptake of this Fe^{3+}, siderophore complex requires a very specific uptake mechanism; this binding makes this essential element unavailable for many other rhizomicroorganisms. These microorganisms, including deleterious species, then are unable to obtain sufficient iron for optimal growth since they produce either no siderophores at all or less efficient ones (Raaijmakers et al. 1995). Thus, the population of deleterious microorganisms is reduced, creating a favorable environment for the development of the plants (De Weger et al. 1986).

Several species of fluorescent pseudomonads produce siderophores, and there is evidence that a number of plant species can absorb bacterial siderophore complexes (Bitter et al. 1991).

Pyoverdines (PVDs) or pseudobactins are fluorescent yellow-green siderophores (Budzikiewicz 1997). *P. aeruginosa* produces siderophore pyochelin having lower affinity for iron. Fluorescent pseudomonad species, viz., *P. fluorescens*, *P. stutzeri*, and *P. putida*, produce siderophore named as pseudonlonine (Lewis et al. 2000; Mossialos et al. 2000; Mercado-Blanco et al. 2001).

15.7 Scope of *Pseudomonas* as Biocontrol Agent

The prospect of manipulating crop rhizosphere microbial populations by inoculation of beneficial bacteria, i.e., *P. fluorescens*, to increase plant growth has shown considerable promise in laboratory and greenhouse studies. The potential environmental benefits of this approach, leading to a reduction in the use of agricultural chemicals, fit with sustainable management practices. We can expect to see new *P. fluorescens* products becoming available to farmers as a biofungicides. The success of these products will depend on our ability to manage the rhizosphere to enhance survival and competitiveness of these beneficial microorganisms. Sequencing the genome provided further information of its environmental interactions and its metabolic capabilities, which can be used to control plant diseases. Though *P. fluorescens* is the most widely used biocontrol agent, the major limitation is not only its shelf life but also inconsistent field performance.

15.8 Conclusion

Unlike chemical pesticides, biocontrol agents need support even after their application to get established in targeted niche. Therefore, for the success of biological control, one has to ensure not only the quality of biocontrol agent applied but also its establishment in natural ecosystem to thrive and compete well with the pathogens. Development of better formulations to ensure survival of activity in the field and compatibility with chemical and biological seed treatments is another area of focus. *P. fluorescens* as bioagent has good prospectus in the future as it gives very high cost-benefit ratio. In view of this, the first assumption is to isolate the *P. fluorescens* bacteria from the rhizosphere of various field crops with enhanced antagonistic activity against soil-borne fungal pathogens under native environmental conditions and determine the ability of selected bacterial isolates to suppress the soil-borne fungal pathogens under in vitro conditions.

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Isolation, Characterization of Nematode-Controlling Bacteria and Fungi from Nature

16

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Abstract

The root-knot nematodes (genus *Meloidogyne*) are a major endoparasitic pest affecting the production of many economically valuable annual and perennial crops worldwide in tropical and subtropical climatic zones. The infected plants show typical symptoms which include root galling, lack of vigor, stunting growth, nutrient deficiency particularly nitrogen deficiency, yellowing of leaves, and wilting under water stress conditions. The root-knot nematodes are one of the most destructive and difficult diseases to control in agricultural sector. These nematodes cause billions of US dollars in yield loss annually every year. The use of chemical nematicides is usually effective and has been used for over 50 years, but they cause significant environmental pollution as most nematicides are highly toxic compounds. Among the various strategies advocated to manage root-knot disease is the use of native biocontrol agents as an integral component of integrated disease management. The use of biocontrol agents from bacteria and fungi has been the focus of many researchers, mostly for development of microbial biopesticides against diseases and pests. The efforts have resulted in several microbial insecticides being marketed in many countries.

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A comprehensive understanding of mechanisms of disease inhibition by bacterial or fungal pathogens remains limited. In this chapter, we investigated the uses of some biocontrol agents to control root-knot nematodes if not totally eliminate them from our agricultural fields. The results showed that certain groups of bacteria and pathogenic fungi have been intensively studied, and some were developed for use as microbial bioinsecticides. The success of these bioinsecticides relies mainly on the activities of the fungus and bacteria, which can be affected by various environmental factors along with the interaction between the pathogen and its host insect pest.

Keywords
Nematode • Endoparasite • Biological control • Bacteria • Fungi

16.1 Introduction

The soil around plant roots that forms the rhizosphere is dynamic and a very complex zone. All plant-parasitic nematodes are obligate parasites and must enter this zone to reach their host and cause damage. Plant-parasitic nematodes are recognized as major agricultural pathogens and are known to attack plants and cause crop losses throughout the world. Root-knot nematodes, *Meloidogyne* spp., are recognized as the most economically important genus of plant-parasitic nematodes worldwide. Different physical or abiotic factors like temperature, soil pH, soil texture, soil moisture, and soil type influence the development and distribution of *Meloidogyne* spp. Soil temperature is one of the critical factors for the survival of the *Meloidogyne* spp. as it influences all aspects of nematode: life cycle, behavior, hatching of the eggs, invasion in the root tissue, motility, and over all development. Lower temperature of the soil will kill the egg and larvae of *Meloidogyne* spp. The nematode causes severe damage and yields loss to a large number of cultivated plants and especially vegetable crops in the tropics and subtropics (Magdy 2002). The root-knot nematodes (genus: *Meloidogyne*; Greek word means melon, apple, or gourd-shaped female) are among the most severe and damaging sedentary endoparasitic pest of many cultivated plants worldwide. The food produced by plants is one of the basic requirements for sustaining life, and agriculture is the main source of human diet on the face of the earth. Thus, many efforts were made to improve agricultural productions by developing and expanding agricultural fields, and in the process many problems have emerged such as the spread of plant pests and diseases, prompting human to try to control the spread of the diseases caused by fungal, bacterial nematodes, and various kinds of destructive insects, acari, snails, etc. (Khalil 2013).

Among the top five plant pathogens affecting the cultivated crop and economical crop production in the world, root-knot nematodes with more than 150 species are one of the most destructive and difficult diseases to control, mainly in tropical and subtropical agriculture. They cause millions of dollars in agricultural crop loss annually and also cause problems in the urban areas by damaging turf grasses, ornamental plants, and kitchen gardens. They are distributed worldwide over a wide range of geographical conditions of tropical, subtropical, and temperate regions of the world. They are more prevalent in tropical and subtropical climatic zones where summer is longer than the winter. The various nematode species within the genus have an overall host range covering approximately 5500 plant species (Trudgill and Blok 2001). The members of phylum Nematoda (round worms) have been in existence for an estimated one billion years, making them one of the most ancient and diverse types of animals on

earth (Wang et al. 1999). Nematodes evolved from much simpler organisms about 400 million years before explosion in the Cambrian era (Poinar 1983). There are many identified root-knot nematode species that are responsible for high economic damage to varied crops, and the *Meloidogyne incognita* (Kofoid & White) Chitwood is the most important pest under economic aspects that can also interact with fungal pathogens and infect 1700 plant species (Sasser et al. 1983). In the infective juvenile (J2) stage of development, *M. incognita* enters the elongation zone of the root and burrows through the apoplast to the root tip (Fig. 16.1a) where it enters the vascular cylinder, moving up to the zone of root differentiation. The nematode then inserts its stylet into the plant cell, and secretion from the stylet enhances the activity of KRP2 gene in cytoplasm and induces nuclear division without cytokinesis, creating multinucleate giant cells to efficiently nourish the parasitic nematode. Infection is associated with the reprogramming of plant cell development rather than host cell death (Caillaud et al. 2008; Vieira et al. 2013).

The introduction of agricultural and commercial crops into the field in many cases showed that a threat was posed by the root-knot nematode, *Meloidogyne incognita* (Fig. 16.1b). As this species is endoparasitic, infected suckers or roots that are used in the vegetative propagation of the crop facilitate its spread. The visible effect of nematode infection on plant root induces typical symptoms and is popularly known as "root knot" or "root gall" of various sizes depending on the species of root-knot nematode and the host plant. The common symptoms of the infestation with root-knot nematode are lack of vigor, stunted growth, yellowing of leaves, and wilting under water stress conditions. The nematode fungus interaction was first noted by Atkinson, who observed that infection by root-knot nematodes is always associated with infection of *Fusarium* wilt due to injury of the roots when the nematode inserts the stylet (Atkinson 1892). The severity of such interactions often result in a disease complex causing synergistic yield losses (Hussey and McGuire 1987) as described for root-knot nematodes and soil-borne fungal pathogens (Back et al. 2002). The control and elimination of root-knot nematode infections in plants is more difficult than that of other pests because they mostly inhabit the soil and attack only the underground roots of the plants (Stirling 1991).

The most common methods of controlling root-knot nematodes in cultivated crops for over

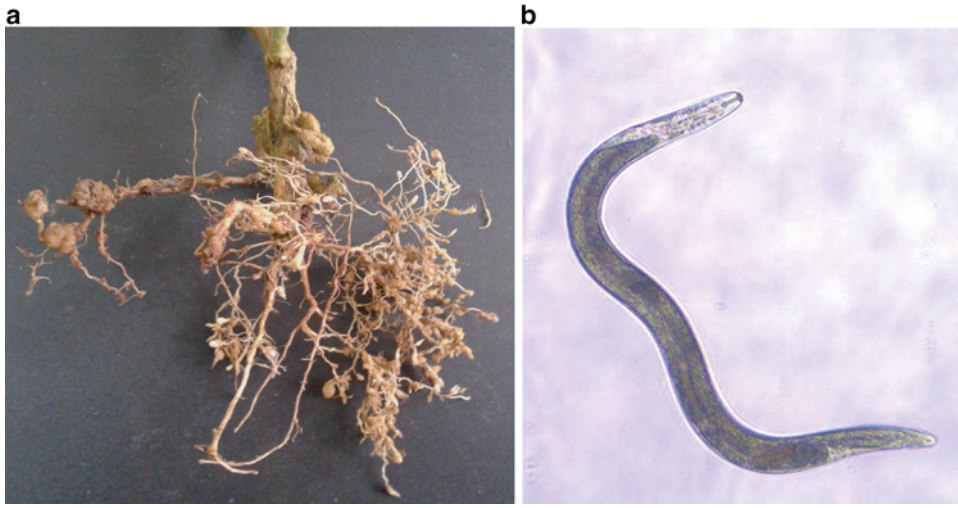

Fig. 16.1 (**a**) Nematode-infected root. (**b**) *Meloidogyne incognita*

50 years have been by nematicides, which are inexpensive chemicals that effectively kill nematodes in soil. There are two types of nematicides that are used to control nematodes. These are the soil fumigants (gas) and non-fumigants (liquid or solid) with the former being more popular and extensively used because they do not rely on alternative host crops for rotation; they drastically reduced nematode populations in the soil and are cost-effective for most crops. The nematicides are effective in controlling nematodes and are only practical for use on high-value crops. While non-fumigant nematicides reduce nematode populations, their effectiveness is not as consistent as that of fumigant nematicides. Most fumigant nematicides have been banned in most of the countries as they are environmental toxins. The nematicide method has undesirable effects due to their negative impact on the environment and is considered as major threat to human health, with leaching of toxic contaminants to food sources and drinking water, adverse effect on useful organisms, and depletion of stratospheric ozone. The method to control root-knot nematodes and other plant diseases and pests by nematicidal and fungicidal treatments has also caused environmental pollution and resistance of disease-causative organisms to fungicides. Therefore, an alternative method for nematode management is urgently needed for safe alternatives, cheap and effective methods that are environmentally friendly but which will minimize or eliminate plant-parasitic nematode populations and thus ensure high crop production and food security (Khalil 2013). Nematode control is far more complex than any other kind of pathogens because nematodes mainly attack underground parts of plants (Sikora and Fernandez 2005). A range of management strategies studied, including crop rotation, soil amendments, and nematicides, could be collectively used to enhance the activity of naturally occurring biological control methods (Sikora 1992). The alternative method for nematode management could be the use of some beneficial microorganisms with dual antagonism against both the nematode and the fungal pathogen.

16.2 Biological Control

The attempts made so far for commercial production and use of biocontrol agents in the most developed parts of the world has faced many challenges. This is because the growers do not generally use biocontrol products due to lack of rapid and adequate control (Felde et al. 2006). Inconsistent performance of applied biocontrol agents has been the main problem in exploring this mode of management due to some abiotic and biotic factors. The biotic factors include interactions with nontarget organisms, damage caused by nontarget pathogens and pests, degree of rhizosphere and/or soil colonization by a biocontrol agent, initial population levels of the target organisms, and susceptibility of the host plant species and host plant cultivar. The abiotic factors include climate, and physical and chemical composition of the rhizosphere (Meyer and Roberts 2002; Sikora and Hoffmann-Hergaten 1993). The rhizospheric soil is a storehouse of many different soil organisms that have to compete for nutrients and food sources. The biological control exploits the natural beneficial organisms to either protect the host plant from infections or to reduce the severity of the disease (Janja et al. 2013). The biological control of diseases of plants is eco-friendly and is a potential component of integrated disease management. Thus, biological control uses microbes to control plant pathogens. The first pioneer of nematode biocontrol was Duddington (1951).

Biochemical and microbial pesticides are considered less toxic than conventional pesticides and generally affect only the target pest and closely related organisms, in contrast to broad-spectrum, chemical pesticides that may affect not only the target organisms but also different wildlife such as birds, insects, and mammals. The biopesticides are often more effective even in small doses and often decompose more quickly, resulting in lower exposure and less environmental pollution than conventional chemical pesticides. The biopesticides, when used as a component of integrated pest management (IPM) programs, will also decrease the use of conventional chemical pesticides (Halbrendt 1996).

The research on biological control has led to a production of various commercial biopesticide products containing live microorganisms or their metabolites that target specific nematode hosts, but their low efficacy on the field conditions is not encouraging (Janja et al. 2013). These products based on microbial metabolites are classified as biopesticides and resemble that of chemical pesticides.

In recent years, there are several microbial pathogens that are mostly studied and commonly applied to effectively control root-knot nematodes worldwide and have been commercially produced in the USA, Canada, India, and other countries. The microbial agents include the different bacterial species and the antagonistic fungi such as predacious fungi, endoparasitic fungi, and fungi that produce antibiotics and toxins (Khalil 2013).

The advances in the last decades produced a number of biopesticides and biocontrol products that are marketed by some companies for organic farming to wean out chemical fertilizers. Soil organism lives in a very complex ecosystem and competes for food sources. This interaction of microbes in the soil, i.e., environment and competition for food, is the major factor in which biological control exploits. Most of the commercial biocontrol products available in the market contain live microorganisms like *Bacillus firmus* and *Pasteuria penetrans* and fungus like *Purpureocillium lilacinus* and/or their metabolites that target specific nematodes (Janja et al. 2013). The antagonistic activity of bacteria and fungi as biocontrol of root-knot nematodes has been carried out by different research groups worldwide. The research on natural bio-agents that work against root-knot nematodes and do not have a detrimental impact on the environment is an ongoing process.

The most serious problems faced by cultivated crops are infestations by pests and diseases; among them root-knot nematodes are considered to be the most common and destructive diseases. The root-knot nematodes attacks several important plants such as vegetables, leguminous crops, oil- and fiber-yielding crops, food grain, and fruit trees including weeds which are the secondary host to parasitic nematodes (Khalil 2013).

The nematodes in soil, like any living organisms, are also susceptible to infections by bacteria and fungi, and thus there are possibilities of using soil microorganisms to control plant-parasitic nematodes (Mankau 1980; Jatala 1986). The nematophagous bacteria as natural enemies of nematodes exhibit diverse modes of action which includes parasitizing; producing toxins, antibiotics, or enzymes; competing for nutrients; inducing systemic resistance of plants; and promoting plant health. They also act synergistically on nematodes through the direct suppression of nematodes and facilitating the rhizosphere colonization and activity of microbial antagonists (Tian et al. 2007).

Bacteria being the most abundant organisms in soil have shown great potential for the biological control of nematodes. Extensive investigations conducted over the last 20 years to assess their potential to control plant-parasitic nematodes have shown that nematophagous bacteria are widely distributed, possess various modes of action, and have broad host ranges (Emmert and Handelsman 1999; Siddiqui and Mahmood 1999; Meyer 2003).

A variety of nematophagous bacterial groups isolated from soil, host plant tissues, and nematodes and their eggs and cysts can control nematodes by parasitizing; producing toxins, antibiotics, or enzymes; interfering with nematode–plant host recognition; competing for nutrients; inducing systemic resistance of plants; and promoting plant health (Thomason and Lear 1961). The bacteria have wide range of suppressive activities on different nematode species whether free-living or predatory on animals or plant-parasitic nematodes (Kerry 2000; Meyer 2003; Mankau 1980; Stirling 1991; Siddiqui and Mahmood 1999). The biological control of nematodes is considered to encompass control that results from the action of soil microorganisms and microfauna, which is mediated through mechanisms such as parasitism, predation, competition, and antibiosis. There are three major groups of organisms that are antagonistic to nematodes which differ in their mode of action: (a) predators which actively seek out nematodes and then consume them, (b) parasites which grow

within their host and obtain nutrition from the host and are capable of causing disease in the host (pathogens), and (c) organisms that influence nematode abundance through mechanisms other than predation and parasitism (Stirling 1991).

The bacteria being the most abundant microorganisms and because of their close association with nematodes in the rhizosphere continuously destroy them in all soil types (Akhtar et al. 2012). Similarly, it had been reported that rhizobacteria, *Bacillus* sp., and *Pseudomonas* sp. inhibits egg hatching and can also affect the nematode juveniles by production of exotoxic compounds in response to cellular metabolism. The permeability changes of the juvenile cuticle which is characterized by selective permeability during molting inside the eggs cause antagonistic effect against *M. incognita* (Westcott and Kluepfel 1990).

There are several bacteria and fungi that are natural enemies of nematodes and have been isolated from the soil that keeps nematode populations at low levels. Some bacteria and fungi have been used to reduce populations of some kinds of nematodes under laboratory conditions, but have not been successful at field level (Stirling 1991). The organisms showing characteristic control of one or more nematode pests are specific on which nematodes they will attack and are very difficult to culture in sufficient quantities to be useful for field application. The conditions under which each is most effective are often quite specific. The possibility of nematode tolerance by the plant associated with AMF leads to the interest of plant scientists to study the interaction between them (Hol and Cook 2005; Brundrett 2002; Khalil 2013).

The application of biological sources for plant disease control is an important potential alternative to replace chemical pesticides. This method has been proposed for the replacement of chemical control of plant diseases. The use of microorganisms as biological control agents has been studied intensively as there are no other alternatives left to control plant pathogens (Ozbay and Newman 2004; Kotan et al. 2009; Oskay 2009). AFM might be protecting plants by physical and physiological response to the pathogen attack or alternatively by a direct suppressive effect on nematodes by not allowing them to share root space and suitable feeding sites on the root (Francl 1993; Graham 2001).

The rhizosphere is the site in the soil ecosystem where most microbial interaction occurs as different gases and root exudates are released in the surrounding environment. Rhizosphere becomes more complex when different microbial communities interact with plant pathogens in the soil, influencing on the growth and development of the plant (Ozbay and Newman 2004). Antagonism activity of different microorganisms can be attributed to the production of secondary antimicrobial metabolites (antibiosis), lytic activity of different enzymes, or different effectors (Alabouvette et al. 2006; Ozbay and Newman 2004). The production of different secondary metabolites by one microorganism that is toxic to other microorganism results in antibiosis which is responsible for the activity of many biological control agents in *Pseudomonas* spp., *Bacillus* spp., *Streptomyces* spp., *Trichoderma* spp., and *Gliocladium* spp. (Kubicek et al. 2001; Alabouvette et al. 2006). The production of several hydrolytic enzymes that degrade cell walls of pathogenic fungi results in parasitism (Alabouvette et al. 2006; Ozbay and Newman 2004). Many types of antibiotics are produced by different soil bacteria. Extracellular lytic enzymes produced by bacteria can also act at some distance from the site of production and its activity may degrade bacterial wall (Downing and Thomson 2000; Anitha and Rabeeth 2010).

Plant growth-promoting rhizobacteria (PGPR) are bacteria inhabiting the rhizosphere, are directly or indirectly beneficial in promoting plant growth, and induce resistance to different disease-causing pathogens like fungi, bacteria, viruses, and nematodes. PGPR can physical or chemical changes related to plant defense and induced systemic resistance (ISR) that can suppress a broad spectrum of plant diseases caused by a range of pathogens (Kloepper et al. 2004; van Loon et al. 2004).

The PGPR in general increases plant growth, promotes root development, and alters root architecture by the production of indole acetic acid (IAA). Diverse bacterial species and especially plant-associated bacteria have got the ability to produce IAA. Bacteria use this ability to colonize including phytostimulate by increasing root surface area and root tip numbers for uptake of nutrients from the soil. The stimulation of roots helps plant to circumvent basal plant defense mechanism (Kloepper et al. 2007; Mantelin and Touraine 2004).

16.3 Bacteria as Biocontrol Agent against Nematode

Biological control using microbial agent against plant-parasitic nematodes is an alternative means, which received immense importance among nematologists in last decades. The targeted nematodes can be controlled without any environmental pollution (Zeinat Kamel et al. 2010). Bacteria, because of their association in the rhizosphere and by their parasitic behavior, can destroy nematode present in soil. The important genera of rhizobacteria including *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Clostridium*, *Desulfovibrio*, *Pseudomonas*, *Serratia*, and *Streptomyces* are known to reduce nematode population in soil (Siddiqui and Mahmood 1999). The parasitic bacteria *Pasteuria penetrans* is considered as a potential biocontrol agent especially against *Meloidogyne* species (Mankau 1975a, b; Brown et al. 1985). It includes *P. sensu* and *P. thornei* that parasitizes root-lesion nematodes such as *Pratylenchus brachyurus* (Starr et al. 1983). *Clostridium butrycum* is an another important bacteria that produced butyric acid, and *Desulfovibrio desulfuricans* bacteria produced hydrogen sulfide which resulted in reduced nematode multiplication (Hollis and Rodriguez-Kabana 1966; Rodriguez-Kabana et al. 1965).

Among all bacteria, the plant growth-promoting rhizobacteria (PGPR) are those strains that generally improve plant growth by colonizing the root system and preempting the establishment of, or suppressing, deleterious rhizosphere microorganisms (Schroth and Hancock 1982). The systemic resistance induced by these bacteria has also been considered as a mechanism for biocontrol of pathogens (Wei et al. 1996). Mechanisms for induced systemic resistance may be due to increase in activity of chitinases, β-1.3 glucanases, peroxidases, and other pathogenesis-related proteins (Lawton and Lamb 1987); accumulation of phytoalexin (Kuc and Rush 1985) and formation of protective biopolymer isolates are highly specific (Lawton and Lamb 1987; Kuc and Rush 1985). Most of the rhizobacteria act against plant-parasitic nematodes by means of producing lytic enzymes such as chitinase, cellulase, lipase and protease, and toxins that suppress nematode population in the rhizosphere and promote plant growth (Tian et al. 2007; Zuckerman and Jasson 1984; Siddiqui and Mahmood 1999).

Exposure of root-knot nematode to culture filtrates of *P. fluorescens* under in vitro conditions significantly reduced egg hatch and caused substantial mortality of *M. javanica* juveniles (Siddiqui and Shaukat 2003). *P. fluorescens* has induced systemic resistance and inhibited early root penetration of *Heterodera schachtii*, the cyst nematode in sugar beet (Oostendorp and Sikora 1989, 1990). Application of the bacterium *P. chitinolytica* reduced the root-knot nematode infection in tomato crop (Spiegel et al. 1991). For the biological control of plant-parasitic nematodes, rhizobacteria have been proved to be a very effective means. Many types of aerobic endospore-forming bacteria like *Bacillus* spp. and *Pseudomonas* spp. are dominant in the soil. *Pseudomonas fluorescens* not only antagonizes nematodes but also improves plant growth and development, reduces galling and nematode multiplication (Siddiqui et al. 2009), and, in combination with *P. aeruginosa* reduces *M. javanica* juvenile penetration into tomato plants (Shaukat and Chahal 2002). *P. aeruginosa* reduces infection of *M. javanica* in various crops (Perveen et al. 1998) and significantly reduces gall formation in root-treated chilies (Siddiqui et al. 1999).

Bacillus thuringiensis (Bt) produces one or more type of crystal toxins that are known to be toxic and is widely used microorganism for the

biological control of insect pests (Bhau and Koul 1998; Samsonov et al. 1997). *B. thuringiensis* has been used as a biological insecticide for many years due to its effectivity against wide range of insect species and is found to be safe for higher animals and mammals (Tailer et al. 1992; Borgonie et al. 1996; Nester et al. 2002; Wei et al. 2003).

B. thuringiensis produces parasporal crystals composed of protein molecules or delta endotoxins that are toxic to insect pests (Höfte and Whiteflеy 1989). *M. javanica* and *M. incognita* population was effectively suppressed by *B. thuringiensis* (Chahal and Chahal 1993; Zuckerman et al. 1993), and *B. subtilis* induces protection against *Meloidogyne incognita* and *M. arenaria* infection in cotton (Sikora 1988). *B. thuringiensis* was also shown to produce exotoxins in the culture medium. The α-exotoxin, for example, is a lecithinase, and it may be labile because it is a protein and its molecular weight has been thought to be 40,000–50,000 (Asano et al. 1994). On the other hand β-exotoxin (thuringiensis) is a thermostable nucleotide derivate that inhibits DNA-dependent RNA polymerase, subsequently blocking cell mitosis (Sebesta et al. 1981).

Application of a mixture of three PGPR strains, viz., *Bacillus pumilus* strain INR 7, *B. subtilis* strain GB03, and *Curtobacterium flaccumfaciens* strain ME1 as a seed treatment, has resulted in much more intensive plant growth promotion and disease reduction when compared to strains tested singly. This might be due to different mechanisms of action for each PGPR strain (Raupach and Kloepper 1998).

The genus *Bacillus* consists of a big group of gram-positive bacteria, is able to produce endospores, and can suppress nematode invasion in the plant root system (Kloepper and Ryu 2006). It was reported that *B. subtilis*, *B. cereus*, and *B. pumilus* are widely distributed and exhibited larvicidal activity against the second stage juveniles (J2) of *Meloidogyne incognita* in vitro (Gokte and Swarup 1988). Because of its high shelf life, *B. subtilis* when used as seed treatment in pot experiments reduces *M. incognita* multiplication on tomato. *B. thuringiensis* was used as a potential biocontrol agent to many plant-parasitic nematodes, including *Meloidogyne* sp. (Gautam et al. 1995; Borgonie et al. 1996; Marroquin et al. 2000; Khyami-Horani and Al-Banna 2006).

Successful biocontrol combinations have been recorded against root-knot nematodes (Table 16.1). The combination of the bacterium *Bacillus subtilis* and the fungus *Paecilomyces lilacinus* suppressed nematode populations beyond the individual application of the agents (Gautam et al. 1995).

16.4 Fungi as Biological Control Agents of Nematodes

Nematophagous fungi have been found in all regions of the world, from the tropics to Antarctica. They have been reported from agricultural, garden, and forest soils and are especially abundant in soils rich in organic material. Environmental and health concerns over the use of chemical pesticides have increased the need for alternative measures in the control of nematodes. Biological control is considered to be ecologically friendly and a possible alternative in pest and disease management. Fungi as biological control are an exciting and rapidly developing research area, and there is growing attention in the exploitation of fungi for the control of nematodes. Like other microbes, fungi can directly parasitize nematodes or secrete nematicidal metabolites or enzymes that affect nematode viability and toxicity (Desai et al. 1972). Sophisticated technique to capture live nematodes or infect them makes nematophagous fungi one of the most destructive and natural enemies of the nematodes. In addition to nematodes, these fungi can colonize plant root, and this makes them a very potential and effective biocontrol agent. The parasitic habit of nematophagous fungi has evolved among the fungi having cellulolytic and lignolytic activity as a response to limited availability of nitrogen in the surrounding soil. Nematodes in the soil become one of the important sources of nitrogen for nematophagous fungi (Barron 1992). Plants belonging to the Leguminosae family is the favorite of the nema-

Table 16.1 The uses of some bacteria as biocontrol agents against root-knot nematode infection in agricultural crops

Sl no.	Bacterial species	Targeted nematode	Uses	References
1.	**(A) *Bacillus* sp.** *Bacillus thuringiensis*	*Meloidogyne*	Prevented *M. incognita* from forming galls on tomato	Ignoffo and Dropkin (1977)
2	*Bacillus subtilis*, *B. cereus*, *B. pumilus*, *Pseudomonas* sp.	*M. incognita*, *Heterodera cajani*, *H. zeae*, *H. avenae*	The most effective isolates were *B. subtilis* and *B. pumilus* against all tested species. The noncellular extract exhibited a high degree of larvicidal properties	Gokte and Swarup (1988)
3	*Bacillus subtilis*	*Meloidogyne* spp. *Rotylenchulus reniformis*	Reduced nematode reproduction and galling on cotton, tomato, and peanut	Sikora (1988)
4	*Bacillus licheniformis, Pseudomonas mendocina*	*M. incognita*	*B. licheniformis* caused higher reduction in nematode multiplication than *P. mendocina* on tomato. *P. mendocina* had adverse effect on plant growth	Siddiqui and Husain (1991)
5	*Bacillus licheniformis, Alcaligenes faecalis*	*M. incognita* race 3	*B. licheniformis* caused higher reduction in nematode multiplication than *A. faecalis* on chickpea	Siddiqui and Mahmood (1992)
6	*Bacillus cereus*	*M. javanica*	Inhibited penetration of nematodes on tomato roots	Oka et al. (1993)
7	*Bacillus subtilis*	*M. incognita* race 3	Treatment of *B. subtilis* reduced nematode multiplication and improved growth of chickpea plants	Siddiqui and Mahmood (1993)
8	*Bacillus thuringiensis*	*C. elegans*, *M. incognita*, *R. reniformis*, *P. penetrans*	Isolate 371 of bacterium-reduced nematode populations on tomato and strawberry	Zuckerman et al. (1993)
9	*Bacillus subtilis*	*M. incognita*	Nematode multiplication was reduced on tomato in pot test	Gautam et al. (1995)
10	*B. subtilis*	*M. incognita* race 3	Greater growth in chickpea plants and reduced nematode multiplication when seeds were treated with bacteria	Siddiqui and Mahmood (1995a)
11	*B. subtilis*	*H. cajani*	Reduced multiplication of pigeon pea cyst nematode in the presence of bacteria	Siddiqui and Mahmood (1995b)

(continued)

Table 16.1 (continued)

Sl no.	Bacterial species	Targeted nematode	Uses	References
12	**(B) *Pseudomonas* spp .** *P. aureofaciens*	*Criconemella xenoplax*	One strain inhibited nematode multiplication in greenhouse test	Westcott and Kluepiel (1992)
13	*P. fluorescens*	*Panagrellus* sp.	Bacteria cultivated in plant count broth for 24 h at 30 °C reduce nematode up to 57.4 %	Weidenborner and Kunz (1993)
14	*P. solanacearum*	*R. reniformis*	Resulted in slight inhibition of nematode activity on aubergine roots	Kermarrec et al. (1994)
15	*P. fluorescens*	*M. javanica*	Reduced nematode multiplication and morphometrics of root-knot females on tomato in different soil types	Siddiqui and Mahmood (1995)
16	**(C) Other bacteria** *Streptomyces* sp. isolate CR-43	*Caenorhabditis elegans*, *M. incognita*, *Pratylenchus penetrans*	Reduced multiplication of all the species in different test	Dicklow et al. (1993)
17	*Streptomyces* sp.	*Belonolaimus longicaudatus*	Isolation of aromatic nitro compounds and griseulin had nematicidal effect against nematode	Nair et al. (1995)
18	*Clostridium butyricum*	*Tylenchorhynchus martini*	Nematicidal acids produced by the bacteria reduced nematode population	Johnston (1958)
19	*Clostridium* sp.	*T. martini*	Nematicidal concentrations of n-butyric acid and lesser amount of propionic acid were quickly formed in treated pots 4 days after flooding which resulted in rapid killing of nematodes	Hollis and Rodriguez-Kabana (1966)
20	*Serratia marcescens*	*M. incognita*	Bacteria produced a volatile metabolite and were nematoxic	Zavaleta-Mejia (1985)
21	*Agrobacterium radiobacter*	*Globodera pallida*	Resulted in reduced nematode infection by 40 % when sprayed on seed pieces of potato	Sikora et al. (1989)

tophagous fungi as compared to other plants. This can be due to it being suitable ecological niche for nematophagous fungi (Birgit et al. 2006). The fungi involved in the biological control of nematodes are broadly of three types: i.e., predator, parasite, and antagonist. The antagonistic effects of fungi on nematodes may be either physical or physiological in nature. Several fungi are known to regulate the nematode densities in soil by exhibiting a range of antagonistic activity including production of nematoxic compounds (Kerry 2000; Lopez-Llorca and Jansson 2006)

and play a major role in recycling carbon, nitrogen, and other important elements from the rather substantial biomass of nematodes.

Close association between nematodes and fungi in all types of soil is the main reason for the destruction of the nematodes. Seventy genera and 160 fungi species identified so far are associated with nematodes, but only a few proved to be successful as commercial biocontrol agents in the fields (Qadri 1989). O'Bannon and Nemec (1979) reported that the combined application of a mycorrhizal fungus and the nematode *Tylenchulus semipenetrans* in lemon seedling shows reduced growth suppression. Application of *Glomus fasciculatum* or *G. etunicatum* to the cuttings of *Piper nigrum* cv. Panniyur reduced root-knot nematode (*Meloidogyne incognita*) population both in roots and soil and showed increased plant growth even in the presence of nematode (Sivaprasad et al. 1990). The mycorrhizal tobacco seedling when transplanted into root-knot nematode-infested soil showed that plant growth and yield were qualitatively and quantitatively better than in non-mycorrhizal plants. It was reported that tomato root tissues forming galls after inoculation with *Meloidogyne incognita* had no arbuscular mycorrhizal (AM) fungi, while roots without nematode galls had vesicles and arbuscules of *Glomus fasciculatum*, which inhibits the formation of nematode (Mittal et al. 1991). The cowpea plant roots when infested with AM fungi stimulated growth by improving host nutrition and minimized the damage caused by *Meloidogyne incognita* after changes in root exudates causing fewer nematodes to be attracted to the plant roots (Ahmed and Alsayed 1991). The major contribution of arbuscular mycorrhizal (AM) fungus is to reduce root disease and to increase nutrient uptake, resulting in more vigorously growing plants that are able tolerate root disease. Plants' association with mycorrhizae may also help tolerate environmental stresses, such as drought, that make them more vulnerable to fungal pathogen infection (Bethlenfalvay and Newton 1991). It had been reported that inoculation of arbuscular mycorrhizal (AM) fungus (*Glomus* sp.) to tomato, bean, chickpea, Banana tree and peach has significantly reduced galling and nematode multiplication of root-knot nematode, *M. incognita* (Sundararababu et al. 1993; Zombolin and Oliveira 1986; Diederichs 1987). *Harposporium* produces conidia which initiate infection by lodging in the buccal cavity or the gut of the nematode host, and the plant-parasitic nematodes are unable to ingest these conidia.

The genus *Arthrobotrys Corda* is one of the most interesting genera of nematode-trapping fungi and produces specialized adhesive networks to capture nematodes (Dackman and Nordbring-Hertz 1992; Tunlid et al. 1994; Zhao et al. 2004). *Arthrobotrys conoides* Drechsler is an autochthonic fungus, which can immobilize the free-living nematode *Panagrellus redivivus* and the pine wood nematode *Bursaphelenchus xylophilus* by adhesive network (Yang et al. 2007), and several fungi such as *Pochonia chlamydosporia* and *Paecilomyces lilacinus* have been developed as commercial biological nematicides. The fungus *Omphalotus olearius* produced omphalotin A, a nematicidal compound that demonstrated greatest activity against the root-knot nematode (RKN) *Meloidogyne incognita* (Buchel et al. 1998; Mayer et al. 1999). It was reported that fungal endophytes of fescue grass induced production of compounds such as loline alkaloids, pyrrolopyrazine, and organic acids that may have activity against some phytoparasitic nematodes (Rowan and Gaynor 1986; Porter 1994; Bush et al. 1997). The fungus, *Esteya vermicola*, has been used as biocontrol agent against pine wilting disease caused by *Bursaphelenchus xylophilus* (Feng et al. 2013; Xue et al. 2014). It has been reported that fungal metabolites such as aspyrone (Kimura et al. 1996), peniprequinolone (Kusano et al. 2000), $\beta\gamma$-dehydrocurvularin (Kusano et al. 2003), penipratynolene (Nakahara et al. 2004), 5-hydroxymethyl-2-furoic acid (Kimura et al. 2007), and fumiquinones A and B (Hayashi et al. 2007) act as nematicides against the pine wood nematode *Bursaphelenchus xylophilus* (Fukuda 1997; Kuroda et al. 1991) which causes pine wilt disease for the Japanese black pine (*Pinus thunbergii* Parl.) and Japanese red pine (*P. densiflora* Sieb. et Zucc.). In the management of *Pratylenchus goodeyi*, a banana root-lesion

nematode in Kenya includes the use of endophytic fungi that cause no damage to the host but gives benefits, such as enhanced protection against various biotic and abiotic constraints (Waweru et al. 2013). The fungus *Clonostachys rosea* (syn. *Gliocladium roseum*) suppresses the sporulation of the plant-pathogenic fungus *Botrytis cinerea* and kills pathogenic nematodes and is a potential biocontrol agent, but the process of nematode infection is not clear (Zhang et al. 2008). The efficacies of three nematophagous fungi, *Paecilomyces lilacinus*, *Plectosphaerella cucumerina*, and *Pochonia chlamydosporia*, are used for controlling potato cyst nematodes (Jacobs et al. 2003), and also *Penicillium oxalicum* acts as a biocontrol agent against fungal diseases and the potato cyst nematodes, *Globodera pallida* and *G. rostochiensis* (Martinez-Beringola et al. 2013).

The antagonistic fungus *Paecilomyces lilacinus* proved its activity against root-knot nematodes on varied crops. Several reports clarified that using formulated *P. lilacinus* reduced the formation of galls and egg masses (Udo et al. 2013). Also, Kiewnick and Sikora (2006) reported that the fungal biocontrol agent *P. lilacinus* strain 251 (PL251) has potential to control the root-knot nematode *Meloidogyne incognita* on tomato (Table 16.2).

Although several compounds with nematicidal activity have been reported from fungi, no major commercial product based on these natural fungal compounds has been developed yet for wide use (Li, et al. 2007). Secondary metabolites from fungi associated with rhizosphere and rhizoplane of crop plants offer an exciting area of research for the discovery of potential nematicidal compounds.

16.5 *Trichoderma*: An Antagonist Agent against Nematodes

Trichoderma is a genus of fast-growing fungi widely existing in the soil, which play an important role in biotic control. *Trichoderma* species frequently are predominant over wide geographic regions in all climatic zones, where they are significant decomposers of woody and herbaceous materials and are considered to be common fungi found in almost any type of soil, which interact with other fungi, including plant-pathogenic species. *Trichoderma* spp. act as opportunistic, avirulent plant symbionts and can produce metabolite to inhibit soil pests (Harman et al. 2004). The antagonistic nature of these species was first demonstrated over 60 years ago by Weindling (1932), who suggested their potential use as biocontrol agents for plants diseases.

The use of *Trichoderma* or *Trichoderma-based* products on plants has both short-term effects (immediate control of disease and growth enhancement) and long-term effects (decrease in fungal pathogen in field) that are due to its ability to colonize and grow in association with the host plant and induce localized or systematic resistance in them (Harman 2000). Various mechanisms have been reported for different *Trichoderma* species on how they are able to degrade. This included antibiosis, competition, mycoparasitism, and enzyme hydrolysis (Harman and Kubicek 1998; Harman et al. 2004). *Trichoderma* spp. can produce secondary metabolites that are bacteriostatic and nematicidal agents and are used as biocontrol agents, which are able to suppress *Meloidogyne* sp. populations (Suarez et al. 2004). Several reports recognized fungi as antagonist to antibiotic produced by bacteria, but there have been a limited number of observations of antibiotic production by *T. harzianum*. Numerous studies (Spiegel and Chet 1998; Susan et al. 2000; Haggag and Amin 2001; Sharon et al. 2001; Howell 2003; Siddiqui and Shaukat 2004; Santhosh et al. 2005) showed that *Trichoderma* are used for inhibiting the growth of plant-parasitic nematodes. Windham et al. (1989) reported reduced egg production in the root-knot nematode *M. arenaria* after soil treatments with *T. harzianum* (T-12) and *T. koningii* (T-8) preparations. It was reported that different *Trichoderma* spp. are capable of producing either antibiotics or intracellular lytic enzymes that are responsible for antagonism as this species have many effects on plant physiology making it challenging to understand the interaction (Dennis and Webster 1971; Elad and Henis 1982). *Trichoderma har-*

Table 16.2 Uses of some fungi as biocontrol agents against root-knot nematodes in agricultural crops

Sl no	Host plants	Nematodes	Fungal species	Effect on nematodes	References
1	Peach	*Meloidogyne incognita*	*Gigaspora margarita*	Suppressed nematodes reproduction	Strobel et al. (1982)
2	Pepper	*Meloidogyne incognita*	*Glomus fasciculatum*	Significantly reduced galling and nematode population when pre-inoculated with arbuscular mycorrhizal fungi	Sivaprasad et al. (1990)
3	Black pepper	*Meloidogyne incognita*	*Glomus mosseae, Acaulospora laevis, Glomus fasciculatum, Gigaspora margarita*	Individually all the VAM fungi reduced nematode reproduction, but the highest reduction is caused by *A. laevis*	Anandraj et al. (1990)
4	Bean	*Meloidogyne incognita*	*Glomus* spp.	Severity of nematode disease reduced due to reduction in nematode reproduction	Osman et al. (1990)
5	Tomato	*Meloidogyne* spp.	Verticillium *chlamydosporium*	Fungus reduced multiplication of nematodes on tomato plant when 2000 propagules/g of the fungus were added to the soil in the pot test	De Leij and Kerry (1990)
6	Watermelon	*M. incognita* *R. reniformis*	P. lilacinus	Reduced number of nematodes	Vicente et al. (1991)
7	Tomato	*Meloidogyne arenaria*	*Verticillium chlamydosporium*	Reduce nematode multiplication	De Leij and Kerry (1991)
8	Yellow pitaya	*Meloidogyne incognita*	*G. manihotis*	Reproductive capacity of nematode reduced	Palacino and Leguizamon (1991)
9		*Meloidogyne incognita acnta*	*Paecilomyces lilacinus*	Medium and high doses as seed dressings significantly reduced root galling	Khan et al. (1992)
10	Black pepper	*Meloidogyne incognita*	*G. fasciculatum*	Reduction in root-knot index and nematode count in the root tissue and rhizosphere soil	Sivaprasad et al. (1992)
11	Brinjal	*M. incognita*	*P. lilacinus*	Reduced nematode population on brinjal	Trivedi (1992)

(continued)

Table 16.2 (continued)

Sl no	Host plants	Nematodes	Fungal species	Effect on nematodes	References
12	Tomato	*M. javanica*	*G. fasciculatum*	Inhibit nematode population	Sundarababu et al. (1993)
14	Tomato	*Meloidogyne incognita*	*G. fasciculatum*	Reduction in nematode infestation	Mahaveer et al. (1994)
15	Tomato, aubergine	*M. javanica*	*P. lilacinus*	Suppressed 65–83 % nematodes	Ibrahim (1994)
16	Greenhouse plants	*R. reniformis*	*P. lilacinus*	This fungus had detrimental effects on nematodes both in greenhouse and field conditions	Walters and Barker (1994)
17	Clover	*M. incognita*	*Glomus* spp.	Gradual reduction in nematode population	Kassab (1995)
18	Banana	*M. incognita*	*G. mosseae*	Suppressed nematode reproduction and galling	Jaizme-Vega et al. (1997)
19	Brinjal	*M. incognita*	*G. fasciculatum*	Lowers the number of galls and egg masses	Borah and Phukan (2000)
20	Tomato	*M. incognita*	*G. mosseae*	Suppressed nematode multiplication	Bhat and Mahmood (2000)
21	Tomato	*M. javanica*	*G. mosseae*	Reduced galling and nematode multiplication	Siddiqui and Mahmood (2000)
22	Okra	*M. incognita*	*G. mosseae*	Reduced nematode population	Jothi et al. (2000)
23	Chili	*M. incognita*	*G. mosseae*	Reduced the nematode multiplication	Sundarababu et al. (2001)
24	Pearl millet and green gram	*M. incognita*	*G. mosseae*	Adversely affect the nematode population	Jothi and Sundarababu (2001)
25	Okra	*M. incognita*	*G. mosseae*	Suppressed root galling	Sharma and Mishra (2003)
26	Ginger	*M. incognita*	*G. fasciculatum* *G. mosseae*	Suppressed the nematode population	Nehra (2004)
27	Tomato	*M. incognita*	*G. fasciculatum*	Reduced nematode population	Kantharaju et al. (2005)
28	*Mentha arvensis*	*M. incognita*	*G. aggregatum*	Significantly reduced nematode population	Pandey (2005)
29	Tomato	*M. incognita*	*G. fasciculatum*	Reduced nematode population, number of galls, and root-knot index	Shreenivasa et al. (2007)

(continued)

Table 16.2 (continued)

Sl no	Host plants	Nematodes	Fungal species	Effect on nematodes	References
30	Tomato	*M. incognita*	AM fungi + organic fertilizers	Less galling and nematode multiplication	Siddiqui and Akhtar (2007)
31	Tomato	*M. incognita*	*G. intraradices* *Rhizobium etli*	Reduction in root galling	Reimann et al. (2008)
32	Cucumber	*M. incognita*	*Glomus* spp.	Decreased the number of females, eggs, and egg masses in the root	Zhang et al. (2008)
33	Cucumber	*M. incognita*	*G. intraradices*	Decreased the number of galls, egg masses, and eggs g^{-1} root	Zhang and Zhang (2009)
34	Tomato	*M. incognita*	*G. aggregatum*	Reduced RKI, NRR, number of galls, and egg masses	Serfoji et al. (2010)
35	Tomato	*M. incognita*	*G. clarum*, *Gigaspora albida, and Acaulospora scrobiculata*	Significantly reduced the gall index and number of egg mass	Da Silva-Sousa et al. (2010)
36	Tomato	*Meloidogyne* spp.	20 strains of AM fungi	Suppressed nematode development, multiplication, and root galling	Affokpon et al. (2011)
37	Barley tomato roots	*Meloidogyne javanica*	*Pochonia chlamydosporia*	Promote plant growth and affect management of root-knot nematode infestations	Escudero and Lopez-Llorca (2012)

zianum or *T. atroviride* could produce glucose oxidase in addition to anti-nematodal compounds that directly affect nematodes or make the roots less attractive and antagonize *Meloidogyne incognita* eggs. This antagonizing mechanism leads to the limiting nematodes' capacity to penetrate the host plant roots. The effectiveness of *Trichoderma* spp. varies with the host plant and nematode species. The eggs, larvae of *Meloidogyne javanica*, and immature female of *Rotylenchulus reniformis* can be effectively controlled by *T. harzianum* and *T. hamatum*.

Different types of volatile and nonvolatile low molecular weight diffusible compounds are released when *Trichoderma* spp. interacts with the host plant. These compounds include antibiotics (harzianic acid, alamethicins, tricholin, peptaibol) and cell wall-degrading enzymes (chitinolytic enzymes, glucanases, proteases). Different species of *Trichoderma* utilize these compounds to degrade the pathogen cell wall, thus reducing the incidence of disease (Lorito et al. 1993; Haggag and Amin 2001; Jin et al. 2005).

The successful biocontrol potential of *Trichoderma* isolates against many plant species has been reported, and it has been proved beyond doubts that they can enhance the plant productivity and induce resistance in the plants both in greenhouse and in field conditions (Papavizas 1985). The genus *Trichoderma* comprises of numerous species, some of which

have got the unique potential as biological control agents through different mechanisms like growth enhancement by reducing the root-knot nematode damage in the rhizosphere (Windham et al. 1989; Meyer et al. 2001). *Trichoderma harzianum* Thu (ATCC-PTA 3701) is a nematode-inhibiting strain that has strong anti-nematode activity against *M. incognita* in tomato, basil, and chamomile and has plant growth-promoting capabilities, therefore considered a suitable biocontrol agent (Pandey et al. 2011). *T. harzianum* strain Thu when inoculated with vermicompost can be an environmentally friendly strategy to reduce the population of *M. incognita* on *W. somnifera* and to obtain higher root yields. Besides providing a complete nutrition to the plant, vermicompost supports the growth of *T. harzianum* in the rhizosphere (Kalra et al. 2010), thus substantially restricting the nematode population buildup. Such approach involving *T. harzianum* and vermicompost may also be suitable in other disease management strategies for minimizing the yield losses caused by phytopathogens even in the subsequent crops. *Trichoderma viride* acts as antagonist against the root-node nematodes *Meloidogyne incognita* and improves growth of mulberry with increased leaf yield and reduced nematode population (Muthulakshmi et al. 2010). *Trichoderma harzianum* BI was evaluated for its capacity to reduce the incidence and pathogenicity of the root-knot nematode *Meloidogyne javanica* on tomato (Naserinasab et al. 2010; Gupta et al. 2015). Moreover, this study demonstrates the biocontrol activities of *Trichoderma* isolates and their parasitic capabilities on *M. javanica*, elucidating the importance of the gelatinous matrix in the fungal parasitism (Sharon et al. 2007).

A number of *Trichoderma* isolates are now used commercially for the control of fungal pathogens in the soil. Judicious use of *Trichoderma* in combination with other plants symbionts and rhizosphere microorganisms can serve as a model for the introduction and implementation of biocontrol means for the management of root-knot nematode (Table 16.3).

16.6 Conclusion

Plant-parasitic nematodes, especially root-knot nematodes, *Meloidogyne* spp., are considered the most economically important group worldwide. They attack a wide range of crops, especially vegetable crops, and cause severe damage and high yield lose. During the last few decades, nematode control has been based on the use of chemical pesticides applied to soil or the plant. New efforts are being made to develop management strategies that do not rely on nematicides or are aimed at reducing the use of pesticide materials. There are many promising biocontrol agents against root-knot nematodes such as vesicular-arbuscular mycorrhizal fungi (AMF), mutualistic fungal and bacterial endophytes, egg pathogenic fungi, obligate parasites, and antagonistic plant growth-promoting rhizobacteria (PGPR). Although a large number of fungi have been reported to reduce nematode density, only a few of them have shown their efficacy as efficient parasites of nematodes. Even the efficient parasites do not have all the desired characteristics of a good biocontrol agent, but their application has given promising results. Such abilities make them of great value in ecology and agricultural economy, such as controlling plant- and animal-parasitic nematodes. Recently, an increasing number of bacteria have been reported to infect nematodes and shown potential application in biocontrol of nematodes. Although the pathogenic mechanism of fungi and bacteria are different, extracellular enzymes (especially serine proteases) identified from them are important virulence factors in the infections of these microorganisms against nematodes. Over the last few years, an increasing number of nematicidal enzymes were identified from different microorganisms, and these enzymes showed immense practical potential, especially as biocontrol agents. Meanwhile, strain improvement by using biotechnology has received increased attention in recent years. Moreover, the structures of proteases and chitinases were resolved, which provided a basis for improving the catalytic activity of these enzymes. In summary, studies of

Table 16.3 Uses of some *Trichoderma* species as biocontrol agents against root-knot nematode infection in agricultural crops

Host plants	Nematodes	Biocontrol agents	Effect on nematodes	References
Eggplant	*M. incognita*	*Trichoderma harzianum*	Reduced nematode population and galling	Rao et al. (1996)
Tomato	*M. incognita*	*T. virens* + *Burkholderia cepacia*	Suppressed egg hatching and J2 mobility	Meyer et al. (2000)
Tomato	*M. javanica*	*T. harzianum*	Second stage juveniles immobilization and reduced root galling	Sharon et al. (2001)
Tomato	*M. hapla* and *M. incognita*	*T. viride* + *Glomus intraradices*	Reduced the number of galls and egg sacs	Masadeh et al. (2004)
Soybean	*M. incognita*	*T. pseudokoningii* + *G. mosseae* + *Bradyrhizobium japonicum*	Suppressed nematode reproduction and galling	Oyekanmi et al. (2007)
Tomato	*M. incognita*	*T. harzianum*	Reduction in galling and nematode multiplication	Siddiqui and Akhtar (2008)
Tomato	*M. javanica*	*T. harzianum*	Significantly decreased nematode egg hatching level, number of galls and eggmasses per plant, and number of eggs per eggmass	Sahebani and Hadavi (2008)
Soybean	*M. incognita*	*T. pseudokoningii* + *G. mosseae* + *B. japonicum*	Reduction in nematode density in roots	Oyekanmi et al. (2007)
Soybean	*M. incognita*	*T. harzianum*	Suppressed final nematode population, root galling, and eggmass	El-Sharif and Ismail (2009)
Patchouli	*M. incognita*	*T. harzianum*	Reduction in the severity of root-knot disease and nematode population	Pandey et al. (2009)
Tomato	*M. incognita*	*T. harzianum* + waste material	Reduction in galling and nematode multiplication	Siddiqui and Shakeel (2009)
Balloon-flower	*M. incognita*	*T. viride* + plant pesticide residues	Inhibited egg hatching and reduced root galling and nematode population	Zhang and Zhang (2009)
Tomato	*M. incognita*	*T. harzianum* + other PGPR	Reduction in nematode galling and multiplication	Siddiqui and Akhtar (2009)
Tomato	*M. incognita*	*T. harzianum*	Decreased nematode development and reproduction parameters	Abd-Elgawad and Kabeil (2010)

(continued)

Table 16.3 (continued)

Host plants	Nematodes	Biocontrol agents	Effect on nematodes	References
Mulberry	*Meloidogyne incognita*	*Trichoderma viride*	Improved growth of mulberry with increased leaf yield and reduced nematode population	Muthulakshmi et al. (2010)
Tomato	*Meloidogyne javanica*	*Trichoderma harzianum* BI	Reduced the incidence and pathogenicity of the root-knot nematode	Naserinasab et al. (2010)
Tomato	*Meloidogyne incognita*	*Trichoderma* isolates	Inhibited nematode reproduction, suppressed second stage juvenile densities in roots, egg production, and root galling	Affokpon et al. (2011)
Withania somnifera	*Meloidogyne incognita*	*Trichoderma harzianum*	Inhibited nematode population and helped to obtain higher root yield of *Withania somnifera*	Pandey et al. (2011)
Tomato	*Meloidogyne javanica*	*Trichoderma* sp.	Reduced nematode population	Golzari et al. (2011)
Tomato	*Meloidogyne javanica*	*Trichoderma longibrachiatum*, *Mortierella* sp.	Enhanced the plant growth by supplying many nutritional elements and induced the systemic resistance in plants	Al-Shammari et al. (2013)
Okra	*Meloidogyne* spp.	*Trichoderma viride*	Showed positive impact on plant growth by improving plant height, fresh shoot weight, and root length	Afzal et al. (2013)
Tomato	*Meloidogyne javanica*	*Trichoderma harzianum* (its teleomorph is *Hypocrea lixii*)	Produced toxic effect on nematodes Enhanced plant growth, supplying many nutritional elements and inducing systemic resistance in the plants	Elgorban et al. (2013)
Bacopa monnieri	*Meloidogyne incognita*	*Trichoderma harzianum* ThU	Enhanced bacoside contents and reduced nematode population	Gupta et al. (2015)

nematicidal enzymes identified from nematophagous fungi and bacteria will enhance the potential application of these novel biochemical processes. Besides the general challenges of biocontrol, the farmer is faced with peculiar conditions. It is established that the efficiency of biocontrol agents varies with soil type; therefore, for microbial agents to be very effective, they have to be isolated from the surrounding environment. Identification of biocontrol agents largely involves the manipulation of naturally occurring microbial organisms rather than the introduction of identified and researched agents. Giving the existing scenario, the prognosis for the farmer

assessing biocontrol, as a part of nematode disease management, is very poor. Government and other research-funding bodies must be committed to investing in manpower development and funding of research in this area.

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Eco-friendly Plant Growth Promoting Rhizobacteria for Crop Improvement

17

K.V.B.R. Tilak and C. Manoharachary

Abstract

Soil microorganisms are important in the geobiochemical cycles of inorganic and organic nutrients in the soil and maintenance of soil health and quality. The rhizosphere of plants is inhabited by complex and dynamic communities of microorganisms, notable among which are plant growth-promoting and soil-supporting bacteria. Soil-plant-rhizobacteria interactions are complex, and there are many ways in which the outcome can influence the plant health and productivity. The PGPRs are also potential biocontrol agents of several soilborne plant pathogens. The rhizosphere provides the frontline defense of roots against attack by pathogens. The present review provides the interaction of PGPR with other microbes in the rhizosphere, thus contributing for sustainable crop production.

Keywords

Rhizosphere • PGPR • Mycorrhizae • Bioinoculants • Biocontrol agents

17.1 Introduction

Bacteria inhabiting the rhizosphere and beneficial to plants are termed as plant growth-promoting rhizobacteria (PGPRs) (Kloepper et al. 1980a, b). Plant growth-promoting rhizobacteria (PGPRs) or plant health-promoting rhizobacteria (PHPRs) (Kloepper et al. 1989) were first defined by Kloepper and Schroth (1978) to describe such soil bacteria that colonize the roots of plants following inoculation onto seed and that enhance plant growth. About 2–5 % of rhizosphere bacteria are PGPR (Antoun and Prévost 2005) which are free-living bacteria. However, some researchers have coined a broader definition of PGPR to include symbiotic microorganisms like nitrogen-fixing rhizobia. Vessey (2003) and Gray and Smith (2005) designated rhizobia and *Frankia* species that are involved in symbiotic associations with

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higher plants as intracellular PGPRs or symbiotic PGPRs. Dinitrogen-fixing associative symbiotic bacteria which do not cause any morphological modification of the host plant are also considered as PGPRs.

PGPR may enhance plant growth through direct or indirect mechanisms (Kloepper 1993; Lazarovits and Nowak 1997). Direct mechanisms of enhancement in plant growth include production of phytohormones, increased availability of nutrients to plants, stimulation of disease resistance mechanisms, and others. Indirect mechanisms include control of plant diseases, stimulation of other beneficial symbioses, degradation of xenobiotics in contaminated soils, and increasing immunity and protection from disease and abiotic stresses (Jacobsen 1997). Based on their functions, PGPRs may be classified as biofertilizers (increasing availability of nutrients to plants), biopesticides (controlling diseases, insect pests, nematodes, etc. by production of antibiotics, antifungal metabolites, etc.), phytostimulators (production of plant growth hormones) and rhizoremediators (degradation of pollutants), and others (Somers et al. 2004, 2005).

In most cases, a single PGPR exhibits multiple growth-promoting attributes including biocontrol ability (Vessey 2003). PGPRs are commonly used to improve crop yields and help in sustainable agriculture (Fig. 17.1). Further, they possess potential in solving environmental problems including phytoremediation to decontaminate soils and waters.

A considerable number of soil and rhizospheric fungi and bacteria collectively known as plant growth-promoting microorganisms (PGPMs) have demonstrated their ability to colonize plant roots and provide benefits to their respective hosts (Manoharachary and Tilak 2015; Tilak 2015a, b). Among these benefits, many authors documented improved root hydraulic conductance and alleviation of abiotic stresses such as drought and salinity. It is accepted that movement through aquaporins represents a quite faster pathway of water movement across biological membranes. Groppa et al. (2012) reviewed the PGPM effects on plant water status and root hydraulic conductance, with special emphasis on the experimental data that proved or suggested an impact of PGPM on root aquaporins under both normal and water-limiting conditions.

In recent years, the role of the rhizosphere as an ecosystem has gained importance in understanding the functioning of biosphere and also mechanisms of action of PGPR (Barriuso et al. 2008). The earlier studies on PGPR laid emphasis on biological control of plant diseases confined to bacteria like fluorescent pseudomonads and *Bacillus* spp. In recent years, with the elucidation of many mechanisms of plant growth promotion involving a large number of plant and microbial species, knowledge about very diverse bacterial taxa has been obtained (Lucy et al. 2004).

Fluorescent pseudomonads and bacilli form a major group among PGPRs along with other bacteria like *Acetobacter*, *Actinoplanes*, *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Cellulomonas*, *Clostridium*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pasteuria*, *Serratia*, *Xanthomonas*, etc. The rhizosphere microorganisms also include rhizobia and bradyrhizobia and establish symbiotic relationship with leguminous plants. These bacteria generally improve the plant growth through direct effects on plant by producing plant growth-promoting substances, thus increasing the availability and uptake of nutrients besides suppressing soilborne plant pathogens (Dutta and Podile 2010; Tilak et al. 2010; Wu et al. 2009; Lugtenberg and Kamilova 2009; Nautiyal and Tilak 2009).

Over the last decade, understanding of rhizosphere biology has progressed with the discovery of PGPR that colonizes plant roots and promotes plant growth. These PGPRs could compete with other rhizosphere microorganisms most effectively leading to increased plant growth (Kloepper et al. 1980b). Application of plant growth-promoting rhizobacteria has also shown to increase legume growth and development in terms of plant nodulation and nitrogen fixation under normal growth conditions along with an increase in plant yields (Angaw et al. 2011a, b;

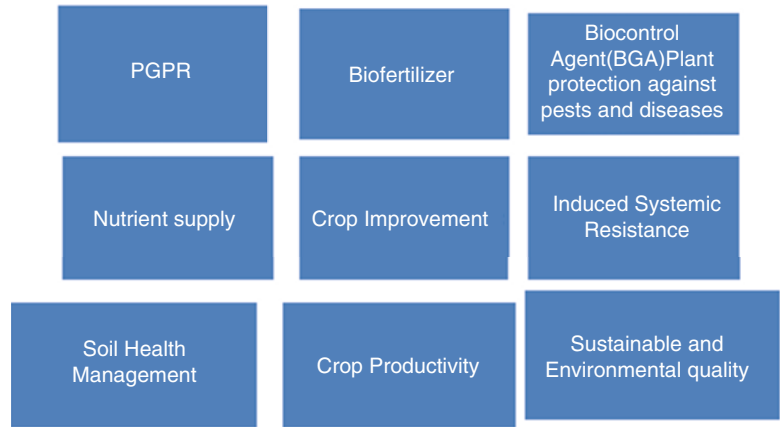

Fig. 17.1 Functions of plant growth-promoting rhizobacteria (PGPRs)

Tilak et al. 2010; Vogeti et al. 2009; Podile and Kishore 2006; Gupta et al. 2003; Tilak 2015a, b).

17.2 PGPRs as Bioinoculants

Huge amount of literature is available on the application of bacteria for improvement of plant performance (Saxena and Tilak 1994; Malik et al. 1996, 1999; Saxena et al. 2000; Gupta et al. 2003; Shende et al. 2010; Tilak et al. 2010; Angaw et al. 2011a, b; Tilak 2015a, b), but few bacteria like *Azotobacter* and *Azospirillum* have been employed in commercial production. The organisms under scrutiny for potential use in agriculture are the bacteria belonging to the genera *Pseudomonas* and *Bacillus* species (Nautiyal et al. 2002, 2006; Tilak et al. 2006; Tilak and Reddy 2006; Podile and Kishore 2006; Nautiyal and Tilak 2010). Positive response of plant growth has been recorded by promoting chitinolytic *Paenibacillus elgii* in tobacco (Das et al. 2010). In the last two decades, several examples of rhizobacteria capable of providing substantial disease control in the field have been reported (Saxena et al. 2000; Nautiyal et al. 2002, 2006). Many bacterial genera have shown their potential for biocontrol both under in vitro and in vivo conditions. The usefulness of *Bacillus* as a source of antagonist for many plant pathogens is well known. Several potent strains from different species of *Bacillus* have been tested on a wide variety of plant species for their ability to control several diseases. *Bacillus* has ecological advantages because it produces endospores that are tolerant to extreme environmental conditions such as heat and desiccation (Nautiyal et al. 2006).

Fluorescent pseudomonads have revolutionized the field of biological control of soilborne plant-pathogenic fungi. During the last decade, PGPRs have emerged as the largest potentially promising group involved in the biocontrol of plant diseases. They have received the much needed attention for several reasons, such as their colonization ability in roots, their simple nutritional requirements, and particularly their ability to use many carbon sources that exude from roots and to compete with the indigenous microflora in the rhizosphere. Apart from these qualities, pseudomonads are amenable to genetic manipulation. These characteristics make them ideal as a promising bioinoculant. There are numerous examples of biocontrol agents that control devastating fungal plant pathogens of important crops including fluorescent pseudomonads (Nautiyal 1997a, b; Anith et al. 1998; Pal et al. 2000; Saxena et al. 2000; Nautiyal et al. 2006).

17.3 PGPR: Arbuscular Mycorrhizae

More than 80 % of all terrestrial plant species form mycorrhizal associations (Sylvia 2005). Arbuscular mycorrhiza (AM) is the most common mycorrhizal association and has a widespread

distribution throughout the plant kingdom forming mutualistic relationship with most of the vascular plants. In Cruciferae, Chenopodiaceae, Polygonaceae, and Cyperaceae, either there is very little mycorrhization or no mycorrhizae. Families that do not form arbuscular mycorrhiza include Pinaceae, Betulaceae, Fumariaceae, Commelinaceae, and Urticaceae. The fungal partner belongs to Glomeromycota forming vesicles within or between cortical cells that act as storage or reproductive organs and arbuscules that are formed within the cortical cells providing a large surface area of contact between host and fungus mycelium which is formed inside and outside the root. The genera, which form arbuscular mycorrhizal (AM) fungal association, are *Acaulospora*, *Ambispora*, *Aracheospora*, *Diversispora*, *Entrophospora*, *Geosiphon*, *Gigaspora*, *Glomus*, *Intraspora*, *Kuklospora*, *Pacispora*, *Paraglomus*, and *Scutellospora* (Schenck and Perez 1990; Schluβler et al. 2001; Oehl and Sieverding 2004; Sieverding and Oehl 2006; Walker et al. 2007).

Mycorrhizal association helps in increased nutrient and water uptake by absorption through improved absorptive area, translocation of elements to host tissues, and their accumulation. The unique ability of mycorrhiza helps to increase the uptake of phosphorus (P) and other nutrients by plants, suggesting that mycorrhizal fungi have the potential for utilization as a supplement for phosphatic fertilizers. Ectomycorrhizal fungi permeate the F and H horizon of forest floor, and minerals get mobilized in these zones by hyphal network followed by their absorption before they reach the subsoil system. AM fungi are known to degrade complex minerals and organic substances in the soil and thus make essential elements available to host plants. Mycorrhizal association is known to offer resistance to drought and plant pathogens and tolerance to adverse conditions, release growth hormones like auxins and gibberellins and growth regulators such as vitamin B, and also contribute to organic matter turnover along with nutrient cycling in forest and cropland ecosystems. Mycorrhiza is known to help in soil aggregation and soil stabilization and add strength to soil fertility. Mycorrhizae are symbiotic, and hence they live hand in hand with other living organisms and are non-pollutants besides sustaining competition.

AM fungi are geographically ubiquitous and are commonly associated with plants in agriculture, horticulture, pastures, and tropical forests. About 90 % of vascular plants establish mutualistic relationship with AM fungi (Kendrick and Berch 1985).

The occurrence of AM fungi in roots has been reported from an exceptionally wide range of plants. Besides roots, the colonization has been reported in other plant parts also, for example, in leaves of *Salvinia* (Bagyaraj 1984; Bagyaraj et al. 1979), in senescent leaves of *Funaria hygrometrica* (Park and Linderman 1980), in decaying peanut leaves, and rhizomatous tissue of *Zingiber officinale* (Taber and Trappe 1982). Colonization has also been reported from scales of *Colocasia antiquorum*, *Elettaria cardamomum*, and *Musa paradisiaca* and *Sansevieria trifasciata*, garlic, and ginger (Kunwar and Manoharachary 1998, 1999).

Arbuscular mycorrhizal interactions bring about certain changes in the host metabolism and physiology. These include inhibition of increased production of cytokinins as evidenced by the presence of two gibberellin-like substances in culture extracts of *Glomus mosseae* and increased nitrate reductase activity.

Mycorrhizal symbioses are important considering the fact that 70–80 % of terrestrial plants are mycorrhizal, thus helping in the acquisition of water and minerals, besides offering protection from diseases. The development and formation of mycorrhizae cause changes not only in host plant but also in the rhizosphere microbial community, resulting in interaction among rhizosphere microorganisms (Bianciotto and Bonfante 2002). Bianciotto et al. (1996) suggest that bacteria present in/on spores or hyphae of AM fungi release extracellular soluble factors which mediate the bacterial-fungal interactions and AM fungi. These beneficial organisms serve as vehicles for colonization of plant roots by rhizobacteria. Rhizobacteria showing a beneficial effect on mycorrhizae are often termed as "mycorrhiza helper bacteria." Bianciotto et al.

(2004) observed strong evidence of a vertical transmission of endobacteria through the vegetative generation of AM fungus which offers effective nutritional security to the host plant.

Studies have shown that inoculation with PGPR and diazotrophs along with AM fungi may increase plant growth and yield. Chanway and Hall (1991) estimated that associative nitrogen fixation by *Bacillus* could contribute in part to the growth promotion effect observed with *Pinus contorta* inoculated with the mycorrhizal fungus, *Wilcoxina mikolae.* Colonization by AM fungi may modify the root exudate pattern, which may act as chemoattractants for the soil bacteria. In a dual inoculation study with *Glomus mosseae*, *Bacillus coagulans* was superior to a single inoculant, i.e., *Azotobacter chroococcum*, in enhancing plant biomass of *Simarouba glauca* (Sailo and Bagyaraj 2003). Wu et al. (2009) reported increased growth and nutrient uptake in maize, enhanced root colonization by the AM fungus, and improved soil properties when inoculated with a biofertilizer containing N-fixer (*A. chroococcum*), P-solubilizer (*B. megaterium*) and K-solubilizer (*B. mucilaginous*), and AM fungus (*G. mosseae* or *G. intraradices*).

PGPR beneficial effect was also observed in *Eucalyptus diversicolor* along with an unidentified bacterium resulting in 49 % more shoot dry weight than the uninoculated control.

The effects of combined inoculation with PGPR, AM fungi, and rhizobia have been tested by many workers. Extracellular metabolites produced by the above organisms could possibly be the reason for the synergistic effects. This is documented by the addition of cell-free culture filtrate of PGPR to the mycorrhizal and nodulated legume *Hedysarum coronarium* resulting in maximum plant growth and nutrient uptake in comparison to washed cells of PGPR or the whole bacterial cultures (Azcon 1993).

The interactive effects of PGPR, AM fungi, and rhizobia have resulted in bioremediation effect of heavy metal-contaminated and polluted soils (Vivas et al. 2003a, b). In a lead-contaminated soil, co-inoculation with *Brevibacillus* sp., an indigenous PGPR strain, and a mixture of indigenous AM fungal species enhanced plant growth, mycorrhizal infection, and N and P content in clover, along with a decrease in the amount of lead absorbed (Vivas et al. 2003b).

Different mechanisms allow AM fungi and PGPR to increase stress tolerance in plants. This includes the intricate network of fungal hyphae which block pest access to roots and various biocontrol mechanisms of PGPR. Inoculation of apple tree seedlings with *Glomus fasciculatum* and *G. macrocarpum* suppressed the apple replant disease (ARD) caused by phytotoxic micromycetes (Catska 1994). After 12-month cultivation, plant biomass (height, shoot, and root dry masses) had increased by inoculation with *G. fasciculatum*. The number of colony-forming units (CFUs) per unit soil of phytotoxic micromycetes decreased, whereas CFU of the genus *Azospirillum* was higher. It may be assumed that the use of some AM fungi and such bacteria can replace the chemical treatment of the soil with ARD. AM fungi protect the host plant against root-infecting pathogenic bacteria. The damage due to *Pseudomonas syringae* on tomato is significantly reduced when the plants are endomycorrhizal (Garcia Garrido and Ocampo 1989). The mechanisms involved in these interactions include physical protection, chemical interactions, and indirect effects (Fitter and Garbaye 1994).

The rhizosphere is thus influenced by the plant roots as well as by mycorrhizal fungus. The mycorrhizosphere is the zone influenced by both the root and the mycorrhizal fungus, and it includes the more specific term "hyphosphere" which refers only to the zone surrounding individual hyphae (Johansson et al. 2004). Bacterial communities associated with plant roots may be affected by root colonization with AM fungi. This may be due to metabolic products of AM fungi and their resultant changes. The hyphal exudates might have been detrimental or stimulatory effect on rhizosphere bacteria. Sood (2003) reported greater attraction of the PGPRs *Azotobacter chroococcum* and *Pseudomonas fluorescens* toward tomato roots colonized by *Glomus fasciculatum* compared to non-arbuscular

mycorrhizal tomato roots. Rhizosphere bacteria remain in close association with AM fungi. Endosymbiotic bacteria closely related to the genus *Burkholderia* have been found in symbiotic AM fungi *Gigaspora margarita*, *Scutellospora persica*, and *Scutellospora castanea* (Bianciotto et al. 2000). PGPR and AM fungi interactions have shown synergistic effects. In a Petri plate system, roots of carrot (*Daucus carota* L.) inoculated with phosphate-solubilizing bacteria *Pseudomonas aeruginosa* showed substantial increase in P-solubilization when inoculated with *G. intraradices* (Villegas and Fortin 2001).

17.4 Rhizobacteria as Biological Control Agents of Plant Pathogens

The suppression of growth of soilborne and rootborne plant pathogens by the use of antagonistic microorganisms to reduce diseases is termed as biocontrol. The National Academy of Sciences (USA) defined biocontrol as "the use of natural or modified organisms, genes or gene products to reduce the effects of undesirable organisms (pests), and to favour desirable organisms such as crops, trees, animals and beneficial insects and microorganisms." Wilson (1997) defined biological control as "the control of a plant disease with a natural biological process or with the product of a natural biological process." This definition allows the inclusion of biological chemicals produced by living organisms and extracted from them, host resistance (constitutive and induced), and antagonistic microorganisms (Singh et al. 2004).

The rhizosphere bacteria are the ideal biocontrol agents as they can provide the frontline defense for plant roots against the attack by various root-/soilborne plant pathogens (Dey et al. 2014; Manoharachary and Tilak 2015). Disease suppression by biocontrol agents occurs due to interactions among the biocontrol agents and the members of the spermosphere and rhizosphere or phyllosphere community (Singh et al. 2004). The microbes used in biocontrol have various advantages, namely:

1. These organisms are considered safer than the chemicals that do not accumulate in the food chain.
2. Self-replication circumvents repeated applications.
3. Unlike chemical agents, target organism seldom develops resistance to target organisms.
4. Chemical control agents along with biocontrol agents are advocated in integrated plant disease control management.
5. Properly developed biocontrol agents are not considered harmful to the population and functional dynamics of the soil microorganisms in the rhizosphere.

The major disadvantages include variability of field product. Also, the effectiveness of a given biocontrol agent may be restricted to a specific location, due to the effects of soil and climate. Moreover, biological control depends upon the establishment and maintenance of a threshold population of bacteria on planting material or in soil, and a drop in viability below that level may eliminate the possibility of biological control (Weller 1988). Many soil edaphic factors, including soil temperature, moisture, pH, clay content, and interactions of biological disease control microorganisms with other rhizosphere bacteria and with pathogens, also affect their viability and tolerance to abiotic stresses once applied (Dey et al. 2012). Concentration of O_2 and CO_2 in the soil is also one of the major factors that affects activity of biocontrol agents in the rhizosphere (Nautiyal 1997a, b; Goel et al. 2001).

Several rhizosphere bacteria have been demonstrated to possess biocontrol potential which include potential *Pseudomonas* spp. which make up a dominant population in the rhizosphere and seem to be one of the most appealing for the biological control of plant diseases (Fig. 17.2). The worldwide interest in the *Pseudomonas* spp. as biocontrol agents was started in the late 1970s with the studies conducted at the University of California, Berkeley, USA (Weller 1988), and several companies now have developed biocontrol agents as commercial products. Fluorescent pseudomonads possess several properties like

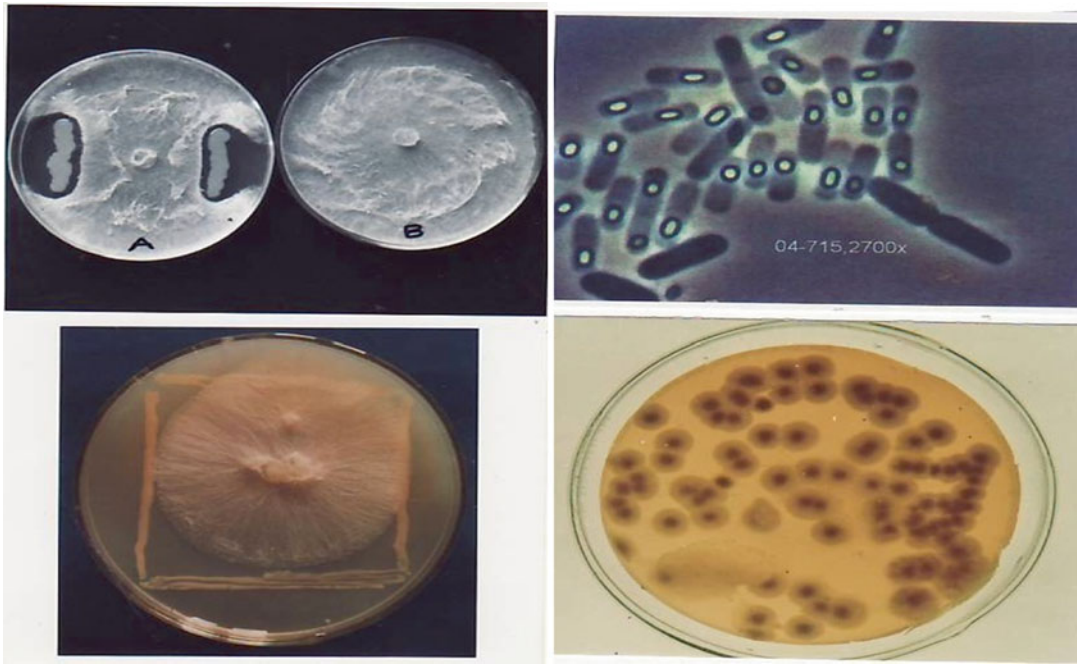

Fig. 17.2 *Right: Top*, growth inhibition of soilborne root pathogens by *Pseudomonas putida*; *bottom*, inhibition of *Sclerotium oryzae* by *Pseudomonas putida. Left: Top*, cells of *Bacillus cereus*; *bottom*, cultural characteristics of pseudomonads (Direct isolation from soil on PIA medium)

relatively easily culturable under laboratory conditions, production of a variety of secondary metabolites which are toxic to bacterial and fungal pathogens, and compatibility with commonly used pesticides and other biocontrol agents which have made them as ideal biocontrol agents (Vidhyasekharan and Muthamilan 1995).

Despite the extensive research where biological agents have been used to control plant diseases, there has been limited commercial success. An efficient biocontrol agent must meet the requirements of a good colonizer and critical competitor in the rhizosphere besides being viable and non-contaminant along with good shelf life and quality. Root colonization is a prerequisite for a strain to act as successful as a biocontrol agent. Colonization is an active process, which involves the proliferation of microorganisms in/on and around the growing roots (Johri et al. 1997). Microorganisms compete with each other for carbon source, mineral nutrients at infection sites on the roots. Competition between the biocontrol agent and pathogen can result in displacement of the latter (Osburn et al. 1989), provided environmental variables are cooperative/favorable.

Pseudomonads have revolutionized the field of biological control of soilborne plant-pathogenic fungi. Most of them fall either in *fluorescens* or *putida* group (Fig. 17.2). During the last three decades, they have emerged as the largest potentially most promising group of plant growth-promoting rhizobacteria involved in the biocontrol of plant diseases (Cook 1993; Pierson and Weller 1994; Barbosa et al. 1995; Gomes et al. 1996; Wei et al. 1996; Compant et al. 2005). Fluorescent pseudomonads have received much attention as they readily colonize roots in nature, besides being common among microorganisms (Weller 1988). The simple nutritional requirement and the ability to use many carbon sources that exude from roots and to compete with indigenous microflora may explain their ability to colonize the rhizosphere (Mazzola and Cook 1991). Additionally, pseudomonads are amenable to genetic manipulation. These characteristics

make them useful vehicle for the delivery of antimicrobial and insecticidal compounds and plant hormones to the rhizosphere. The traits of fluorescent pseudomonads such as production of antibiotics, hydrogen cyanide, and siderophore which are involved in suppression of plant root pathogens have been reviewed (O'Sulivan and O'Gara 1992; Kloepper et al. 1980a).

There are numerous examples of biocontrol of several devastating fungal plant pathogens of important crops by fluorescent pseudomonads that have been reviewed from time to time (O'Sulivan and O'Gara 1992; Kumar and Dube 1992; Weller and Thomashow 1993; Krishna Murthy and Gnanamanickam 1997; Pierson and Weller 1994; Saxena et al. 2000; Pal et al. 2001; Duffy et al. 2004; Compant et al. 2005). Natural disease suppression involving pseudomonads has been reported by many workers. It was noticed that in disease-suppressive soils, continued cropping fails to suppress the disease. The results indicate that naturally occurring soil pseudomonads are important elements in these soils to suppress the diseases.

A number of *Pseudomonas* strains have been used as biological control agents in greenhouse and field conditions against an array of plant pathogens. In many cases, they not only help in suppressing the pathogens but also improve the plant yield by acting as plant growth promoters (O'Sulivan and O'Gara 1992; Dowling and O'Gara 1994). *Pseudomonas* spp. have great potential in biological control of plant pathogens (Pal et al. 2001; Manoharachary and Tilak 2012). Few examples of PGPRs as biocontrol agents against plant pathogens are enlisted in Table 17.1.

17.5 Conclusion

Soil microorganisms are important in the geobiochemical cycles of inorganic and organic nutrients in the soil and maintenance of soil health and quality. The rhizosphere of plants is inhabited by complex and dynamic communities of microorganisms, notable among which are plant growth-promoting and soil-supporting bacteria. Soil-plant-rhizobacteria interactions are complex, and there are many ways in which the outcome can influence the plant health and productivity. The PGPRs are also potential biocontrol agents of several soilborne/root-borne plant pathogens. The rhizosphere provides the frontline defense of roots against attack by pathogens.

PGPR and other beneficial microorganisms including AM fungi and rhizobia have vast potential of their exploitation as beneficial inoculants for crop productivity and establishment of forest seedlings besides their utility in disease suppression and biocontrol agents.

There are problems preventing the commercial use like quality, viability, shelf life, and acceptance by the farmer. Improvement of the biocontrol mechanisms of these bacteria by ecological or genetic means is an important approach for enhancing their performance as bioinoculants. However, inconsistency of the bioinoculants demonstrates that there is still a considerable need for extensive studies on rhizobacterial and mycorrhizal populations to understand the different criteria, which influences the composition of the microflora and their diversity.

Recent advances in our understanding of the ecology and molecular biology of the systems responsible for effective and competitive PGPR bioinoculants are opening the ways for strain improvement. The new tools such as recombinant DNA technology, mathematical modeling, and computer technology combined with a continuation of the more classical approaches such as crop rotation, various tilling strategies, addition of organic amendments, etc. may help to harness the power of PGPRs to improve the soil, the plant, human health, and the environment.

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Table 17.1 Biocontrol of pathogens by PGPR

Biocontrol organism	Suppressed pathogen	Crop
Pseudomonas fluorescens	*Erwinia* spp.	Potato
	Erwinia carotovora	Cassava
	Fusarium spp.	Radish
	Thielaviopsis basicola	Tobacco
	Rhizoctonia solani	Peanut
	F. oxysporum f. sp. *ciceris*	Chickpea
	Pythium ultimum	Pea
	Xanthomonas malvacearum	Cotton
	Botrytis cinerea	*Petunia*
	Macrophomina phaseolina	Chickpea
	G. graminis var. *tritici*	Wheat
	Sarocladium oryzae	Rice
Pseudomonas putida	*Fusarium* spp.	Radish
	Erwinia carotovora	Potato
	F. oxysporum	Flax
	F. oxysporum f. sp. *lycopersici*	Tomato
	F. solani	Beans
	Xanthomonas campestris	Potato
P. aureofaciens	*G. graminis* var. *tritici*	Wheat
	Phytophthora megasperma	*Asparagus*
Pseudomonas (Burkholderia) cepacia	*Fusarium* spp.	Tomato
	F. graminearum	Wheat
	F. moniliforme	Maize
	Rhizoctonia solani	Cotton
	Botrytis cinerea	Apple
	Penicillium expansum	Apple
	Sclerotinia sclerotiorum	Sunflower
	Heterodera glycines	Soybean
	Meloidogyne incognita	Soybean
Pseudomonas spp.	*Fusarium oxysporum*	Carnation
	F. moniliforme	Maize
	Pythium ultimum	Sugar beet
	Rhizoctonia solani	Cowpea
	Agrobacterium tumefaciens	Grapevine
Bacillus subtilis	*Fusarium roseum*	Corn
Bacillus spp.	*G. graminis* var. *tritici*	Wheat
	Pythium spp.	Wheat
	Rhizoctonia spp.	Wheat
Rhizobium	*Macrophomina phaseolina*	Soybean
Bradyrhizobium spp.	*Rhizoctonia solani*	Mung bean
	Fusarium solani	Sunflower

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Microbial Inoculants as Biofertilizer

18

Deep Chandra Suyal, Ravindra Soni, Santosh Sai, and Reeta Goel

Abstract

Increasing application of chemical fertilizers in agriculture make country self dependent in food production but it depreciate environment and cause harmful impacts on living beings. The excess uses of these fertilizers in agriculture are costly and have various adverse effects on soil fertility. Further, soil microorganisms play an important role in the plant growth and development by various means *viz*. nitrogen fixation, phosphate solubilisation, phytohormone production etc. Therefore, bio-inoculants for agriculture purpose i.e. bio-fertilizers could be a better alternative to chemical fertilizers for agricultural as well as environmental sustainability.

Keywords

Microbial inoculants • Biofertilizers • PGPRs • Phytohormones • Nitrogen fixation

18.1 Introduction

Soil microorganisms are important component of integrated nutrient management and soil biodiversity system. They play a crucial role in the plant growth and development. In recent years, it is being noticed that excessive exposure to chemical fertilizers and pesticides which not only deteriorate soil health but also create several environmental impacts as global threat. Beneficial microorganisms offer the potential to meet our agricultural needs and thus, are better alternatives for sustainable agriculture practices. As compared to the chemical fertilizer, biofertilizers are

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safer with reduced environmental damage, has more targeted activity and effective in smaller quantities. Furthermore, they are able to multiply but simultaneously controlled by the plant and indigenous microbes. Moreover, microbial inoculants have quicker decomposition procedures and are less likely to induce resistance by the pathogens and pests.

Bio-inoculants for agriculture purpose are also known as bio-fertilizers. They can broadly defined as formulations of active or latent strains of microorganisms mainly bacteria either alone or in combination with algae or fungi components which, directly or indirectly, stimulate microbial activity and thereby increase mobilization of nutrients from soil. They are customized formulations employing functional attributes of the microorganisms to a range of soil systems and cropping patterns for attaining agricultural sustainability. PGPR includes many well known genera *Rhizobia*, *Azospirillum, Klebsiella*, *Bacillus*, *Burkholderia, Azotobacter, Enterobacter,* and *Pseudomonas* etc, but some of these genera include endophytic species as well. The best-characterized endophytic bacteria include *Azoarcus* spp, *Gluconacetobacter diazotrophicus*, and *Herbaspirillum seropedicae* etc. The practical use of biological fertilizers is well below its full potential, mainly due to non-availability of suitable inoculants. Therefore, further studies on bioinoculant formulations and their exploration will definitely help to understand the complexity and dynamism of microbial functioning and interactions in soils.

18.2 Plant Growth Promoting Rhizobacteria

The rhizosphere, the zone surrounding and influenced by plant roots, is a hot spot for several organisms and one of the most composite ecosystems on Earth (Mendes et al. 2013). The rhizosphere is the habitat for several bacteria, archaea, fungi, algae, viruses, oomycetes, nematodes, arthropods and protozoa. Mendes et al. (2013) described the rhizosphere microbiome in terms of "the good" (beneficial microorganisms), "the bad" (plant pathogens) and "the ugly" (human pathogens). Plant beneficial microorganisms not only promote their growth but also protect them from pathogen attack by a range of mechanisms.

PGPRs can induce plant's growth either directly or indirectly. Direct mechanisms comprise the production of substances like phytohormones, liberation of nutrients and stimulation of induced systemic resistance. For example, diazotrophs, Phosphate (P) solubilizing bacteria (PSB) *viz*. Rhizobia group, *Azospirillum*, *Agrobacterium*, *Pseodomonas & Dyadobacter*, etc (Singh et al. 2012; Rani et al. 2013; Kumar et al. 2014; Suyal et al. 2014). Furthermore, indirect mechanisms include stimulation of symbiotic relationships, stimulation for root growth and biocontrol ability. For example, bacterial genera like Azospirillum, Bacillus, and Pseudomonas can enhance plant growth by legume symbioses (Podile and Kishore 2006). Moreover, it is also important to know that in some cases, numerous mechanisms are involved when it comes to beneficial plant microbial interactions (Nihorimbere et al. 2011). Thus, the identification of the mechanisms accountable of plant growth represents a big challenge in present scenario.

18.2.1 Diazotrophs

Diazotrophs are able to reduce N_2 to NH_3, whereas others, including plants and animals must rely on a fixed form of nitrogen for survival *viz*. rhizobia, *Frankia*, *Azospirillum Pseudomonas, Dyadobacter* (Kumar et al. 2014; Suyal et al. 2014) etc. Though biologically fixed nitrogen has been found in a small number of non-legumes, this activity could have a great impact on the ecology of wild and cultivated ecosystems. Some of the well known diazotrophic genera are described below.

18.2.1.1 Rhizobia

Soil rhizobia are bacteria best known for their symbiosis with leguminous plants. Rhizobia include a range of genera, including *Rhizobium,*

Bradyrhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium, and *Azorhizobium*. Symbiotic nitrogen fixation is a major source of nitrogen, and the various legumes crops and pasture species have ability to fix as much as 200–300 kg nitrogen per hectare (Peoples et al. 1995). Inoculation of these rhizobial strains selected for high N_2-fixing capacity with legumes can improve N fixation in agriculture, mainly when local rhizobia are absent from soils or less effective.

18.2.1.2 Azotobacter

The genus *Azotobacter* belongs to the gama -subclass of the Proteobacteria. These are gram–negative, nitrogen–fixing soil bacteria that have extremely high respiration rates. The first species of the genus *Azotobacter*, named *Azotobacter chroococcum*, was isolated from the soil in Holland in 1901 and thereafter, six other species:, *A. vinelandii, A. beijerinckii, A. paspali, A. armeniacus, A. nigricans* and *A. salinestri* has been reported.

They benefits plants in multiple ways such as by producing ammonia, vitamins, growth substances, indole acetic acid, gibberllins, cytokinins etc. (DeLuca et al. 1996). The genus *Azotobacter* has a high respiratory rate, and its ability to fix atmospheric N_2 in O_2 stress at and above air saturation levels has intrigued researchers for many years (Verma et al. 2001).

18.2.1.3 Azospirillum

Azospirillum belong to the facultative endophytic diazotrophic group and has been reported to colonize the surface and/or the interior of roots of many grasses and cereals. It shows various plant growth promoting activities *viz*. N_2 fixation, production of plant growth-promoting substances etc.

18.2.1.4 Acetobacter

Presently, Acetobacteraceae family includes ten genera: *Acetobacter, Gluconacetobacter, Gluconobacter, Acidomonas, Asaia, Kozakia, Saccharibacter, Swaminathania, Neoasaia, and Granulibacter.* Among them, only three are N_2-fixing genera: *Gluconacetobacter, Swaminathania and Acetobacter. A. diazotrophicus*-sugarcane relationship, first observed in Brazil, was the first report of a beneficial symbiotic relationship between grasses and bacteria through nitrogen fixation (Cavalcante and Döbereiner 1988).

18.2.1.5 Pseudomonas

Several pseudomonas species have been studied for their plant growth promotion activities. Recently, plant growth promoting of Himalayan cold adapted diazotrophs *P. jesenii* MP1 (Kumar et al. 2014) and *P. migulae* S10724 (Suyal et al. 2014) has been revealed. These indigenous diazotrophs are particularly well adapted to the fluctuating temperatures of the hills and could be used effectively as a bioinoculant in high altitude agricultural lands.

18.2.2 Phosphate Solubilising Bacteria

Phosphorus is a plant macronutrient that has a vital role in plant metabolism, ultimately affects on crop yields. It is also important for the functioning of key enzymes that control the metabolic pathways. It is expected that about 98 % of Indian soils contain insufficient amounts of available phosphorus, which is essential to support plant growth (Vassilev and Vassileva 2003). P fertilizers are required for crop production, but only a small part of P is utilized by plants, rest is converted into insoluble fixed forms (Rodriguez and Fraga 1999). Solubilization of insoluble P by microorganisms was firstly reported by Pikovskaya (1948). Now days, many bacterial and fungal species are reported to have the potentials to solubilize inorganic phosphates and commonly known as phosphate solubilizing microorganisms (PSM). Among microbial populations present in soils, phosphate solubilizing bacteria (PSB) constitute P solubilization potential of between 1–50 %, while phosphorus solubilizing fungi (PSF) exhibit only 0.1–0.5 % solubilization (Chen et al. 2006). The commonly known P-solubilizers include *Pseudomonas,*

Bacillus, *Arthrobacter, Rhodococcus, Serratia, Gordonia, Phyllobacterium, Delftia* sp. (Wani et al. 2005), *Azotobacter* (Kumar et al. 2001), *Xanthomonas*, *Chryseobacterium* (Singh et al. 2012), *Enterobacter, Pantoea*, Klebsiella (Chung et al. 2005), *Xanthobacter agilis, Vibrio proteolyticus* (Vazquez et al. 2000), *Rhizobium leguminosarum* bv. *Trifolii* (Abril et al. 2007), *Pseodomonas* sp. (Rani et al. 2013).

18.2.3 Mycorrhiza

Arbuscular mycorrhizal fungi (AMF), are the member of phylum Glomeromycota and can establish mutualistic symbiosis with several land plants. AMF are categorised into seven main groups: arbuscular (AM), ecto- (EcM), ectendo-, arbutoid, ericoid, monotropoid, and orchid mycorrhiza. AM and EcM are the most widespread and ecologically important mycorrhiza and the only ones commercially exploited in agriculture/forestry. The main benefit to use mycorrhiza is its greater soil exploration and increasing uptake and supply of N, P, K, Zn, Cu, S, Fe, Ca, Mg and Mn to the host roots (Mallik 2000).

18.3 PGPR Supporting Plant Growth under Abiotic Stress

It has been assumed that the rhizosphere microbial communities contributes to the ability of some plant species to survive under extreme environment (Jorquera et al. 2012; Mendes et al. 2013). For example, halotolerant bacteria thrive under salt-stress conditions and in association with the host plant are able to express qualities that promote plant growth (Jorquera et al. 2012). Upadhyay et al. (2009) isolated 24 halotrolerant bacteria from the rhizosphere of wheat plants grown in a saline zone, which showed the capability of producing indole-3-acetic acid, P solubilization, siderophores production and N_2 fixation. Similarly, regardless of the impact of low temperatures on nodule formation and nitrogen fixation, local legumes in the high arctic can nodulate and fix N at rates comparable to those reported for temperate climate legumes. There is great interest in agriculture and horticulture for bacterial and fungal inoculants that enhance growth of plants under low temperature (Mendes et al. 2013). For example, *Burkholderia phytofirmans* PsJN increased grapevine root growth and physiological activity at 4 °C (Barka et al. 2006; Mendes et al. 2013). When co-inoculated with *Bradyrhizobium japonicum*, *Serratia proteamaculans* stimulated soybean growth at 15 °C, the temperature at which soybean nodule infection and nitrogen fixation are normally repressed (Zhang et al. 1995, 1996). To identify mechanisms involved in plant growth promotion in cold environment, Katiyar and Goel (2003) selected cold-tolerant mutants of different *P. fluorescens* strains to solubilize phosphorus and to promote plant growth. They also identified two cold-tolerant mutants that were more efficient in P solubilization at 10 °C than their respective wild types (Katiyar and Goel 2003). Trivedi and Sa (2008) reported two phosphorus solubilizing mutants (of 115) that were more efficient than their wild-type strain within a temperature range from 4 to 28 °C (Mendes et al. 2013).

Other abiotic factors that may badly affect plant growth are pH and high concentrations of toxic compounds. Low pH soils or contaminated soils are main challenges in many production systems worldwide. Kawasaki et al. (2012), used a split-root model and a combination of T-RFLP, DGGE, and 16SrRNA gene pyrosequencing and showed that *Trifolium* and other legumes respond to polycyclic aromatic hydrocarbons contamination in a systemic manner. Similarly, Rani et al (2013) explored cadmium (Cd) resistant *P. putida* 710A for *Vigna radiata* (L.) Wilczek plant growth promotion and metal sequestering in Cd polluted soils. Also, fungi play an important role in rhizoremediation, for example, inoculation of the endophytic fungus *Lewia* sp. in the rhizosphere of *Festuca arundinacea* (Cruz-Hernandez et al. 2012).

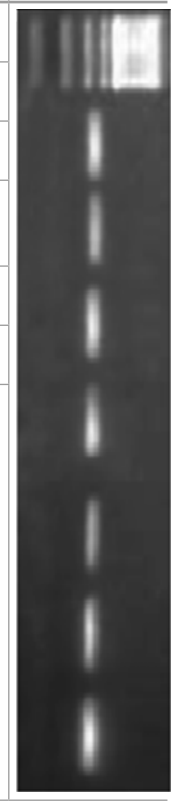
18.4 Himalayan Cold Adapted Diazotrophs for Sustainable Hill Agriculture

Isolation and characterization of the diazotrophs adapted to temperature is central to understanding the ecology of cold adaptive nitrogen fixers and their cold adaptive mechanisms. Previous reports highlighted the prevalence of *nif* and *csp* from the Indian Himalayas (Prema Latha et al. 2009; Singh et al. 2010). Predicted proteins look to be beneficiary in the agronomic practices at ice-cold heights of the Himalayas (Prema Latha et al. 2009). Recently, Suyal et al. (2014) isolated seven cold adapted bacteria from the rhizosphere of Red Kidney bean (*Phaseolus vulgaris* L.) from Western Indian Himalaya (Table 18.1). Furthermore, proteomics of S10724 strain revealed the up-regulation of stress proteins under cold diazotrophy, while most of the down regulated proteins were related to cell division (Suyal et al. 2014). In subsequent studies, net house studies were performed to determine the plant growth promoting ability of strain S10724 on native Green gram (*Vigna radiata* (L.) wilczek) (Suyal et al. 2014). The strain significantly ($p<0.05$) stimulated the growth of roots (45.3 %) and shoots (45.6 %) of Green gram plants (Table 18.2). Furthermore, other growth related parameters *viz*. fresh and dry weight was also found to be increased significantly. The total chlorophyll and nitrate reductase activity was also found to increase in S10724 inoculated plant as compared to their untreated control. Moreover, S10724 treatment increase the germination efficiency of the seeds by 22 % at 25 °C while 25 % at 12 °C unlikely to respective controls (Table 18.2). Similarly, Plant growth promoting properties of Himalayan psychrotroph *Pseudomonas jesenii* MP1 were tested against five native crops *viz. Cicer arietinum* L. (Chickpea), *Vigna mungo* (L.) Hepper. (Black gram), *Vigna radiata* (L.) Wilczek. (Green gram), *Cajanus cajan* (L.) Millsp. (Pigeon pea) and *Eleusine coracana* (L.) Gaertn. (Finger millet) (Kumar et al. 2014). The strain significantly ($p<0.05$) stimulated the growth of shoot length, root length, plant fresh weight and plant dry weight of each crop, over their respective untreated controls. Moreover, MP1 treatment significantly increases chlorophyll content, nitrate reductase activity and P content of the plants. MP1 inoculation showed better effect on Chickpea and Black gram in comparison to other crops. Further, total bacterial and diazotrophic count of MP1 treated soils along with their available Phosphorus (P) and Nitrogen (N) content were found to increase significantly, in comparison to their respective untreated controls (Kumar et al. 2014). These results suggest that *P. migulae* S10724 and *P. migulae* MP1 can be potential plant growth promoting diazotrophs under fluctuating temperature ranges and therefore, could be used effectively as a low cost bioinoculant in high altitudes agro-ecosystems successfully. The exploration of the psychrophilic diazotrophs for the agricultural purpose is in its infancy and therefore, further studies will definitely contribute to the understanding of low temperature diazotrophy mediated agriculture practices.

18.5 Bioinoculants as Biofertilizers

The majority of bio-inoculants used in last few years are mostly *Rhizobia*, constituting ~79 % of the global demand. Phosphate-mobilising bio-inoculants are ~15 %, with other bio-inoculants, such as mycorrhizal products, making up 7 % (Transparency Market Research 2014; Owen et al. 2014). *Azospirillum* species heads a long list of commercial free living PGPR products that are applied to crops in formulations. Some of them are good biocontrol agents and some improve plant growth as well. Additionally, one of the most important species of PGPR used for commercial products is *Bacillus subtilis* under the trade names Serenade, Kodiak, etc. The beneficiary crops are beans, cotton, legumes, pea, rice and soybean. Moreover, well known commercial product is *Agrobacterium radiobacter*, under the trade names Diegall, Nogall, etc. In this case, the beneficiary crops are: fruit, nuts, ornamentals and trees. Finally, *Pseudomonas fluorescens* has also been used to produce commercial inoculants under the trade names Conquer and Victus.

Table 18.1 Characterization of the N_2 fixing psychrophilic bacterial strains isolated from Himalaya (Suyal et al. 2014).

S. No.	Strain ID	Gram reaction and morphology	Accession no.	Nearest phylogenetic neighbour (NCBI-BLAST/EzTaxon) with % similarity	Temperature optima (°C)	*nif*H amplicon (Poly et al. 2001)
1.	S10103	Gram + ve, Rods	JX173281	*Bacillus megaterium* strain SVC4 (100 %)	15	
2.	S10105	Gram + ve, Rod/coccus	JX173282	*Arthrobacter* sp. BA51 (2011) (100 %)	15	
3.	S10107	Gram + ve, Rods	JX173283	*Rhodococcus qingshengii* (100 %)	15	
4.	S10501	Gram + ve, Rod/coccus	JX173284	*Arthrobacter nicotinovorans* strain KNUC2107 (100 %)	15	
5.	S10504	Gram + ve, Rods	JX173285	*Bacillus* sp. IPPBC p001 (100 %)	15	
6.	S10724	Gram −ve, Small rods	JX173286	*Pseudomonas migulae* (100 %)	12	
7.	S10725	Gram + ve, Rod/coccus	JX173287	*Arthrobacter* sp. bB6 (2011) (100 %)	15	

Despite their established economic and ecological benefits the application of such PGPR as biofertilizer must be carefully assessed because of their importance as opportunistic pathogens in nasocomial infections and in patients with diverse diseases (Mendes et al. 2013).

18.6 Conclusion

Besides promoting plant growth, bioinoculants can also alleviate biotic as well as abiotic stresses on crops, thus, providing an environmental friendly sound alternative for sustainable agriculture. However, successful implementation of microbial bioinoculants is dependent on shelf-life, variable efficacy across environments and different plants species other than soil forms. Moreover, the inconsistency of bio-inoculant performance and lack of independent validation does little to build confidence in their efficacy. Therefore, more elementary knowledge is required about microbial behavior and interactions along with dynamics of edaphic and biotic factors for sustainable agriculture. Nevertheless, targeted microbial inoculant for particular soil type is a better approach than uniform formulation.

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Table 18.2 *T*-test analysis depicting the effect of psychrophilic diazotroph *Pseudomonas migulae* strain S10724 on mung bean under net-house conditions after 60 days of germination (Suyal et al. 2014)

Treatment	*in vitro* seed germination assay		Pot trial data					
	% germination of the seeds							
	12 °C[a]	25 °C[a]	Shoot length (cm)[a]	Root length (cm)[a]	Plant fresh weight (g plant^{-1})[a]	Plant dry weight (g plant^{-1})[a]	Chlorophyll content (mg g^{-1} fr. wt)[a]	Nitrate reductase activity (mmol NO_2 g^{-1} fr. wt h^{-1})[a]
Control	30 ± 0.51 (6)[b]	70 ± 0.26 (3) [b]	7.22 ± 0.84	5.67 ± 0.65	0.44 ± 0.13	0.19 ± 0.43	0.40 ± 0.07	0.25 ± 0.16
P. migulae S10724	40 ± 0.32 (6) [b]	90 ± 0.12 (3) [b]	13.26 ± 0.55 (45.6)[c]	10.36 ± 1.02 (45.3)[c]	0.86 ± 0.21 (48.8)[c]	0.59 ± 0.61 (67.79)[c]	0.69 ± 0.16 (42)[c]	1.27 ± 0.16 (80.3)[c]
f-value	3.18	5.35	1.29	7.28	7.97	5.86	5.11	1.18
t-value	5.21	4.01	5.62	3.84	5.61	5.39	3.76	6.71

Note:
[a]Each value is mean of five replicates
[b]Values in parentheses indicate germination time in days
[c]Values in parentheses indicate percent increase over treatment
Data were analyzed statistically at the 5 % (p50.05) level of significance

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Microbial Inoculant: Modern Era of Fertilizers and Pesticides

19

Hemant J. Patil and Manoj K. Solanki

Abstract

In past few decades of agriculture history, chemical fertilizers and pesticides contributed significantly to boost agro-production even in few years of introduction. Their special attributes such as quick and nonspecific action and less expensive, low-cost production and storage make them more acceptable widely. However, their lethal effects on plants, animals, humans, and the environment diverted attention toward eco-friendly alternative. In addition, developing resistance in pests becomes an unresolved puzzle in current time frame and a raising demand for reliable and environment-friendly tool for plant disease management. In view of the growing concern toward safe and nutritious food, biofertilizers and biopesticides seem crucial component of modern agriculture.

Keywords

Biofertilizer • Biopesticide • ISR • Bioinoculants • SAR

19.1 Introduction

In the global scenario, agricultural trade is playing a major role in securing food needs of mankind and concurrently affects the growth and development of a country. In an analysis of the Food and Agriculture Organization (FAO) of the United Nations, 20 % of the population of developing countries was undernourished during 1990–1992, and further it was reduced up to 17 % in 1997–1999 (Bruinsma 2003). It indicates that the role of evolving agricultural practices from conventional to advance will help improve yield and economy as well. In the past, human

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ancestors used minerals and manures like wood ash as nutritional factors in addition to soil toward improving crop yield (Fertilizer 1998). Thereafter, in the eighteenth century, crop rotation has been practiced to see its effect on plant growth and improved yield. In the same time frame, it was recommended by the Humboldt to amend guano, i.e., seabird excreta, in agricultural soil as it contains nitrogen, phosphate, and potassium, three nutrients vital for plant growth. In this way, an era of amending agricultural soil with additives was started.

In growing years, the soil additives, which represent the group of substances that when added to the agricultural land results enhanced plant growth as well as yield, were practiced and known as fertilizer. Generally, it includes nitrogen, phosphorous, and potassium compounds with some secondary nutrients, which significantly improve the quantity and quality of the agricultural food. Most common and widely used fertilizers are categorized as (a) nitrogenous fertilizers like synthetic ammonia, nitric acid, ammonium nitrate, urea, etc. and (b) phosphatic fertilizers like phosphoric acid, ammonium phosphate, normal superphosphate, triple superphosphate, etc. However, the excessive and long-term use of such chemical-based fertilizers is criticized by conservationist for the betterment of the environment and mankind (Parr et al. 1994; Fertilizer 1998), as it causes the air as well as groundwater pollution through eutrophication of water bodies (Youssef and Eissa 2014). In this context, agriculturist have focused on "nutrient-rich high-quality food" using sustainable agricultural practices, channelizing their interests more toward biofertilizers, ensuring biosafety (Selvakumar et al. 2014), etc. Biofertilizers are basically microorganisms, especially nitrogen fixers (N-fixer), phosphorus solubilizer (P-solubilizer), and potassium solubilizer (K-solubilizer). Particularly, these are microbes such as soil bacteria or fungi having potential to solubilize insoluble phosphate in soil through secreting organic acids and make phosphate available for easy uptake by plants (Gupta 2004). Sometimes, these beneficial microorganisms are used in combination with fungi or those bacteria associated with plant roots such as rhizobium, which have symbiotic interaction with legume roots or rhizobacteria, which inhabit on root surface or in rhizosphere soil (Mohammadi and Yousef Sohrabi 2012).

In simultaneous approach, pesticides play a key role in protecting plants from damaging influences by other organisms, insects, and weeds. They are either chemical or biological agents having ability to restrict or kill the unwanted pests, organisms, or insects causing damage to plant or animal. Similar to fertilizers, pesticides are too practiced in agriculture since ancient time by human being. Initially, natural compounds or extracts like salt, sulfurous rock, tobacco extracts, red pepper, etc. have been used as pesticides (Joshi 2006). In following years, petroleum oils, heavy metals, and arsenic were used very commonly to control unwanted pests and weeds until the 1940s. After that it was replaced by organic, synthetic pesticides, of which the most common was chlorinated hydrocarbon, viz., dichlorodiphenyltrichloroethane, i.e., DDT (Hilborne et al. 2005). Considering environmental damage and human health, some classes of the pesticides mainly chemical based (Cropper et al. 1992) were banned to be used on agricultural land by US Environmental Protection Agency (EPA). This concurrently led consumer interest in proficient organic products and sustainable agricultural practices as discussed above, which ultimately results in demand for biological products especially microbial inoculants. The biofertilizers and biopesticides are popular due to its being less toxic, target specific, easily degradable, and subsequent application as compared to conventional counterparts.

19.2 Bioinoculant

In ancient time, it was practiced to transfer productive soil from one field to another considering it positively affects the crop productivity (Bashan 1998). This is how bioinoculation was initiated in farming culture, which in further years evolutionized as mixing of "naturally inoculated" soil and became a recommended activity of legume inoculation in the USA (Smith 1992). The first

use of pure culture as bioinoculant was reported and patented by Nobbe and Hiltner (1896) with *Rhizobium* spp. Bioinoculants represent those living microbes which when amended to the agricultural soil result plant growth promotion through providing plant nutrition and plant protection, stimulating plant hormone production, raising minerals uptake, weathering of soil minerals, etc (Bashan and Holguin 1997; Sullivan 2001). It generally comprises either individual microbial strain or a group of different beneficial microorganisms as consortia having positive impact on plant growth. These formulations are customized according to the requirement and depend on soil type, cropping systems, and microorganism function for better outcome (Roesti et al. 2006; Ahmad et al. 2013). Preliminary these bioinoculants can be categorized as bacteria and fungi followed by subcategories as intracellular and extracellular for bacteria (Gray and Smith 2005) and root-associated fungi (RAF), ectomycorrhizas (EcM), and arbuscular mycorrhizas (AM) for fungi (Owen et al. 2015). On soil amendment, bioinoculant encounters immediate response from established native microflora, especially symbiotic and nonsymbiotic plant growth-promoting bacteria (PGPB) and/or rhizobia. These responses vary depending on the bacteria used in inoculant, its density, plant species, soil type, and also the environmental conditions (Bashan 1998). To overcome such responses and support survival, microbial inoculant needs an empty niche, which is difficult in agriculture land except sterile soil and may result rapid fall down in bacterial population of inoculants.

19.2.1 Biofertilizers

Soil fertility refers to the plant growth, particularly an ability of soil to allow seedling emergence and root penetration by providing nutrients and suitable soil structure to support the plant growth (CMG garden notes 2014). It is the prime quality of soil on which the good farming practices are based. Fertile soil is composed of minerals, organic matter, and beneficial microorganisms, where key components are nitrogen, phosphorous, potassium, and minerals such as boron, chlorine, cobalt, copper, iron, manganese, magnesium, molybdenum, sulfur, and zinc. These components have great impact on physical, chemical, and biological properties as well as processes in the soil. Biofertilizers are the agents which contribute in two important processes, viz., mineralization and immobilization, toward increasing mineral and organic content and eventually fertility of the soil. Mineralization results availability of soluble plant nutrients through decomposition of organic matter in the soil, while immobilization means conversion of atmospheric nitrogen to ammonium for easy uptake by plants (Paul 2014). Biofertilizer is basically the product composed of live or latent cells of beneficial microorganisms as monoculture or mixed culture, which can be introduced to the soil, seed, or plant surface. These microbes have the ability to promote plant growth on colonizing rhizosphere or plant interior and consequently increase supply of primary nutrients in assimilated form to the host plant.

On the other side, there are few pitfalls making biofertilizers less competitive, such as small shelf life, suitable carrier material, sensitive to high temperature, complicated in transportation and storage, etc. In addition, unskilled production, inappropriate technology, and improper use of abundant waste made it more expensive compared to chemical fertilizers. Being biological formulations, its performance depends on the environment surrounding the application area, and hence it exerts slow effect and requires special care for storage and mixing with carrier material in view of mitigating effects for extended use.

The practice started with small-scale compost production and further evidently proved the ability of biofertilizer. It was recognized then that the cultures accelerate the decomposition of organic residues and agricultural waste through various processes and give healthy harvest of crops (Abdul Halim 2009).

As per the records, the industrial-scale microbial inoculants were started in Malaysia in the late 1940s and peaking up in the 1970s taking

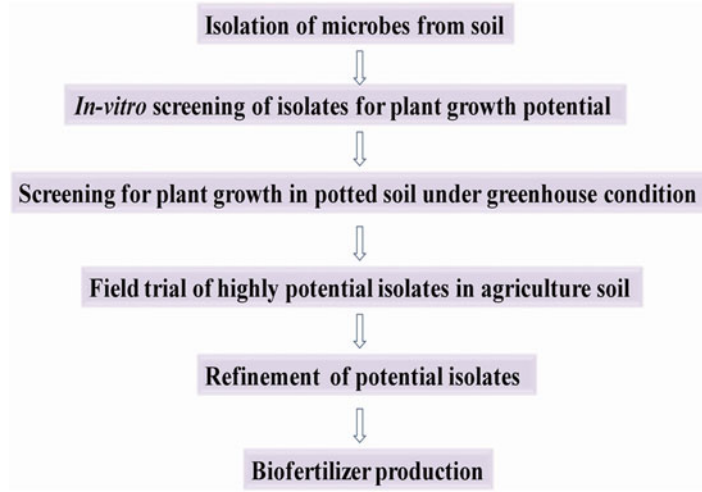

Fig. 19.1 Biofertilizer production

lead by *Bradyrhizobium* inoculation on legumes. However, *Rhizobium*, blue-green algae (BGA), and *Azolla* are being used as crop-specific agents, while members like *Azotobacter*, *Azospirillum*, phosphorus solubilizing bacteria (PSB), and vesicular-arbuscular mycorrhiza (VAM) could be regarded as broad spectrum biofertilizers (Gupta 2004). Biofertilizer production can be achieved through simple steps (Fig. 19.1) for general use, and little modification may help for specific application.

Besides satisfying the nutrient requirement and improved plant growth, biofertilizers help overcome the agricultural problems and poor cropping systems arising due to intensive use of agrochemicals. Biofertilizer is a cost-effective, environment-friendly, as well as renewable source of land nutrient, and also they play a key role in maintaining a long-term soil fertility and sustainability. They can be seen as a significant entity for enhancement and maintenance of soil fertility in the coming future.

19.3 Biopesticides

In the modern era of agriculture, various sophisticated agricultural practices have been implemented considering better results and eco-friendly approaches aiming better future of sustainable agriculture. Organic farming is one of these practices focusing maximum yield of superior quality, and it relies on traditional approaches such as crop rotation, green manure, compost, biological pest control, etc. These eco-friendly production systems help to promote and/or enhance soil biodiversity and soil biological activity. These practices also have positive impact on the balanced biological cycles. The use of biopesticide is also an eco-friendly and the most widely accepted practice in evolving agriculture. Biopesticides are well defined by "the US-EPA" as "certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals" (Raudales and McSpadden Gardener 2008). On the basis of formulation or active ingredients, biopesticides are categorized as microbial pesticides, plant-incorporated protectants, and biochemical pesticides (US-EPA). In the past, plant extracts were likely to be reported as the earliest biopesticides, such as nicotine, which were used to control plum beetles most likely in the seventeenth century. However, the use of chemical-based pesticides was increased dramatically during the twentieth century due to quick results and host-nonspecific effectivity. After few decades, the downside of chemical-based pesticides was initiated as control of pests with synthetic chemicals results in the development of resistance in insects, which eventually

affects human/animal health and environmental concerns (Usta 2013). It addresses the need of an effective alternative with biodegradable and environment-friendly properties and results in the rise of biopesticides. The biopesticides derived from beneficial microorganisms having ability to control the pest are termed as microbial pesticides, which facilitate one of the sophisticated and better alternates for plant disease management. However, the first and most widely used microbial pesticide till date includes the bacteria *Bacillus thuringiensis* (Bt). It was first isolated in 1901 from a diseased silkworm by Japanese biologist Shigetane Ishiwata and further rediscovered 10 years later by Ernst Berliner in Thuringen, Germany, in a diseased caterpillar of flour moth (BPIS-2015). In recent years, few members of the following genera have been reported to be used as biopesticide all over the world: *Bacillus*, *Pseudomonas*, *Streptomyces*, *Agrobacterium*, *Coniothyrium*, *Paecilomyces*, *Beauveria, Trichoderma*, *Cydia pomonella* granulovirus (CpGV), etc.

The ability of biopesticides to kill target pests with high specificity indirectly facilitates the survival of beneficial organisms in treated crops and becomes one of the major reasons for biopesticides to take over its chemical counterparts.

There is a large need to make end user more aware about biopesticides especially its formulation, way of action, and best time to use, which may help them more acceptable in current time frame. The following are few lighter and darker sides of biopesticides (Usta 2013).

Benefits

- Usually nontoxic and nonpathogenic to human, animal, or any other organism except target pest.
- The target specificity is very high and has no any direct effect on predators or parasites of target pest.
- Mostly effective in very small quantities and often decompose quickly, which results significantly less exposure as well as pollution.
- Nonhazardous residue allows application even at prior to harvesting stage.
- In Integrated Pest Management (IPM) programs, biopesticides can greatly decrease the use of conventional pesticides, keeping crop yields high.
- User needs to be aware about managing pest and must carefully follow all label directions to use biopesticides effectively and safely.
- Sometimes beneficial microbes (used in biopesticide) become established in the treated habitat and remain active during subsequent pest generations or seasons.
- Besides pest control may encourage beneficial soil microflora, which leads to increased crop yield.

Drawbacks

- Due to specific target range, unable to control broad range of pests present in the field and cause damage to crop.
- Proper timing and procedures are essential for effective application, as sensitive to heat, desiccation, and UV exposure.
- Special formulation and storage procedures are necessary in some cases and may complicate the production and distribution for certain products.
- Due to target specificity, the potential market for some products may be limited, which results in less availability or higher cost in the different corners of the world.

19.4 Why Microbes in Agriculture?

Agriculture is one of the earliest profitable sectors for the mankind, which depends majorly on fertile soil and stable environmental conditions. It has great impact on the ecological balance, biological diversity, water, soil quality, etc. As discussed earlier, microbes play essential role in agriculture in terms of raising agribusiness significantly. Involvement of microbes in agriculture can be defined as integral part of agriculture (Russo et al. 2012). The microorganisms living in the vicinity of plant roots are generally known as rhizobacteria (in ancient Greek, rhizome means roots). These microorganisms have various potentials such as atmospheric nitrogen fixation,

phosphate solubilization, production of antibiotic, secondary metabolite, plant growth regulators, auxins, siderophores, HCN, ammonia, etc., which are considered to be crucial for plant growth. Microorganisms act as natural scavengers due to their ability to degrade dead plant and animal matter, pollutants like pesticides, hydrocarbons, dyes, paints, etc. These microbes can perform more effectively when added with desired type of microorganism in its active form and appropriate quantity (Higa and Parr 1994). The compatibility of one strain with another also affects performance, when microbes are introduced as consortia of more than one strain. In earlier studies, it has been evidenced that microbes have the ability to trigger plant immune systems, viz., induced systemic resistance (ISR) and systemic acquired resistance (SAR) in plant especially in the presence of plant pathogens (van Loon et al. 1998; Patil et al. 2011). These microbes help plant to suppress the plant pathogens and indirectly promote plant growth.

Soil-root interface provides strata for interactive association of soil microbes and plant roots. This heterotrophic microbial population utilizes root exudates and decaying plant matter as carbon source (Barea et al. 2005; Bisseling et al. 2009). The rhizosphere and rhizoplane are considered to be surrounded with higher microbial population compared to the soil having no vegetation due to elevated levels of tempting substances such as sugars, organic acids, amino acids, vitamins, etc. secreted by plant roots. These substances induce competition and attracts microbes of various species (Okon and Labandera-Gonzales 1994), which leads to diverse microbial population at different rhizospheres (Bisseling et al. 2009).

19.5 Plant Health

Plants represent indispensable environmental asset having significant contribution in the world food supply and economy in terms of cereals, fruits, vegetable, timber, etc. Also plants play unique role in maintaining biodiversity and ecological balance of the planet and share our heritage. Since ancient time, human health has given special attention and can reflect from advancements in medical diagnostics and treatments for human illnesses (Döring et al. 2012). Due to unavailability of defined health parameters, it is difficult to describe plant health; hence, the absence of disease may simply be considered as plant with good health. However, Schlosser (1997) described that a plant can be considered as healthy as long as its physiological performance, which is determined by its genetic potential and environmental conditions, is maintained. The beneficial microbes present in the rhizosphere secrete plant growth-promoting substrates and confer plant protection from plant pathogens, ultimately resulting in good plant growth and vigor.

Plant health management was defined by Cook (2000) as the science and practice of understanding and overcoming the succession of biotic and abiotic factors that limit plants from achieving their full genetic potential as crops. According to Cook (2000), if the term biological control includes the genetic manipulation of plant in a way that the plant itself defends against the threats, then biological control would be the most significant approach in plant health management.

Healthy and vigorous plants are less susceptible to attack by pathogens and insects, which reduces chances of acquiring diseases. There are few recommended practices to be followed toward maintaining good plant health. It involves (a) suitable plantation site, (b) proper mulching, (c) enough watering, (d) fertilizers as per requirement, (e) pruning, etc. Plant health can be compromised by many ways such as arising unusual pests, stresses due to change in climate, land use, etc. There are possibilities of arriving pests with imported goods and travelers, as well as by natural means. This can be best explained with an example of discovery of *Chalara fraxinea* (ash dieback) in England in 2012, which raised the fact about danger to plant health from new pests and diseases and made aware the general public as well as British government (policy paper). Plants may have concern about their health and may behave in a manner to maintain good health.

The same was suggested by Cook et al. (1995) that plants may modulate the rhizosphere microbiome to their benefit by selectively stimulating microorganisms with traits that are beneficial to plant growth and health.

19.6 Soil Health

Soil is a nonrenewable resource of natural composition made up of mixture of weathered rock and organic matter, which form the Earth's surface (Nielsen and Winding 2002). It facilitates the base for all plants as well as habitat for soil microbes and various insects. Soil health is considered as an ability of soil to be productive in terms of plant growth and yield. It is generally based on interaction of physical, chemical, and biological properties of soil, which has direct effect on plant health and maintains environmental quality. The balance and stability of these health parameters keep soil health good. However, it is sometimes considered that the soil health depends on biological activities held in the soil. There are few controversies about the role of soil in plant growth and yield, as few beliefs on its direct effect on plant growth and yield, while the rest consider that it merely provides physical support and the growth and yield are the result of non-soil components, such as fertilizer and pesticide. Soil organisms contribute significantly in soil health through various ways such as humus formation, decomposing dead plant and animal residues, enrichment of soil organic matter, etc. They produce carbon dioxide in soil to be dissolved in water and further converted in carbolic acid, which breakdowns insoluble rock minerals (Edwards et al.). These soil microbes convert organic nutrients to minerals which are easier to uptake by plant roots. They produce extracellular polysaccharides and other cellular debris which have cementing effect to hold soil aggregates together toward improving water holding capacity of soil. Their presence in soil naturally poses competition for soil-borne pathogens and reduces plant disease probability. Therefore, it is important to maintain the soil health in terms of balancing integrity of terrestrial ecosystem and to recover the same after uncertain disturbances such as drought, climate change, pollution, human anthropogenic activities, etc.

19.7 Plant Immunization: ISR and SAR

Plants are often exposed to diverse environmental stresses and have acquired specific mechanisms to combat these stresses. Every year billions of dollars worth of crop yield and quality are vanished by phytopathogens and pests (Adesemoye et al. 2009). Modern agriculture system adopted pesticides against pathogens, but their success rates are often limited, and there are potential risks to environmental health as discussed above (Fravel 2005; Bhattacharyya and Jha 2012; Sahoo et al. 2013). Resistant strains of pathogens rapidly arise to many new systemic pesticides, and the difficulty of developing "environment-friendly" biopesticides has increased their cost and reduced the number that is available. Many recommended pesticides are being removed from the market, and the others can only be used on particular crops for restricted periods of time. Importers of agricultural products are setting strict limits for pesticide residues on food crops, and for some pesticides there is a zero tolerance. Consumers and consumer groups are also becoming increasingly concerned about pesticide use and residues on food products. In recent years, it is a great social and biological interest to better understand the underlying mechanisms by which plants defend themselves in order to better manage both natural and crop plant populations. Due to the constant threat of predation, and the fact that plants cannot physically remove themselves from unfavorable soundings caused by adverse environmental conditions, lack of nutrients, or predation, plants have evolved many physical and structural mechanisms to defend themselves and survive in housie conditions. Advancement in biotechnology, including the introduction and development of transgenic plants, biocontrol, ISR (plant immunization), and increased use of disease-resistant plants utilizing new technologies developed by

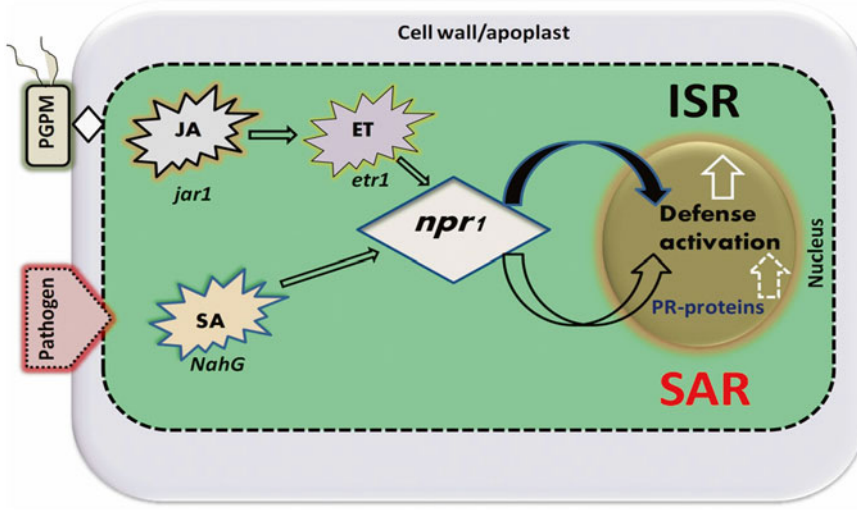

Fig. 19.2 SAR and ISR triggered by beneficial soil-borne microbes in *Arabidopsis*

plant breeders, offers promise of providing alternative means of disease control that are effective and economical and reduce the dependence on pesticides. Since the same mechanism for resistance is activated in immunized and resistant plants, the effect is systemic and often lasts for the life of an annual plant. Induced resistance is defined as an enhancement of the plants' defensive capacity against a broad spectrum of pathogens and pests that is acquired after appropriate stimulation. The resulting elevated resistance due to an inducing agent upon infection by pathogen is called ISR and/or SAR (van Loon et al. 1998; Pieterse et al. 2014) (Fig. 19.2).

The induction of systemic resistance by rhizobacteria is referred to as ISR, whereas that by other agencies is called SAR. It is the generalized (systemic) resistance which is naturally present in plant but induced or enhanced by plant-associated nonpathogenic plant growth-promoting microbes (PGPM). It is independent of salicylic acid (SA), and hence no pathogenesis-related (PR) proteins are synthesized, but it is plant specific and is dependent on plant genotype. Host specificity plays an important role in the activating resistance response, and hence PGPM distinguish their host plant before stimulating them.

Plant hormones, jasmonic acid (JA), and ethylene (ET) are playing obligatory role in ISR (Fig. 19.2). JA regulates plant response to biotic and abiotic stresses including pathogen attack. Sometimes it is formed as volatile compound which can reach to plant parts and nearby plants to warn off pathogen attack and trigger plant defense responses. Wounding or pathogen attack also stimulates the production of ethylene which then onsets defense responses in favor of plant. Typical ethylene-induced visual defense responses include rapid senescence, ripening, and abscission of infected tissue like sudden fall of leaves or fruits. SAR is a direct defense activation process initiated by the necrotizing pathogens and activates the resistance for secondary infection. SAR is triggered up against a broad spectrum of pathogens including viruses, bacteria, fungi, and oomycetes (Pieterse et al. 2014). Necrosis is followed by accumulation of salicylic acid in phloem tissue that triggers first hypersensitive response and induction of SAR. Salicylic acid is plant hormone required for the production of PR proteins that inhibits induction of virulence factors and is also known to control another plant hormone ethylene. These proteins are products of pathogen-activated PR genes. They are necessary to induce plant defense, while some of them function as antimicrobials by degrading cell wall of pathogens. Some of these proteins possess lytic potential such as chitinase, lysozyme, and peroxidase enzyme, which are effective against

various pathogens (Solanki et al. 2011, 2012). They also act as messengers to signal the pathogen attack, which further activates lignin formation and deposition creating efficient barrier to infecting agents. Inhibition of SA accumulation or biosynthesis impairs SAR (van Loon et al. 1998). ISR resembles SAR, but is induced by root colonization of specific strains of nonpathogenic plant growth-promoting rhizobacteria in contrast to SAR that is induced by necrotizing pathogens. Unlike SAR, ISR is dependent on JA and ET, independent of SA, and not associated with PR-gene expression (van Loon et al. 1998). At molecular level, both SAR and ISR in *Arabidopsis* are knotted through NPR1 gene (Fig. 19.2). A crucial step in plant defense is the timely perception of the stress in order to respond in a rapid and efficient manner. Once resistance is induced, it offers nonspecific protection against pathogenic fungi, bacteria, nematodes, and viruses as well as against insect pests. A large number of defense enzymes that have been associated with ISR include phenylalanine ammonia lyase (PAL), chitinase, β-1,3-glucanase, peroxidase (PO), polyphenol oxidase (PPO), superoxide dismutase (SOD), catalase (CAT), lipoxygenase (LOX), ascorbate peroxidase (APX), and protease inhibitors (Kavino et al. 2008; Vanitha and Umesha 2011). These enzymes also bring about liberation of molecules that elicit the initial steps in induction of resistance, phytoalexins, and phenolic compounds (van Loon et al. 1998; Singh et al. 2002; Patil et al. 2011; Solanki et al. 2011). ISR by PGPM has been achieved in large number of crops including apple (Dimkic et al. 2013), *Arabidopsis* (Ryu et al. 2004; Elsharkawya et al. 2012), banana (Kavino et al. 2008; Wang et al. 2013), cotton (Dong et al. 2003), cucumber (Cao et al. 2011), groundnut (Asadhi et al. 2013), rice (Wan et al. 2008; Yoshioka et al. 2012), sorghum (Gopalakrishnan et al. 2011), sugar beet (Bargabus et al. 2002), and tomato (Mizumoto et al. 2007; Park et al. 2011; Solanki et al. 2014b) against the broad spectrum of pathogens including fungi (Fernando et al. 2007; Saravanakumar et al. 2009), bacteria (Fontenelle et al. 2011; Jogaiah et al. 2013), nematodes (Siddiqui and Shaukat 2004a), and viruses (Kavino et al. 2008; Elsharkawya et al. 2012) (Table 19.1).

19.8 Crop Productivity

The disproportionate use of chemical fertilizers in agriculture has caused several environmental problems like global warming, decreased soil quality, imbalanced soil microflora, and polluted water resources. To conquer these problems, application of bioinoculums has been found most effective substitute. Bioinoculums play a very significant role in improving soil fertility by fixing atmospheric nitrogen, both in association with plant roots and without it, solubilizing insoluble soil phosphates, and producing plant growth substances in the soil. They are in fact being promoted to harvest the naturally available biological system of nutrient mobilization (Singh et al. 2011b; Ahemad and Kibret 2014). Bioinoculums are an additional constituent of soil and crop rotation, organic amendments, tillage protection, utilization of crop waste, soil fertility restoration, and management of pathogens; these processes can extensively useful in maintaining the sustainability of crop productions (Sahoo et al. 2013). Nitrogen and phosphorus are key components in the plant growth, and hence symbiotic nitrogen fixer and phosphate solubilizing microorganism-based bioinoculums play vital role for them (Ahemad and Kibret 2014). In one report, Wani and Khan (2010) observed that chickpea (*Cicer arietinum*) inoculated with *Mesorhizobium* sp. RC3 had enhanced dry weight, number of nodules, grain yield, and protein up to 86 % as compared to control plants and fix more nitrogen content into the plants. While, Valverde et al. (2006) also reported that grain yield enhanced with the seed treatment of *Pseudomonas jessenii* PS06 and *Mesorhizobium ciceri* C-2/2, and also there are many reports available on nitrogen-fixing bacteria which enhance productivity and regulate the biotic and abiotic stress (Wani et al. 2008; Ahemad and Khan 2010; Tank and Saraf 2010; Wani and Khan 2010). Nitrogen-fixing bacteria *Azospirillum brasilense* enhanced the growth of maize plant significantly as compared

Table 19.1 Bioinoculums with their bio-protective action under specific crop systems against specific pathogen

Bacterial strain	Plant species	Mechanism involve	Disease/pathogen	Reference
B. amyloliquefaciens MB101	Tomato	Mycolytic enzymes	Root rot/*Rhizoctonia solani*	Solanki et al. (2012)
B. amyloliquefaciens W19	Banana	Iturin A and Bacillomycin D	Fusarium wilt	Wang et al. (2013)
B. cereus	Lilium	Lipoxygenase	Leaf blight/*Botrytis elliptica*	Liu et al. (2010)
B. mycoides strain Bac J	Sugar beet	Peroxidase, chitinase, and β-1,3-glucanase	Leaf spot/*Cercospora beticola* sacc.	Bargabus et al. (2002)
B. subtilis	Tomato	Iturin A	Damping off	Mizumoto et al. (2007)
B. subtilis GB03 and IN937a	Arabidopsis	2,3-butanediol	*Erwinia carotovora* subsp. Carotovora	Ryu et al. (2004)
B. subtilis GB03 and IN937a	Banana	Peroxidase, chitinase, and β-1,3-glucanase	Banana bunchy top virus	Kavino et al. (2008)
B. subtilis SQR9	Cucumber	Iturin A, surfactin, fegycin, bacillomycin	*Fusarium* wilt	Cao et al. (2011)
Bacillus spp. SS 12.6 and SS-13.1	Apple fruits	Iturin A, bacillomycin, and surfactin	Postharvest decay	Dimkic (2013)
P. aeruginosa strain 7NSK2 and *P. fluorescens* strain CHA0	Tomato	Salicylic acid production	Root-knot nematode/*Meloidogyne javanica*	Siddiqui and Shaukat (2004a)
P. chlororaphis strain PA23	Canola	Chitinase and β-1,3,-glucanase	*Sclerotinia sclerotiorum*	Fernando et al. (2007)
P. chlororaphis O6	Tomato	Pyrrolnitrin	Leaf blight/*Phytophthora infestans*	Park et al. (2011)
P. fluorescens	*Arabidopsis thaliana*	2, 4-diacetylphloroglucinol *Pseudomonas fluorescens*	*Pseudomonas syringae* pv. tomato	Weller et al. (2012)
P. fluorescens	Groundnut	2,4-diacetylphloroglucinol	Stem rot/*Sclerotium rolfsii*	Asadhi et al. (2013)
P. fluorescens	Tomato	Phenylalanine ammonia lyase, guaiacol peroxidases, polyphenol oxidase, and lipoxygenase	Bacterial wilt/*Ralstonia solanacearum*	Vanitha and Umesha (2011)
P. fluorescens MPF47	Tomato	Siderophore	Root rot/*Rhizoctonia solani*	Solanki et al. (2014a)
P. fluorescens SS101	Tomato	Massetolide A	*Phytophthora infestans*	Tran et al. (2007)
P. fluorescens strain WCS417r	Carnation	Lipopolysaccharide	*Fusarium* wilt	Van Peer and Schippers (1992)
P. fluorescens strain WCS417r	Radish	Lipopolysaccharide, iron-regulated factor	*Fusarium* wilt	Leeman et al. (1995a)
P. fluorescens strain WCS417r	Tomato	Lipopolysaccharide	*Fusarium* wilt	Duijff et al. (1997)
P. fluorescens strainWCS374	Radish	Lipopolysaccharide	*Fusarium* wilt	Leeman et al. (1995b)
P. plecoglossicida, *B. antiquum*, *E. ludwigii*, *A. tandoii*, *P. monteilii*	Sorghum	Siderophore	Charcoal rot	Gopalakrishnan et al. (2011)

P. putida strains BTP1	Bean	Z,3-hexenal	*Botrytis cinerea*	Ongena et al. (2004)
Penicillium chrysogenum (PEN)	Cotton	Peroxidase	Wilt/*Verticillium dahlia*	Dong et al. (2003)
Penicillium simplicissimum GP17–2	Arabidopsis and tobacco	*JA*, *SA*, and *ET* genes	Cucumber mosaic virus	Elsharkawya et al. (2012)
Pseudomonas spp.	Sugarcane	Siderophores	Red rot	Viswanathan and Samiyappan (2007)
Pseudomonas spp.	Sugarcane	Siderophores	Red rot	Viswanathan and Samiyappan (2007)
Serratia marcescens 90-166	Cucumber	Siderophore	Anthracnose	Press et al. (2001)
Streptomyces platensis F-1	Rice, oilseed rape, strawberry	Volatile metabolites	Leaf blight/seedling blight/fruit rot	Wan et al. (2008)
Streptomyces toxytricini vh6	Tomato	Accumulation of phenylalanine ammonia lyase and total phenol	*Rhizoctonia solani*	Patil et al. (2011)
Streptomyces vinaceusdrappus S5MW2	Tomato	Chitinase	Root rot/*Rhizoctonia solani*	Yandigeri et al. (2015)
Trichoderma harzianum (TriH_JSB27) and *Penicillium chrysogenum* (PenC_JSB41)	Tomato	Peroxidase and β-1,3-glucanase	Bacterial wilt/*R*alstonia *solanacearum*	Jogaiah et al. (2013)
T. asperellum SKT-1	Rice	Increased expression levels of JA/ET	Seed-borne disease	Yoshioka et al. (2012)
T. harzianum	Sunflower	Nutrient absorption and systemic resistance	Downy mildew/*Plasmopara halstedii*	Nagaraju et al. (2012)
T. harzianum + *Pseudomonas fluorescens*	Tomato	Nematicidal activity	*Meloidogyne javanica*	Siddiqui and Shaukat (2004b)
Trichoderma spp.	Tomato	Cell wall-degrading enzymes	Bacterial spot and early blight/*Xanthomonas euvesicatoria* and *Alternaria solani*	Fontenelle et al. (2011)
Trichoderma/Hypocrea species	Tomato	Accumulation of total phenols, peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase	Root rot/*Rhizoctonia solani*	Solanki et al. (2011)

Hayat et al. (2010), Saraf et al. (2014)

to control (Thakuria et al. 2004) and was concurrently supported by Braud et al. (2009) and Gholami et al. (2009). Different nitrogen-fixing bacteria enhanced the plant growth and productivity in common bean (Remans et al. 2008), green gram (Wani et al. 2008), lentil (Ahemad and Khan 2011), *Lupinus luteus* (Dary et al. 2010), pea (Ahemad and Khan 2011), soybean (Gupta et al. 2005), etc. Dryland crops pearl millet and sorghum inoculated with nitrogen-fixing bacteria like *Azotobacter* and *Azospirilla* results 11–12 % increased yields (Wani 1990), and other crops similar to maize, wheat, and rice recorded 15–20 % increased yields due to bioinoculum inoculation. Numerous soil bacteria and fungi, notably species of *Pseudomonas*, *Bacillus*, *Aspergillus*, *Penicillium*, *Streptomyces*, *Trichoderma*, etc., released organic acids and solubilize the "P" from the nearby soil (Patil et al. 2010; Ahemad and Khan 2011; Valverde et al. 2006). In one report, Kathiresan et al. (1995) have observed good cane yield and quality using P-solubilizing bacteria with half of the recommended dose of P_2O_5, indicates efficacy of bioinoculants and appropriate use of resources for better yield. Habibi et al. (2011) strongly suggested that using biofertilizers (mixed strains) with half dose of organic and/or chemical fertilizers has resulted in the greatest grain yield and oil yield in medicinal pumpkin. They revealed that 50 % of required nitrogen and phosphorus fertilizers could be replaced by bio- and organic fertilizers, as it results improved utilization of provided nitrogen and phosphorus fertilizers and ultimately reduced the cost of chemical fertilizers. This also helps in preventing environmental pollution due to extensive application of chemical fertilizers. Canola plant inoculated with (PSB + *Trichoderma* spp.) + application of farm yard manure (FYM) had great influence on growth, height, and grain yield as compared to control (Mohammadi 2010). Also in other report by Mohammadi et al. (2011), it was observed the significant effects on nutrient uptake by chickpea on application of biofertilizers. Moreover, combined application of "P"-solubilizing bacteria and *Trichoderma harzianum* produced the highest leaf "P" content (0.33 %) and grain "P" content (279 mg 100 g^{-1}).

19.9 Eco-friendly Alternative

Microbial bioinoculums contain fungi, bacteria, yeast, viruses, and phages. Farmers often think of microbes as pests that are destructive to their crops or animals (as well as themselves), but many microbes are beneficial (Sahoo et al. 2013). Rather soil microbes are essential for decomposing organic matter and recycling the soil nutrients (Adesemoye et al. 2009). Some soil microbes form relationships with plant roots and provide important nutrients like nitrogen and phosphorus and immobilize the microelements. These microbes can colonize on interior or exterior of plant parts and provide many benefits, including drought tolerance, heat tolerance, and resistance to insects and plant diseases (Pieterse et al. 2014). Chemical fertilizers and pesticides are being used in escalating amounts in order to increase output in high-yielding varieties of crop plants due to the population pressure. However, excessive use of chemicals and pesticides has habitually affected the atmosphere and posed many problems for human and animal directly or indirectly (Fravel 2005; Bhattacharyya and Jha 2012; Sahoo et al. 2013). Consequently, microbial inoculums showed great potential to overcome these problems and environmentalists see it as possible alternatives to chemical-based, conventional agriculture. Modern farming technologists following the ideology of natural ecosystems are now blooming up in all over the world. So many countries have recently been focused on utilization of microbial inoculums and natural farming systems for the food safety issues. Bioinoculum-based farming is the raising of unpolluted crops through the use of bio-manures, biofertilizers, and biopesticides that provide optimum nutrients to crop plants, keeping pests and pathogens under control. Therefore, for the better technological advancement in modern agriculture sector, new concepts such as organic farming, sustainable agriculture, IPM, integrated nutrient management, and bio-fortification are playing important roles (Samoon et al. 2010; Mazid and Khan 2014). Agricultural practices with microbe-dependent techniques have emerged as priority area globally in view of the growing demand for

safe and healthy food and long-term sustainability and overcoming environmental concerns associated with indiscriminate use of agrochemicals (Table 19.2). The bioinoculum-linked methodologies have few limitations, where, one of the major constraint in using microbial based inoculants is the lack of consistent results and slow nutrient immobilization. Sometimes microbial inoculums showed significant growth promotion or disease suppression in control conditions or small-scale field, but when assessed on the pilot scale, they showed unsatisfactory results. Some commercial products have suggested that their particular microbial inoculants are akin to a pesticide that would suppress the general soil microbial population while increasing the population of a specific beneficial microorganism. Bioinoculums lead to beneficial interaction among plant microbes and environment and provide a sustainable agriculture and production inputs for optimum crop and livestock production. As per the environmental safe alternative, farmers would have used bioinoculum- or microbe-based fertilizers; however, they were curious about equal level of improved productivity such as chemical fertilizers and pesticides. This indeed may be true where an abundance of organic materials is readily available for recycling which often occurs in small-scale farming. Nevertheless, it is already proved by the research that a proper way of agriculture system with vital crop rotation, bioinoculums, and no use of pesticides enhanced the beneficial microbial biota and it sustains for a long time in the soil and improved the soil quality and productivity (Adesemoye et al. 2009; Bhattacharyya and Jha 2012). Moreover, different agroclimatic zones require a specific crop, and due to the market pressure/food demand/cost of goods, most of the times farmers need to use farmland to its full productive potential throughout the year. Microbial inoculums, soil preparation, and crop selection play major role in the different ecological zones, and the purpose of crop breeding is to improve production, crop protection, and food quality. Improved crop varieties have contributed significantly to a stable food supply in many countries. Bioinoculums are utilized in agriculture for different purposes: to enhance the organic content, decompositions, and nitrogen fixation and to enhance the disease resistance to improve the productivity and quality with less labor cost. Furthermore, the application of a wide range of different organic amendments to soils can also help to ensure a greater microbial diversity. For example, combinations of various crop residues, animal manures, green manures, and municipal wastes applied periodically to soil will provide a higher level of microbial diversity than when only one of these materials is applied. The reason for this is that each of these organic materials has its own unique indigenous microflora which can greatly affect the resident soil microflora after they are applied, at least for a limited period.

19.10 Rhizoremediation

Environment preservation is one of the aims of the sustainable development of agriculture. Environmental pollution has increased in many regions due to industrialization and overuse of natural resources. These pollutants known as xenobiotic compounds caused the contamination in soil and water system all over the world. Xenobiotics are chemical substances that are alien to the biological system including naturally occurring compounds, drugs, and environmental agents. The classes of xenobiotics include pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated aromatics, solvents, hydrocarbons, and other pollutants like surfactants, silicones, and plastics. Removal of such pollutants requires specific physical, chemical, and biological techniques chosen depending on specific properties of the soil and contaminant (Tripathi et al. 2013). Conventional physicochemical methods to remove pollutants from several environments mainly include chemical reduction, electrochemical treatment, ion exchange, precipitation, and evaporation recovery (Leitão 2009). However, such processes exhibit significant disadvantages, such as the high cost of recovery, incomplete removal, high energy, and chemical consumptions, besides generation of other toxic by-products. Apart from the complete destruction

Table 19.2 Advantages and disadvantages of chemical pesticide and bioinoculums

Chemical pesticides	Bioinoculums
Advantages	*Advantages*
✓ Nutrients are easy to solubilize and direct effects appear, but not in balanced form	✓ Environment friendly
	✓ Improved availability of phosphorous for plant uptake
✓ Lower price and easy to store, and transport and application methods are also easy	✓ Improved biological nitrogen fixation
	✓ Activation of soil biology
✓ They are quite high in nutrient content; only relatively small amounts are essential for plants	✓ Restoration of soil fertility
	✓ Protection against abiotic and biotic stresses
	✓ Stimulation of plant growth
	✓ They provide a balanced nutrient supply to keep plant healthy
	✓ Enhance the soil microbial activity, by nutrient immobilization and nutrient solubilization, and provide better hospitality to decompose the toxic substances
	✓ They support the colonization of mycorrhizae and other plant beneficial biota
	✓ They enhance the root area and uptake the nutrients from the soil structure
	✓ They recycle the organic matters of soil, therefore improving the exchange capacity of nutrients
	✓ Enhance the water holding capacity of soil, improve the soil quality by buffering the soil against acidity, alkalinity, salinity, pesticides, and toxic heavy metals
	✓ They stabilize the soil nutrient cycle and contribute to the residual pool of organic N and P, reducing N leaching loss and P fixation; they can also supply micronutrients to the plant to improve the metabolic activities
	✓ They supply food and promote the growth of beneficial insect, pest, and earthworms
	✓ They enhance the plant defense and vice versa soil immunity to suppress the unwanted plant diseases, soil-borne diseases, and parasites
	✓ They regulate the plant metabolism against the biotic and abiotic stresses
	✓ They play very important role for microbial signal transduction and beneficial plant-microbe interactions

Disadvantages	*Disadvantages*
✓ Excess use of chemicals causes generous effect on the food chain and natural resources, pollutes the ground water resources, unbalances the soil nutrient, creates the stressful environment for the plant, decreases the microbial population, and enhanced the disease-causing substances, acidification, or alkalization of the soil or reduction in soil fertility, thus causing irreversible damage to the global system	✓ They are comparatively low in nutrient content, so larger volume is needed to provide enough nutrients for crop growth
	✓ The nutrient release rate is too slow to meet crop requirements in a short time; hence, some nutrient deficiency may occur
	✓ The major plant nutrients may not exist in bioinoculum in sufficient quantity to sustain maximum crop growth
	✓ Preparation and application are quite different and sensitive to environmental factors
✓ Chemical pesticide enhanced the soil toxicity, and these chemicals are lethal for the birds, insects, household animal, and humans and caused an intense effect on the health of users	✓ Manufacture and transport cost is higher as compared to chemical fertilizers
	✓ Long-term or heavy application to agricultural soils may result in salt, nutrient, or heavy metal accumulation and may adversely affect plant growth, soil organisms, water quality, and animal and human health
✓ They reduce the colonization of plant roots with mycorrhizae and inhibit symbiotic N fixation by rhizobia due to high N fertilization	✓ Success rate is not the same as chemical fertilizer
	✓ Bioinoculums need to be stored in the lower temperature for the long time use
✓ They affect the decomposition of soil organic matters, which leads to degradation of soil structure	✓ Different kind of soil needs different kind of inoculums, and it also changes from crop to crop sometimes
✓ Soil nutrients are easily lost from soils through fixation, leaching, or gas emission and can lead to reduced fertilizer efficiency	✓ Bioinoculum needs more precautions during the application as compared to chemical pesticides
✓ Some chemicals sustain in the soil for a long time and cause the long-time destruction of crop yield	

of the organic compounds, rhizoremediation can provide a low-cost alternative in comparison to other techniques, which only stabilize or dispose off the contaminant (Errasquin and Vazquez 2003). Microorganisms are the most important components of the soil constituting its living part and are responsible for the dynamics of transformation and development of soil structure. To identify the potential of microorganisms in soil remediation is an important step in the recognition of the value of the genetic resources of microbial biodiversity. Rhizoremediation is a process where microorganisms degrade soil contaminants in the rhizosphere. Soil contaminants enhanced the toxicity of soil and cause negative effect on the plants; due to high hydrophobicity, organic compounds cannot enter in the plant cell. Plant themselves are not able to break down these compounds. Rather, the plant creates a niche for rhizosphere microorganisms to do the degradation. Rhizosphere microorganisms are served by the plant acting as a solar-powered pump that draws in water and the pollutant while producing substrates that benefit microbial survival and growth. Root exudates and root turnover can serve as substrates for microorganisms that perform pollutant degradation.

19.11 Role of PGPM in Rhizoremediation

PGPM can degrade the majority of environmental pollutants, and degradation process stops when the microbe is deprived of food. These microbes have access to the best food source available in soil, namely, root exudates. Normally, PGPM are beneficial soil bacteria, which may facilitate plant growth and development both directly and indirectly (Table 19.3). The direct stimulation may include providing plants with fixed nitrogen, phytohormones, iron that has been sequestered by bacterial siderophores, and soluble phosphate, while an indirect stimulation of plant growth includes preventing phytopathogens (through biocontrol) and thus promotes plant growth and development. Plant rhizosphere is a source of nutrient for all kind of microbes, which consits of the narrow zone of soil surrounding plant roots with approximate 1×10^{11} microbial cells per gram of root (Egamberdieva et al. 2008) that too members of various genera, which help improve plant productivity and nutrient cycles (Mendes et al. 2013).

Rhizoremediation by microbial communities, an alternative technique to protect the environment via lethal effect of chemical fertilizers, has become a subject of great interest in sustainable agriculture and biosafety programs. A major focus in the coming decades would be on safe and eco-friendly methods by exploiting the beneficial PGPM in sustainable agriculture (Samoon et al. 2010). PGPM, in general, consist of diverse naturally occurring microbes whose inoculation to the soil ecosystem advances soil physicochemical properties, soil microbe biodiversity, soil health, plant growth, as well as development and productivity. PGPM have the ability of decreasing and/or removing contaminants from soil, water, sediments, and air. In rhizoremediation processes, selected or genetically modified microorganisms have been recently used in order to improve the soil fertility and production (Bhattacharyya and Jha 2012). Numerous studies have demonstrated that PGPM can accelerate these processes efficiently by interacting directly with the host plant (Pieterse et al. 2014). These microorganisms reside sometimes in soil or sometimes inside the specific plant tissues and the root cortex or the xylem (Kavino et al. 2008). The huge variety of the metabolic pathways employed by PGPM makes them valuable tools for rhizoremediation, which can be used for remediation of pollutants and biotransformation of organic substances, for example, propylene to epoxypropane and production of chiral alcohols (Gai et al. 2009; Stępniewska and Kuźniar 2013). On the other hand, PGPM can produce secondary metabolites that may have an influence on antifungal and antibacterial properties, plant hormones, or their precursors such as plant growth factors, vitamins B12 (Ivanova et al. 2006) and B1 (Mercado-Blanco and Bakker 2007), and bioprotectants (Solanki et al. 2011, 2012, 2014b).

Table 19.3 Plant growth-promoting microbes (PGPM) involved in rhizoremediation

Soil microbes	Types of pollutant	Reference
Arthrobacter nicotinovorans HIM	Atrazine	Aislabie et al. (2005)
Aspergillus niger ZHY256	Dimethoate	Liu and Xiong (2001)
B. cereus (DQ002384), *Serratia marcescens* (AY927692), and *Serratia marcescens* (DQ002385)	Pentachlorophenol	Singh et al. (2009)
Bjerkandera adusta	Hexachlorocyclohexane (HCH)	Quintero et al. (2007)
Bjerkandera adusta and *Anthracophyllum discolor*	Pentachlorophenol	Rubilar et al. (2007)
Enterobacter strain B-14	Chlorpyrifos	Singh et al. (2004)
Micrococcus strain CPN1	Cypermethrin	Tallur et al. (2007)
Phanerochaete chrysosporium	Atrazine herbicide	Mougin et al. (1994)
Phanerochaete chrysosporium (BKM-F-1767)	Alkyl halide insecticides	Kennedy et al. (1990)
Pseudomonas aeruginosa	Fenvalerate	Fulekar 2009
Pseudomonas plecoglossicida	Cypermethrin	Boricha and Fulekar (2009)
Pseudomonas sp. strain ADP	Atrazine herbicide	Martínez et al. (2001)
Rhodococcus chlorophenolicus sp. nov.	Chlorophenol	Apajalahti et al. (1986)
Rhodococcus sp.	Triazinone herbicide	Parekh et al. (1994)
Sphingobium chlorophenolicum ATCC 39723	Pentachlorophenol	Dams et al. (2007)
Sphingomonas chlorophenolica RA2 and *Mycobacterium chlorophenolicum* PCP-1	Pentachlorophenol	Wittmann et al. (1998)
Sphingomonas wittichii RW1	Nitrodiphenyl ether herbicides	Keum et al. (2008)
Stenotrophomonas maltophilia M1	Methomyl	Mervat (2009)
Streptomyces sp. M7	Lindane-contaminated soil	Benimeli et al. (2008)
Synechocystis sp. strain PUPCCC	Chlorpyrifos	Singh et al. (2011a)
Trichoderma asperelloides and *T. harzianum*	Fungicides: captan, thiabendazole, and the mixture captan-carboxin	Chaparro et al. (2011)
T. asperellum	Polycyclic aromatic hydrocarbon biodegradation from heavy crude oil-contaminated soils	Zafra et al. (2014)
T. atroviride	Organophosphorus pesticides	Tang et al. (2010)
T. harzianum	Arsenic tolerance in different plants	Arriagada et al. (2009)
T. harzianum, *T. atroviride*, and *T. virens*	Metal (Zn2+, Pb2+, Ni3+, and Cu2+)	Siddiquee et al. (2013)
T. harzianum, *T. hamatum*, and *T. virens*	Metal-containing compounds and fertilizers	Hajieghrari (2010)
T. harzianum, *T. viride*	Pesticide: oxamyl	Afify et al. (2013)
T. koningii	Cyanide and ferrocyanide	Zhou et al. (2007)
T. viride	Metal (chromium)	El-Kassas and El-Taher 2009
T. viride, *T. koningii*, and *T. harzianum*	Soil heavily contaminated with various insecticides from the dieldrin factory yards and apple orchard (dieldrin)	Patil et al. (1970), Bixby et al. (1971) and Katayama and Matsumura (1993)
Trametes versicolor and *Phanerochaete chrysosporium*	Pesticide mixtures	Fragoeiro and Magan (2008)

(continued)

Table 19.3 (continued)

Soil microbes	Types of pollutant	Reference
Trichoderma spp.	Atrazine	Pelcastre et al. (2013)
Trichoderma spp.	Heavy metal- and pesticide-contaminated soil	Kredics et al. (2001)
Trichoderma spp.	Phenanthrene and pyrene	Matsubara et al. (2006)
Trichoderma spp.	Diesel-contaminated soil, cyanide	Ezzi and Lynch (2005)
Trichoderma viride and *Pseudomonas* spp.	Malathion	Matsumura and Boush (1966)
Trichoderma isolate SP2F1	Copper (Cu II) from aqueous solutions	Ting and Choong (2009)

Tripathi et al. (2013)

19.12 Rhizosphere Competence

Competition occurs between microorganisms when space and nutrients are a limiting factor (Howell 2003; Fravel 2005). The rhizosphere is a major concern where competition for space and nutrient occurs (Howell 2003; Viterbo et al. 2007). Competition can be divided into saprobic competition for nutrients in the soil and rhizosphere and competition for infection sites on and in the root (Fravel 2005). Competition between the biocontrol agent and the pathogen can result in displacement of the pathogen. Biological control agents can compete with other fungi for food and essential elements in the soil and around the rhizosphere (Fravel 2005) and can compete for space or modify the rhizosphere by acidifying the soil, so that pathogens cannot grow (Benítez et al. 2004). For example, *Trichoderma harzianum* T-35 controls *Fusarium* species in case of various crops through offering competition for nutrients and rhizosphere colonization (Viterbo et al. 2007). Competition for carbon, nitrogen, and iron has been shown to be a mechanism associated with biocontrol or suppression of *Fusarium* wilt in several systems by nonpathogenic *Fusarium* and *Trichoderma* species (Harman et al. 2004; Wawerua et al. 2014). Shankar et al. (1994) assessed nutrient competition for the thiamine between the *Gaeumannomyces graminis* var. tritici and a sterile red fungus in the rhizosphere of wheat. Many recent studies have shown an association between increased colonization of the nonpathogenic PGPM in rhizosphere, subsequently enhancing disease suppression (Table 19.1).

19.13 Biosafety

Biosafety reflects an idea of taking precaution to avoid huge loss of biological integrity especially for human and ecological well-being. In other words, these are the preventive measures, suppression principals, and practices for appropriate use and to avoid unintended discharge of bioinoculants in the environment (UNEP 2003). In the current time frame, biosafety becomes undetachable part of the society considering betterment of mankind. The prime concern of this perception is risk assessment of genetically modified organisms (GMOs) or living modified organisms (LMOs) obtained through modern biotechnology. The Cartagena Protocol on Biosafety describes that the newly formed biotechnological products should follow biosafety measures in view of further implementation keeping public health and economic benefits on priority, especially in developing nations. This "precautionary standard" utilized only against the harmful organism and different nations has the freedom to legislate on the restrictions that are helpful to secure the population and environment. Individual microbes have been classified by each country on the basis of their pathogenicity, modes of transmission, and host range of the organism, and this classification varied from country to country on

the basis of special factors like existing level of immunity, density/movement of host population, presence of appropriate vectors, and standards of environmental hygiene (Selvakumar et al. 2014).

In the near future, a major focus would be on safe and eco-friendly approaches through involving beneficial microorganisms in sustainable agriculture. Most of these potential bioinoculants are usually isolated from natural treasures such as soil, water, plant, etc. However, in few cases, the beneficial microbes may have harmful effect in the undesired host and/or environment. Few known potential bioinoculants belong to the genera *Acinetobacter*, *Enterobacter*, *Stenotrophomonas*, etc. Although not all members of these genera are pathogenic in nature, some of them are opportunistic pathogen and may cause infection in favorable conditions. Moreover, microbiologists explore the new microbial species from the soil and plant and raise their population on threshold level to achieve optimal plant growth-promoting effect, which may lead to the harmful effect on human and environment health (Selvakumar et al. 2014). In this consent, utilization of novel bioinoculum needs a standard measure to fulfill necessary precautions. The population pressure-driven necessity of higher production and good quality of food tends toward environmental-friendly cropping systems, where regulatory framework should be recommended in view of proper use of novel bioinoculant strains as biofertilizers and biopesticides.

19.14 Conclusion

In the agro-industry, the microbial inoculant as biofertilizer and biopesticide is widely accepted and being used all over the globe. They become prime choice and able to compete their conventional competent, i.e., chemical-based fertilizers and pesticides, due to their environmental-friendly attributes. Although having slow mode of action, bioinoculant holds rank in user choice, may be due to their worth role of natural scavenger, which helps to make environment clean. They also have the potential to help plants to utilize maximum agrochemicals available in the field. In most of the cases, bioinoculants are not harmful to consumers as well as rhizospheric microbiome, which is supposed to be beneficial for plant health and growth. Plants are usually supposed to recruit their specific rhizospheric microbiome and drive them to act in favor of good plant health and against pathogenic microbial population. These bioinoculants also help in utilizing additional organic waste through composting, especially when municipal organic waste is amended with agricultural soil to enrich organic content. While dealing with the bioinoculants, more concern is required for appropriate inoculum density and activity to achieve consistent and estimated level of performance.

In view of sustainable and long-term use of biofertilizers and biopesticides in agro-industry, more concern are required on the following issues:

- Extensive research for screening multifunctional and consistant microbial strains, which can be used in diverse rhizospheres
- Unraveling the advantages of plant-microbe interactions and attempts to make it more beneficial
- Evaluation of beneficial microbial strains for equipotential under biotic and abiotic stresses
- Monitoring of inocula for survival and dispersal in treated soil for assured performance
- Regulated and vigilant use of biofertilizers in terms of production quality and application

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