Soil Biology

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Biofertilizers for Sustainable Agriculture and Environment

Soil Biology

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Biofertilizers for Sustainable Agriculture and Environment

Springer

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Preface

With the introduction of high-yielding varieties and application of chemical fertilizers and pesticides, the agricultural production has increased significantly but gradually becoming dependent on the inputs of cumulative dosages of these menacing chemicals. These chemicals not only are expensive to the farmers but also reduce organic carbon and microbial activities in the agricultural soils and are harmful for human health as they enter the food chain. The increasing dependence upon such chemicals for greater agricultural production compels the scientific community to overcome this problem and find out realistic solutions.

The application of biofertilizers could be a desirable alternative as they make agriculture more sustainable and environmental-friendly; indeed, the growing crops using biofertilizers are worthy for human health. Biofertilizers are consist of plant remains, organic matter, and safe and beneficial microorganisms, which are natural, organic, biodegradable, eco-friendly, and cost-effective. Biofertilizers indeed meet the integrated nutrient demand of the crops, hence ascribed as indispensable for obtaining greater crop yield, and attribute to increased fertility and health of the soil by providing nutrients and natural environment in the rhizosphere. Microbes present in the biofertilizers are important because they produce nitrogen, phosphorus, potassium, zinc, iron, and other nutrients required for the growth of plants. In fact, several microbes produce plant growth-promoting substances like auxins, cytokinins, and gibberellins, which are essential for the growth and development under vital soil conditions. Microorganisms like *Rhizobium*, *Azospirillum*, *Azotobacter*, *Azolla, Piriformospora indica* (*Serendipita indica*), and *Cyanobacteria*/blue green algae have been found to add a significant amount of nitrogen under optimum soil conditions, thereby largely reducing the use of chemical fertilizers. The application of such microbial inoculants showed a robust impact on the crop yield. Furthermore, several microbes exhibit the ability to recover heavy metals from soil, thereby making the soil environment suitable for growing crop plants.

Phosphate-mobilizing or phosphorus-solubilizing microorganisms convert insoluble soil phosphate into soluble forms by secreting several organic acids. Symbiotic fungi enhance the uptake of water and macro- and micronutrients by extending

extra-radical hyphae several meters beyond the depletion zone, thus increasing the nutrient uptake ability of the host plant. Moreover, they protect plant from environmental stresses like salinity and drought and also strengthen the defense system of plant, thereby suppressing the incidence of plant diseases, and thus help in the biocontrol of plant diseases. In general, biofertilizers improve physicochemical properties of the soil. Hence, it is pertinent to state that biofertilizers are a vital and powerful tool for sustainable agriculture and environment.

The book *Biofertilizers for Sustainable Agriculture and Environment* comprises 24 provocative chapters written by the experts of this field, highlighting the latest research on the beneficial microbial inoculants such as phosphate-solubilizing and phosphate-mobilizing fungi; N_2-fixing bacterial inoculants (free living and symbiotic); phosphorus-, potassium-, and zinc-solubilizing bacteria; algal inoculants; microbes for the removal of heavy metals from agricultural fields for sustainable agriculture; microbes for recycling of biodegradable municipal, agricultural, and industrial waste; and biocontrol agents and biopesticides. Though, under current circumstances, the application of microbial inoculants cannot be treated as an alternative for chemical fertilizers and pesticides, indeed, these natural inoculants can largely be utilized to reduce the use of these chemicals. With a fortune of information on the different aspects of biofertilizers, this intensive volume indeed provides useful information, dealing with different groups of microorganisms and their beneficial effects, and is a valuable resource for researchers, academician, environmentalists, and students in the broad field of microbiology, biotechnology, and agriculture and for the industrialists involved in the production of biofertilizers.

We are highly delighted and thankful to all our contributing authors for their endless support and outstanding cooperation to write selflessly these authoritative and valuable chapters. We extend our sincere thanks to all our colleagues who helped us in the preparation and compilation of this generous volume. We thank the Springer officials, specially William F. Curtis, Eric Schmitt, Sabine Schwarz, Isabel Ullmann, Beate Siek, and Anand Venkatachalam, for their generous support and efforts in accomplishing this volume. We specially thank our families for their consistent support and encouragement.

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Chapter 1
Microbial Biofertilizers: Types and Applications

Lebin Thomas and Ishwar Singh

Abstract The increased dependency of modern agriculture on excessive synthetic input of chemical fertilizers has caused several environmental problems related to greenhouse effect, soil deterioration, and air and water pollution. Furthermore, there is an imperative need for viable agricultural practices on a global level with reduced energy and environmental problems, for adequate cost-efficient production of food for the increasing human population. Consequently, biofertilizers containing microorganisms like bacteria, fungi, and algae have been suggested as viable solutions for large-scale agricultural practices which not only are natural, ecofriendly, and economical but also maintain soil structure as well as biodiversity of agricultural land. Besides providing nutrient enrichment to the soil, microbial biofertilizers promote plant growth by increasing efficient uptake or availability of nutrients for the plants and by suppressing soilborne diseases. Biofertilizers supplement nutrients mainly by fixation of atmospheric nitrogen, by phosphorus solubilization, and by synthesizing plant growth-promoting substances. The nitrogen-fixing bacteria of the rhizobia and other groups are used for growth promotion of legumes and additional crops. In addition, blue-green algae (BGA) as well as *Azolla* subsidize in the nitrogen budget of practicable agriculture. Arbuscular mycorrhizal fungi are important for the uptake of phosphorus and several other minerals in many plants. Phosphorus-solubilizing bacteria like *Azotobacter* and *Azospirillum* that fix atmospheric nitrogen can increase the solubility and availability of phosphorus to plants and, thus, crop yield. Further, *Azospirillum* provides additional benefits such as the production of growth-promoting substances, disease resistance, and drought tolerance. Thus, application of microbial biofertilizers is an effective approach in increasing and maintaining the nutrient economy of soil, thereby reducing the use of chemical fertilizers, for a proficient and sustainable agriculture.

Keywords Biofertilizer types · Agrochemicals · Beneficial microbes · Application of biofertilizers

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1.1 Introduction

Fertilizers are natural or man-made chemicals that, when applied on the plant or to soil or by fertigation (applying by irrigation water), can supplement natural soil nutrients and augment crop growth and soil fertility (Edgerton 2009). These make available important macronutrients (nitrogen, phosphorus, potassium, calcium, sulfur, and magnesium) along with numerous micronutrients (zinc, copper, iron, boron, and molybdenum) to plants (Alley and Vanlauwe 2009). A high production demand of standard fertilizers is observed for those that are commonly known as NPK fertilizers and provide nitrogen (ammonia, urea, ammonium sulfate, ammonium nitrate, calcium ammonium nitrate), phosphorus (di-ammonium phosphate, superphosphates, ground rock phosphates), and potassium (potash or potassium chloride, sulfate of potash or potassium sulfate, sulfate of potash magnesia, potassium nitrate, kieserite, Epsom salt). Micro-enriched fertilization, involving the addition of micronutrients to these standard fertilizers, has encouraged agronomic bio-fortification to alleviate malnutrition and micronutrient deficiencies of copper, iron, zinc, iodine, selenium, and fluorine in crop plants (Arnon and Stout 1939). For example, fertilizers with added zinc have been found to increase cereal grain yield by higher seedling establishment and tolerance to environmental stresses (Cakmak 2008). However, one constraint to plant growth is non-availability of nutrients especially nitrogen and phosphorus to plants despite their ample occurrence in soil, as most nitrogen is present in soil organic matter and plants have to compete with soil microbes to obtain it, while phosphorus forms precipitates with iron and aluminum (in acidic soils) or with calcium (in alkaline soils) (Schachtman et al. 1998; Hinsinger 2001).

The exponential growth in human population has demanded a concurrent production and supply of food, particularly from plants. Consequently, a highly productive and intensive agricultural system has been mostly accomplished by the use of synthetic chemical fertilizers of nitrogen and phosphorus (Schultz et al. 1995). However, increased dependence of modern agriculture on an excessive, imbalanced, and steady synthetic input of chemical fertilizers has caused deterioration of soil quality (by making them biologically inert and highly saline) and surface and ground water, and it has further reduced biodiversity and stifled ecosystem functioning (Socolow 1999). The production and transport of chemical fertilizers, which require the use and combustion of fossil fuels, result in airborne carbon dioxide and nitrogen pollution that get deposited into terrestrial ecosystems. Furthermore, excessive supply of chemical fertilizers to soil than used by the crops gets stored in plants and often causes potential losses (by leaching, volatilization, acidification, and denitrification) due to elevated nitrate and phosphorus concentrations in water bodies instigating eutrophication and hypoxia in lakes and estuaries (Vance 2001) and environmental pollution problems by emissions of greenhouse gases like nitrous oxide (N_2O) from fertilizer production and application (Mosier et al. 2004; Nash et al. 2012).

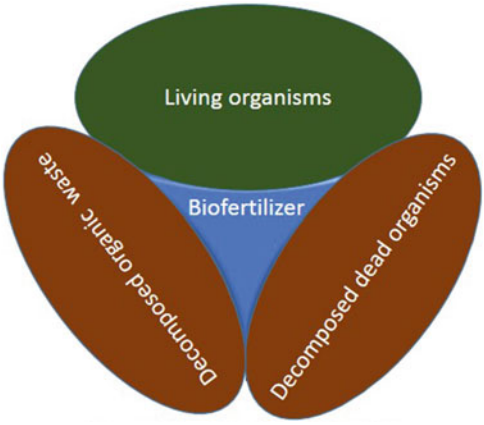

Fig. 1.1 Sources of biofertilizers

Because of the mentioned drawbacks of chemical fertilizers, it is essential to reduce the consumption of chemical fertilizers and pesticides in agriculture without having any adverse effect on crop production by the incorporation and usage of harmless, renewable inputs of fertilizers. The most suitable alternatives for chemical fertilizers are biofertilizers that include organic waste, dead organisms, as well as living organisms (Fig. 1.1). For example, manure and compost are suitable for almost every variety of plants, eggshells have high calcium, and *Stellaria media* (chickweed), *Equisetum* sp. (horsetail), *Azolla pinnata*, *Arctium* sp. (burdock), *Rumex crispus* (yellow dock), *Symphytum officinale* (comfrey), and *Urtica dioica* (nettles) have high nitrogen content. Community waste and sewage sludge provide an inexpensive source of plant nutrition, though these may contain heavy metals and may have adverse effects on crops, consumers, and soil microorganisms (Giller et al. 1998; Graham and Vance 2000). More importantly, biofertilizers can be composed of efficient microbial strains that, by their interactions in rhizosphere, benefit crop plants by the uptake of nutrients. Many bacteria identified as plant growth-promoting rhizobacteria (PGPR), by certain known and unknown mechanisms, can stimulate plant growth. The important known mechanisms exhibited by PGPR that promote plant growth are atmospheric nitrogen fixation, phosphorus solubilization, enhancement of nutrient uptake, or production of plant growth hormones (Bashan et al. 1990; Okon and Labandera-Gonzalez 1994; De Freitas et al. 1997; Bashan 1998; Goldstein et al. 1999). *Achromobacter*, a PGPR, was found to enhance the length as well as number of root hairs and increased nitrate and potassium uptake in *Brassica napus* (oilseed rape), which was evident through the increased dry weights of shoot (from 22% to 33%) and root (from 6% to 21%) (Bertrand et al. 2000). Thus, various types of biofertilizers provide optimum nutrients to crop plant, cause nominal damage to environment, and enhance biodiversity of soil. Their consumption in the future is expected to increase due to overall increase in the demand of fertilizers in order to produce more food on limited arable land and further due to exhausting feedstock/fossil fuels (energy crisis), increasing chemical-fertilizer cost, depleting soil fertility, concerns about environmental hazards, and an increasing threat to sustainable

agriculture. It is predicted that market share of biofertilizers will reach US$1.66 billion by 2022 and will be compounding the annual growth rate of 13.2% during the years of 2015–2022 (Timmusk et al. 2017).

1.2 Microbial Biofertilizers

A biofertilizer of selected efficient living microbial cultures, when applied to plant surfaces, seed or soil, can colonize the rhizosphere or the interior of the host plant and then promote plant growth by increasing the availability, supply, or uptake of primary nutrients to the host. Moreover, in contrast to chemical fertilizers, biofertilizers are more accessible to marginal and small farmers. The most important groups of microbes used in the preparation of microbial biofertilizer are bacteria, fungi, and cyanobacteria, majority of which have symbiotic relationship with plants. The important types of microbial fertilizers, based on their nature and function, are those which supply nitrogen and phosphorus (Table 1.1).

1.2.1 Nitrogen-Fixing Microbes

Nitrogen is most abundant and ubiquitous in the air, yet becomes a limiting nutrient due to difficulty of its fixation and uptake by the plants. However, certain microorganisms, some of which can form various associations with plants as well, are capable of considerable nitrogen fixation. This property allows for the efficient plant uptake of the fixed nitrogen and reduces loses by denitrification, leaching, and volatilization. These microbes can be:

(a) Free-living in the soil (Table 1.1). The assessment of nitrogen fixation by free-living bacteria is difficult, but in some plants like *Medicago sativa*, it has been estimated to range from 3 kg N ha^{-1} to 10 kg N ha^{-1} (Roper et al. 1995). *Azotobacter chroococcum* in arable soils can fix 2–15 mg N g^{-1} of carbon source in culture media, and it further produces abundant slime which aggregates soil. However, free-living cultures of nodulating bacterial symbionts (e.g., *Frankia*) have been found to fix atmospheric nitrogen in the rhizosphere of their host and even non-host plants (Smolander and Sarsa 1990). For *Beijerinckia mobilis* and *Clostridium* spp., inoculation methods of leaf spray and seed soaking stimulated growth in cucumber and barley plants by significant nitrogen fixation and other mechanisms of bacterial plant growth hormone synthesis (Polyanskaya et al. 2002). Free-living cyanobacteria (blue green algae) have been harnessed in rice cultivation in India which can provide up to 20–30 kg N ha^{-1} under ideal conditions (Kannaiyan 2002).
(b) Having symbiotic and other endophytic associations (of rhizobia, *Frankia,* and cyanobacteria) with plants. The nitrogen-fixing efficiency of rhizobia bacteria,

Table 1.1 The important groups of microbial fertilizers

Group of biofertilizers	Sub-group	Examples
Nitrogen-fixing	Free-living	*Anabaena*, *Azotobacter*, *Beijerinkia*, *Derxia*, *Aulosira*, *Tolypothrix*, *Cylindrospermum*, *Stigonema*, *Clostridium*, *Klebsiella*, *Nostoc*, *Rhodopseudomonas*, *Rhodospirillum*, *Desulfovibrio*, *Chromatium*, and *Bacillus polymyxa*
	Symbiotic	Rhizobia (*Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium Mesorhizobium Allorhizobium*), *Frankia*, *Anabaena azollae*, and *Trichodesmium*
	Associative	*Azospirillum* spp. (*A. brasilense*, *A. lipoferum*, *A. amazonense*, *A. halopraeferens*, and *A. irakense*), *Acetobacter diazotrophicus*, *Herbaspirillum* spp., *Azoarcus* spp., *Alcaligenes*, *Bacillus*, *Enterobacter*, *Klebsiella*, and *Pseudomonas*
Phosphorus (microphos)	Phosphate-solubilizing	*Bacillus megaterium* var. *phosphaticum*, *B. subtilis*, *B. circulans*, *B. polymyxa*, *Pseudomonas striata*, *Penicillium* spp., *Aspergillus awamori*, *Trichoderma*, *Rhizoctonia solani*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aereobacter*, *Flavobacterium*, and *Erwinia*
	Phosphate-mobilizing	Arbuscular mycorrhiza (*Glomus* sp., *Gigaspora* sp., *Acaulospora* sp., *Scutellospora* sp., and *Sclerocystis* sp.), ectomycorrhiza (*Laccaria* spp., *Pisolithus* spp., *Boletus* spp., *Amanita* spp.), ericoid mycorrhiza (*Pezizella ericae*), and orchid mycorrhiza (*Rhizoctonia solani*)
Micronutrients	Potassium solubilizing	*Bacillus edaphicus*, *B. mucilaginosus*, and *Paenibacillus glucanolyticus*
	Silicate and zinc solubilizing	*Bacillus subtilis*, *Thiobacillus thioxidans*, and *Saccharomyces* sp.
Growth promoting	Plant growth-promoting rhizobacteria	*Agrobacterium*, *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Actinoplanes*, *Azotobacter*, *Bacillus*, *Pseudomonas fluorescens*, *Rhizobium*, *Bradyrhizobium*, *Erwinia*, *Enterobacter*, *Amorphosporangium*, *Cellulomonas*, *Flavobacterium*, *Streptomyces*, and *Xanthomonas*

Modified from Singh et al. (2014)

an important group of biofertilizers that contains organisms like *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium*, and *Allorhizobium*, can vary till 450 kg N ha^{-1} among different strains and host legume species, in which root nodules are formed (Stamford et al. 1997; Unkovich et al. 1997; Spaink et al. 1998; Vance 1998; Graham and Vance 2000; Unkovich and Pate 2000). The rhizobial biofertilizers can be in powder, liquid, and granular formulations, with different sterilized carriers like peat, perlite, mineral soil, and charcoal (Stephens and Rask 2000). Like rhizobia, *Frankia*, a nitrogen-fixing actinomycete, can also form root nodules in several woody plants (Torrey 1978; Dawson 1986; Benson and Silvester 1993;

Dommergues 1995; Huss-Danell 1997; Wall 2000). This mycelial bacterium forms symbioses with the roots of several non-legume plants like *Casuarina*, *Alnus* (*Alder*) *Myrica*, *Rubus*, etc. These actinorhizal plants are used for timber and fuelwood production, windbreaks, and shelterbelts and in advancing early successional plant community development, mixed plantations, revegetation, and land reclamation (Diagne et al. 2013; Schwencke and Carù 2001). The inoculation of *Frankia* is considered valuable in nurseries and in arid or disturbed environments (Schwintzer and Tjepkema 1990; Sprent and Parsons 2000). Besides, leaves of a few plants (e.g., *Ardisia*) develop special internal cavities harboring symbiotic nitrogen-fixing bacteria like *Xanthomonas* and *Mycobacterium*, and as such, these leaves are source of nitrogen fertilizer to the soil (Miller 1990). Another ecologically important group is that of cyanobacteria—blue green algae (BGA)—some of which like *Trichodesmium*, *Nostoc*, and *Anabaena* contribute to about 36% of the global nitrogen fixation and have been reported to be helpful in enhancing rice-field fertility for the cultivation of rice in many parts of the world (Kundu and Ladha 1995; Gallon 2001; Irisarri et al. 2001). Besides, BGA are also known to be advantageous for possible reclamation of arid environments or ecosystems disposed to flooding (Bashan et al. 1998; Malam Issa et al. 2001). The production and application of BGA is, however, poorly developed, and it should be considered as a biofertilizer for sustainable agricultural practices in various environments (Hashem 2001). Aquatic BGA can further provide natural growth hormones, proteins, vitamins, and minerals to the soil.

(c) Living in rhizosphere (associative/associated) without endophytic symbioses. In comparison to endophytic symbionts, these nitrogen-fixing microbes have less intimate association with roots. These include *Acetobacter diazotrophicus* and *Herbaspirillum* spp. with sugarcane, sorghum, and maize (Triplett 1996; James et al. 1997; Boddey et al. 2000); *Azoarcus* spp. with *Leptochloa fusca* (kallar grass) (Malik et al. 1997); species of *Alcaligenes*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Herbaspirillum*, *Klebsiella*, and *Pseudomonas* with rice and maize (James 2000); and *Azospirillum* with great host specificity comprising a variety of annual and perennial plants (Bashan and Holguin 1997). Several studies have shown that due to nitrogen fixation and production of growth-promoting substances, *Azospirillum* increased the growth and crop yield of wheat, rice, sunflower, carrot, oak, sugar beet, tomato, eggplant, pepper, and cotton (Okon 1985; Bashan et al. 1989; Okon and Labandera-Gonzalez 1994). The inoculum of *Azospirillum* can be inexpensively produced and applied by a simple peat formulation (Vande Broek et al. 2000). The biofertilizer of *Acetobacter diazotrophicus* was found to fix and make available up to 70% of sugarcane crop nitrogen requirement, of about 150 kg N ha^{-1} annually (Boddey et al. 1995).

Thus, the capability of nitrogen fixation in substantial quantity of these microorganisms makes them attractive candidates for their application as biofertilizers.

1.2.2 Phosphorus-Solubilizing Microbes

In soil, the concentration of phosphorus is high, but most of it is present in unavailable forms, which makes it the second most limiting plant nutrient after nitrogen (Schachtman et al. 1998). The phosphorus-solubilizing bacteria (PSB) like *Bacillus* and *Pseudomonas* can increase phosphorus availability to plants by mobilizing it from the unavailable forms in the soil (Richardson 2001). These bacteria and certain soil fungi such as *Penicillium* and *Aspergillus* bring about dissolution of bound phosphates in soil by secreting organic acids characterized by lower pH in their vicinity. The application of the inexpensive rock phosphate with a PSB, *Bacillus megaterium* var. *phosphaticum* to sugarcane, was found to increase sugar yield and juice quality by 12.6%, and it reduced the phosphorus requirement by 25%, thereby further causing a 50% reduction of the costly superphosphate usage (Sundara et al. 2002).

1.2.3 Mycorrhizal Biofertilizers

These are phosphorus-mobilizing biofertilizers or phosphate absorbers. The mycorrhizal fungi form obligate or facultative functional mutualistic symbioses with more than 80% of all land plants, in which the fungus is dependent on host for photosynthates and energy and in return provides a plethora of benefits to its host (Smith and Read 1997; Thakur and Singh 2018). The mycelium of the fungus extends from host plant root surfaces into soil, thereby increasing the surface area for more efficient nutrient access and acquisition for the plant, especially from insoluble phosphorus sources and others like calcium, copper, zinc, etc. (Singh and Giri 2017). Additionally, mycorrhizal fungi are known to enhance soil quality, soil aeration, water dynamics, and heavy metal and drought tolerance of plants and to make plants less susceptible to root pathogens or herbivores (Rillig et al. 2002; Thakur and Singh 2018). This suggests high potential of these fungi for application in agriculture, land reclamation, or vegetation restoration (Menge 1983; Sylvia 1990). Ectomycorrhiza (of Basidiomycetes) forms a mantle on the root surface (of several trees such as *Eucalyptus*, *Quercus*, peach, pine, etc.) and penetrates internally into the intercellular spaces of the cortical region from where it obtains the plant-secreted sugars and other nutrition. The important functions of these fungi are absorption of water and minerals by increasing surface area of roots, solubilizing soil humus organic matter to release and absorb inorganic nutrients, and secreting antimicrobial substances that protect plants from various root pathogens. The importance of ectomycorrhizal symbiosis has been observed for tree plantations in growth and nutrient acquisition, especially for large-scale inoculum practices into nursery or forestry cultivated areas (White 1941; Wilde 1944; Mikola 1970; Smith and Read 1997).

Arbuscular mycorrhizal (AM) fungi like *Glomus* are intercellular, nonspecific obligate endosymbionts (with special structures of vesicles and arbuscules in roots)

that, by functioning as an extended root system, harvest moisture and various micronutrients from deeper and distant niches in the soil, besides increasing the mobility and availability of phosphorus to enhance growth and development in host plants. However, unculturability and the obligate nature of AM fungi have made inoculation incompatible with large-scale industrial-scale agriculture, and thus it might require additional research (Wood and Cummings 1992; Ryan and Graham 2002). Nevertheless, the AM inoculation for production of nursery stocks often results in amended and homogeneous crop growth. For agricultural purpose, the ability of fungi for colonization in specific host plants can vary, which can depend on the inoculum source (Biermann and Linderman 1983; Klironomos and Hart 2002). The production of infective propagules by growing inoculum in symbiosis with living host plants or in root organ cultures is a viable mean, but has limitations of high production cost, slow turnover time, and difficulty in excluding root pathogens. AM inoculum is applied as spores (most reliable), fragments of colonized roots (effective for some taxa), or a combination of these and incorporated soil mycelium mixed with carrier substrate like pumice or clay, sand, perlite, vermiculite, soil rite, and soil or glass pellets (Mallesha et al. 1992; Redecker et al. 1995; Gaur and Adholeya 2000; Klironomos and Hart 2002).

1.2.4 Other Mineral-Solubilizing Biofertilizers

Soil-dwelling microorganisms can further be used as biofertilizers to provide various nutrients other than nitrogen and phosphorus such as potassium, zinc, iron, and copper. Certain rhizobacteria can solubilize insoluble potassium forms, which is another essential nutrient necessary for plant growth (Jakobsen et al. 2005). The higher biomass yields due to increased potassium uptake have been observed with *Bacillus edaphicus* (for wheat), *Paenibacillus glucanolyticus* (for black pepper), and *Bacillus mucilaginosus* in co-inoculation with the phosphate-solubilizing *Bacillus megaterium* (for eggplant, pepper, and cucumber) (Meena et al. 2014; Etesami et al. 2017). Another important mineral is zinc, which is present at a low concentration in the Earth's crust, due to which it is externally applied as the costlier soluble zinc sulfate to overcome its deficiencies in plant. However, some microbes such as *Bacillus subtilis*, *Thiobacillus thiooxidans*, and *Saccharomyces* spp. can solubilize insoluble cheaper zinc compounds like zinc oxide, zinc carbonate, and zinc sulfide in soil (Ansori and Gholami 2015). Similarly, microorganisms can hydrolyze silicates and aluminum silicates by supplying protons (that causes hydrolysis) and organic acids (that form complexes with cations and retain them in a dissolved state) to the medium while metabolizing, which can be beneficial to the plants. For instance, an increase in rice growth and grain yield due to increased dissolution of silica and nutrients from the soil was observed using a silicate-solubilizing *Bacillus* sp. combined with siliceous residues of rice straw, rice husk, and black ash (Cakmakci et al. 2007).

1.2.5 Plant Growth-Promoting Microbes

Besides nitrogen-fixing and phosphorus-solubilizing microbes, there are microbes that are suitable to be used as biofertilizers as these enhance plant growth by synthesizing growth-promoting chemicals (Bashan 1998). For example, rhizospheric *Bacillus pumilus* and *Bacillus licheniformis* were found to produce substantial quantities of physiologically active plant hormone gibberellin (Gutierez-Mañero et al. 2001). However, *Paenibacillus polymyxa* showed a variety of beneficial properties, including nitrogen fixation, phosphorus solubilization, production of antibiotics, cytokinins, chitinase, and other hydrolytic enzymes and enhancement of soil porosity (Timmusk et al. 1999). Further, some species of *Azospirillum* have been reported to produce plant hormones (Bashan et al. 1990; Bashan and Holguin 1997). These indicate the potential of diverse microbes as biofertilizers, which might require additional studies.

The rhizobacterial plant growth-promoting mechanisms of antagonism against phytopathogenic microorganisms include production of antimicrobial metabolites like siderophores and antibiotics, gaseous products like ammonia, and fungal cell wall-degrading enzymes which cause cytolysis, leakage of ions, membrane disruption, and inhibition of mycelial growth and protein biosynthesis (Idris et al. 2007; Lugtenberg and Kamilova 2009). For example, *Pseudomonas* strains can produce antifungal metabolites like phenazines, pyrrolnitrin, pyoluteorin, and cyclic lipopeptides of viscosinamide, which can prevent *Pythium ultimum* infection of sugar beet. *Pseudomonas fluorescens* produces the iron-chelating siderophores like pseudobactin and pyoverdin that bind and take up ferric ions, which makes them better competitors for iron, thus preventing the growth and proliferation of pathogenic microbes like *Pythium ultimum*, *Rhizoctonia batatticola*, and *Fusarium oxysporum* (Cox and Adams 1985; Leeman et al. 1996; Hultberg et al. 2000). *Pseudomonas aeruginosa* produces the siderophores pyoverdine, pyochelin, and salicylic acid and further induces resistance against *Botrytis cinerea* (on bean and tomato) and *Colletotrichum lindemuthianum* (on bean) (De Meyer and Höfte 1997; Audenaert et al. 2002). However, some species of *Pseudomonas* produce extracellular chitinase and laminase that can lyse *Fusarium solani* mycelia. In addition, biofertilizers provide protection against some soilborne diseases, insect pests, and plant diseases; for example, *Azotobacter* pervades the soil with antibiotics which inhibit the spread of soilborne pathogens like *Pythium* and *Phytophthora* (Wani et al. 2013).

1.2.6 Compost Biofertilizers

Compost is a decomposing, brittle, murky material forming a symbiotic food web within the soil, which contains about 2% (*w*/*w*) of nitrogen, phosphorus, and potassium, along with microorganisms, earthworms, and dung beetles. The

microbial organic solid residue oxidation causes the formation of humus-containing material, which can be used as an organic fertilizer that sufficiently aerates, aggregates, buffers, and keeps the soil moist, besides providing beneficial minerals to the crops and increasing soil microbial diversity (Yu et al. 2016). Compost is produced from a wide variety of materials like straw, leaves, cattle-shed bedding, fruit and vegetable wastes, biogas plant slurry, industrial wastes, city garbage, sewage sludge, factory waste, etc. The compost is formed from these materials by different decomposing microorganisms like *Trichoderma viridae*, *Aspergillus niger*, *A. terreus*, *Bacillus* spp., several Gram-negative bacteria (*Pseudomonas*, *Serratia*, *Klebsiella*, and *Enterobacter*), etc. that have plant cell wall-degrading cellulolytic or lignolytic and other activities, besides having proteolytic activity and antibiosis (by production of antibiotics) that suppresses other parasitic or pathogenic microorganisms (Boulter et al. 2002). Another important type (vermicompost) contains earthworm cocoons, excreta, microorganisms (like bacteria, actinomycetes, fungi), and different organic matters, which provide nitrogen, phosphorus, potassium, and several micronutrients, and efficiently recycles animal wastes, agricultural residues, and industrial wastes cost-effectively and uses low energy.

1.3 Application Practices of Microbial Biofertilizers

Biofertilizers are mostly supplied as conventional carrier-based inoculants with the advantage of being cheap and easier to produce. The mass production of biofertilizers involves culturing of microorganisms, processing of carrier material, mixing of carrier material with the broth culture, and packing (Fig. 1.2). The ideal carrier materials used in the preparation of biofertilizers must be cheaper, locally

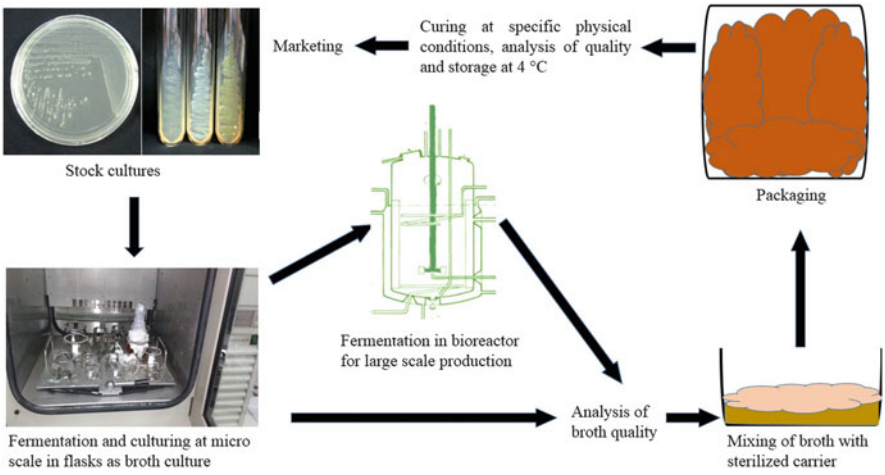

Fig. 1.2 A diagrammatic representation of mass production of bacterial biofertilizers

available, and easier to process; must be non-toxic and organic in structure (so that they remain biodegradable) with high water-holding capacity; and should carry higher bacterial cells and support their survival for longer durations. Some of the commonly used carrier materials in the production of good-quality biofertilizers are neutralized peat soil/lignite, vermiculite, charcoal, press mud, farmyard manure, and soil mixture. However, these can have disadvantages of possessing lower shelf-life, temperature sensitivity, being contamination prone, and becoming less effective by low cell counts. Consequently, liquid formulations have been developed for *Rhizobium*, *Azospirillum*, *Azotobacter*, and *Acetobacter* which although costlier, have the advantages of having easier production, higher cell counts, longer shelf-life, no contamination, storage up to 45 °C, and greater competence in soil (Ngampimol and Kunathigan 2008). Nevertheless, the application practices of microbial biofertilizers include seed treatment, seedling root dipping, and soil application.

1.3.1 Seed Treatment

Seed treatment is a very effective, economic, and most common method implemented for all types of inoculants (Sethi et al. 2014). The seeds are mixed and uniformly coated in a slurry (inoculant mixed with 200 mL of rice kanji) and then shade-dried, before being sown within 24 h. For liquid biofertilizers, depending upon the quantity of seeds, the coating can be done in either plastic bag (if quantity is small) or bucket (if quantity is large). The seed treatment can be done with two or more bacteria (for instance, nitrogen-fixing bacteria such as *Rhizobium*, *Azotobacter*, and *Azospirillum* can be taken along with phosphorus-solubilizing microbes), without any antagonistic effect, and provide maximum quantity of each bacterium on individual seed required for better results (Chen 2006). For example, seed treatment is done for many plants using *Rhizobium* (pulses like chickpea, pea, groundnut, soybean, beans, lentil, lucern, berseem, green gram, black gram, cowpea, and pigeon pea), *Azotobacter* (cereals like wheat, oat, barley; oil seeds like mustard, seasum, linseeds, sunflower, castor; millets like pearl millets, finger millets, kodo millet; forage crops and grasses like bermuda grass, sudan grass, napier grass, para grass, star grass, etc.), and *Azospirillum* or phosphorus-solubilizing bacteria (rice, maize, and sorghum) (Taylor and Harman 1990).

1.3.2 Seedling Root Dipping

This application is common for plantation crops such as cereals, vegetables, fruits, trees, sugarcane, cotton, grapes, banana, and tobacco where seedling roots are dipped in a water suspension of biofertilizer (nitrogen-fixing *Azotobacter* or *Azospirillum* and phosphorus-solubilizing microbial biofertilizer) for sufficient period of time. The treatment time differs for different crops, for instance, vegetable crops are treated for 20–30 min and paddy for 8–12 h before transplantation (Barea and Brown 1974).

1.3.3 Soil Application

In this practice, biofertilizer is applied directly to the soil either alone or in combination. A mixture of phosphate-solubilizing microbial biofertilizer, cow dung, and rock phosphate is kept in shade overnight while maintaining its moisture content at 50% and then applied to the soil (Pindi and Satyanarayana 2012). Some examples of biofertilizers in which soil application is employed are *Rhizobium* (for leguminous plants or trees) and *Azotobacter* (for tea, coffee, rubber, coconuts, all fruit/agroforestry plants for fuelwood, fodder, fruits, gum, spice, leaves, flowers, nuts, and seeds) (Zahran 1999; Hayat et al. 2010).

1.4 Available Microbial Biofertilizers

There are several microbial biofertilizers available as dried or liquid cultures under different trade names in the market, which are used for a variety of purposes including enhancement of plant growth and soil fertility (Table 1.2). For instance, the rhizobia biofertilizers can fix 50–300 kg N ha^{-1} that increases yield by 10–35%, maintains soil fertility, and leaves residual nitrogen for succeeding crops (Davis 1996; Chen 2006). The *Azotobacter* biofertilizer used for almost all crops can fix 20–40 mg N g^{-1} of carbon source that causes up to 15% increase in yield; maintains soil fertility; produces growth-promoting substances such as vitamin B complexes, indole acetic acid, and giberellic acid; and is further helpful in biocontrol of plant diseases by suppressing some of the plant pathogens (Abd El-Lattief 2016; Kurrey et al. 2018). The phosphorus-solubilizing bacterial biofertilizers, which are nonspecific and suitable for all crops, produce enzymes which mineralize the insoluble organic phosphorus into a soluble form, thereby increasing crop yield by 10–30% (Sharma et al. 2013).

1.5 Limitations of Microbial Biofertilizers

Although biofertilizer technology is ecofriendly and possesses a surfeit of advantages, there are some limitations (some of which have been mentioned in Table 1.3) of this technology causing suspicion among stakeholders about its application. The major drawbacks associated with microbial biofertilizers that need immediate attention through further research as well as proper planning include their plant specificity, lower nutrient density (thus, are required in bulk to be made available for most crops), requirement of separate machinery and skill for production and application than that used for chemical fertilizers, difficulty of storage, and more importantly inadequate awareness about their use and benefits among farmers (Malusà et al. 2016). Furthermore, there can be constraints regarding the application or implementation of biofertilizers that affect the technology at stages of production, marketing, or usage (Table 1.3) (Jangid et al. 2012).

Table 1.2 Different microbial biofertilizers available in market and their application

Microbial biofertilizers	Trade names	Application
Azospirillum lipoferum, *Azospirillum brasilense*, and different strains of *Azospirillum*	Biospirillum, Green Plus, Bio-N, Azo-S, ROM, and Spironik	(1) For normal and acidic soils and dry soils (2) For paddy and other crops
Azotobacter chroococcum, different strains of *Azotobacter* (non-symbiotic)	Bioazoto, Bhoomi Rakshak, Kisaan Azotobacter culture, and Azonik	For all crops like wheat, sorghum, barley, maize, paddy, mustard, sunflower, sesamum, cotton, sugarcane, banana, grapes, papaya, watermelon, onion, potato, tomato, cauliflower, chilly, lady finger, rapeseed, linseed, tobacco, mulberry, coconut, spices, fruits, flowers, plantation crops, and forest plants
Gluconacetobacter diazotropicus	Sugar-Plus	For sugarcane
Rhizobium strains (symbiotic, nitrogen fixing)	Biobium, Rhizo-Enrich, Kisaan Rhizobium culture, Rhizoteeka, Green Earth Reap N4, and Rhizonik	Pulses (gram, peas, lentil, moong, urd, cowpea, and arhar), oil legumes (groundnut and soyabeans), fodder legumes (barseem and lucerne), and forest tree legumes (subabul, shisam, and shinsh)
Phosphorus-solubilizing and Phosphorus-mobilizing microbes like *Bacillus megaterium*, mycorrizhal fungi, etc.	Biophos, Get-Phos, MYCO-RISE, Kisaan P.S.B. culture, MycoRhiz, Reap P, and Phosphonive	For all crops
Potassium-mobilizing or potash bacteria like *Bacillus mucilagenosus*	BIO-NPK, Bharpur, BioPotash, Potash-Cure, and Green Earth Reap K	For all crops
Sulfur-solubilizing microbes like *Thiobacillus thioxidans*	Biosulf, Sulf-cure, Sulphonik, S Sol B®, Siron, and MicroS-109	For cereals, millets, pulses, oilseeds, fiber crops, sugar crops, forage crops, plantation crops, vegetables, fruits, spices, flowers, medicinal crops, aromatic crops, orchards, and ornamentals
Zinc-solubilizing microbes	Biozinc, Zinc-Cure, Zinc activator, Zinc extra, and MicroZ-109	For crops like paddy, wheat, pulses, citrus, pomegranate, ginger, etc.
Silica-solubilizing microbes	BioSilica, Silica-Cure, and Silica-109	For crops like cereals, sugar cane, onions, leafy greens, legumes, cucumber, pumpkin, and gourd

Modified from Singh et al. (2014), Biotech International Limited (2018), National fertilizers limited (2018), Biocyclopedia (2018), Indiamart (2018) and International Panaacea Limited (2018)

Table 1.3 The different constraints in biofertilizer technology

Biofertilizer technology constraints	Examples
Technological	(1) Use of less efficient microbial strains and carrier materials (2) Low quality and short shelf-life of microbial inoculants (3) Lack of technically qualified personnel
Infrastructural	(1) Non-availability of suitable production facilities like equipment, space, storage, etc.
Financial and marketing	(1) Non-availability of sufficient funds (2) Less return by sale of products (3) Non-availability of right inoculant (4) Lack of retail outlets or market network for producers
Environmental	(1) Seasonal biofertilizers demand (2) Soil characteristics (3) Simultaneous short-span cropping operations
Human resources	(1) Lack of appropriate training on production practices (2) Unfamiliarity on the quality of the manufactured product (3) Problem in adoption and unawareness of the benefits of technology by farmers (4) Ignorance on the environmental indemnities caused by continuous application of chemical fertilizer

1.6 Conclusion

In modern-day agricultural practices, biofertilizers form an important component of sustainable organic farming in terms of a viable alternative of chemical fertilizers that are associated with various environmental hazards. Biofertilizers can fix and make available atmospheric nitrogen in soil and root nodules, solubilize phosphate (from insoluble forms like tricalcium, iron, and aluminum phosphates) into available forms, sift phosphates from soil layers, produce hormones and antimetabolites to uphold root growth, and decompose organic matter for soil mineralization. This causes increased harvest yields, enhanced soil structure (by influencing the aggregation of the soil particles for better water relation), untainted water sources, and induced drought tolerance in plants (by enhancing leaf water and turgor potential, maintaining stomatal functioning, and increasing root development). However, an increased demand and awareness among farmers and planters about the use of biofertilizers can pave the way for new entrepreneurs to get into biofertilizer manufacturing, which also requires encouragement as well as support from the governments. Biofertilizer technology, which is an inalterable part of sustainable agriculture, has to be appropriate for the social and infrastructural situations of the users, economically feasible and viable, renewable, applicable by all farmers equally, stable in long-term perspective, acceptable by different societal segments, adaptable to existing local conditions and various cultural patterns of society, practically implementable, and productive. Thus, it is apparent that awareness of the significance and economic feasibility of application of biofertilizer technology has to be increased by proper practical training of dealers and farmers.

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Chapter 2
Fungal Inoculants for Native Phosphorus Mobilization

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Abstract More than 96% of the total native phosphorus present in any agricultural soils is in unavailable inorganic or organic forms. They may be utilized by the plants through the activity of efficient fungi which are secreting/producing/releasing huge amount of acid phosphatase, alkaline phosphatase, phytase, and organic acids. The important fungi capable of doing the job are in the groups of *Aspergillus*, *Emericella*, *Gliocladium*, *Penicillium*, *Trichoderma*, and *Chaetomium* besides some AM fungi like *Glomus* and *Gigaspora*. The three efficient fungi already used as inoculums are *Chaetomium globosum*, *Penicillium purpurogenum*, and *Emericella rugulosa*. Seed inoculation using these fungi is mobilizing 45–60 kg P and 16–25% increase in yield of different crops. They are mainly exploiting from labile and moderately labile fractions of phosphorus. Minimum concentration of organic acid of fungal origin required to solubilize P was found between 0.2 and 0.5 mM. In fungal-inoculated plants, microbial contribution was more than the plant contribution. Fungal extracellular enzymes were more efficient than their intracellular counterpart. P uptake occurs around the root tip into epidermal cells with their associated root hairs and into cells in the outer layers of the root cortex. Phosphate can also be taken up by transfer from mycorrhizal fungi to root cortical cells.

Keywords Fungal phosphatases and phytases · Fungal enzymes · Mycorrhizal fungi · Mineral nutrition

2.1 Introduction

Phosphorus is one of the most important nutrients for plant growth and root development. It helps in photosynthesis, energy conservation, carbon metabolism, redox reaction, enzyme activation/inactivation, signaling, and nucleic acid synthesis

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(Vance et al. 2003). In general, P availability in soil is very low due to its easy fixation and immobilization (Yadav and Tarafdar 2010). Phosphorus predominantly presents in the soil as an insoluble inorganic form or an organic form, which are not directly available to plants. A number of mechanisms have been proposed to increase the soil phosphorus in plant rhizosphere. The most important ones are dissolution by organic acids and hydrolysis of organic phosphorus by enzymes like phosphatases and phytases. Both plant and microorganisms may contribute to the processes. Many reports indicated that the changes in rhizosphere pH due to release of different organic acids by plants and soil microorganisms especially fungi may be a major factor of dissolution of soil phosphorus (Hedley et al. 1982; Yadav and Tarafdar 2003). The capability of soil microorganisms to solubilize insoluble phosphorus fractions present in various forms is also well indicated (Richardson 1994; Tarafdar and Yadav 2011). Many research findings have very clearly demonstrated the potential of soil fungi to hydrolyze and solubilize phosphorus and help in plant P availability under field conditions (Yadav and Tarafdar 2007; Tarafdar and Yadav 2011). Tarafdar and Marschner (1995) showed the importance of soil fungi in increasing the available P from organic P like phytate and glycerophosphate to plant roots. It has also been found (Yadav and Tarafdar 2003) that fungal isolates differed in their abilities to hydrolyze different types of organic P compounds. The role of phosphatase and phytase-releasing fungi is well appreciated in exploiting the soil organic P even from very poor P status soils (Yadav and Tarafdar 2003, 2007, 2010). As compared to the plant contribution to P mobilization, fungal acid phosphatase was found to be more efficient in hydrolysis of organic P compounds (Tarafdar et al. 2001). In general, fungi belonging to the genera *Aspergillus*, *Emericella*, and *Penicillium* have more potential to exploit native organic phosphorus for plant nutrition (Yadav and Tarafdar 2003). The plant-unavailable organic and inorganic fractions of P exploited by fungal acid phosphatase and alkaline phosphatase were identified by Tarafdar and Yadav (2011). The fungi of the genera *Aspergillus*, *Emericella*, *Gliocladium*, *Penicillium*, and *Trichoderma* are efficient to mobilize unavailable P from very resistant organic P source like phytin (Yadav et al. 2010) due to their huge production/release of phytase enzymes. Phytate and phytin are metal (Fe, Al, Ca)-associated derivatives and generally constitute up to 50% of the total organic P in the soil (Turner et al. 2002). In the present chapter, I have examined the ability of fungi as inoculants to exploit soil-unavailable P for plant nutrition.

2.2 P Status in the Soil

Almost 96.5% of phosphorus present in the soil is mostly in plant-unavailable inorganic or organic form. Not more than 3.5% of the total P is present in any soil as plant-available form. Plant takes P either as $H_2PO_4^-$ or $HPO_4^=$ or PO_4^{3-} form depending on soil pH. If the soil pH is less than 6.7, then plants mainly take P as the $H_2PO_4^-$ form; between soil pH 6.7 and 9.4, the P is generally available to plants as

Table 2.1 Forms of phosphorus present in the soil (% of total P)

Form of phosphorus	All soils (%)	Arid region (%)
Plant-available form	>1–3.5	0.7–1.6
Unavailable inorganic form	15–79	75–79
Organic form	18–92	18–22

$HPO_4^{=}$ form. If the soil pH is above 9.4, which is generally in rare case, then plants take P as the PO_4^{3-} form. The P status of the world's soil is summarized below in Table 2.1.

In general, 96.5–99% of the total P is present in the soil as plant-unavailable forms that can be exploited for plant nutrition through increasing acid phosphatase, alkaline phosphatase, and phytase activity or increasing organic acid concentration in the soil. That is possible with the introduction of efficient microorganisms, especially fungi including mycorrhizal fungi or efficient plant species. It has been reported (Batjes 1997; Gaume 2000) that 5.7 billion of hectares of worldwide soil contains meager available P for optimum crop production. Generally, P is very less mobile which may be due to the large reactivity of P ions relative to numerous soil constituents and to the consequent strong retention of most of the soil phosphorus onto those. Due to this, negligible proportion of soil phosphorus is present as P ions in the solution. More P ion concentration is only noticed in highly fertilized soils. Their concentration in soil solution varies from 0.1 to 10 micromoles (Frossard et al. 2000).

There are many fractions of inorganic P, some fraction adsorbed by exchange sites generally known as loosely bound, labile, or exchangeable P (Ruban et al. 1999); it is an easily releasable fraction. The other fraction is associated with Al, Fe, and Mn oxides and hydroxides; phosphorus and iron are often bound to sediments, and iron complexes help in the adsorption of P by ligand exchange; here the amount of FeOOH is one of the factors controlling P release. The third fraction is Ca-bound compounds, generally referred to as apatite-P. The novel approach to characterize hydrolysable organic P is the enzymatic hydrolysis in soil (Pant and Warman 2000). The three important enzymes responsible for hydrolysis are acid phosphatase, alkaline phosphatase, and phytase. The available P released through the cleavage of organic bonds by these three enzymes can be taken up by the plants.

Phosphorus (may be both inorganic and organic forms) present in the soil ranges between 100 and 2500 kg/ha, with an average of 1000 kg/ha in the top 20 cm. They may be divided into four categories: P in soil solution as ions and compounds; surface adsorption of P onto inorganic soil constituents; minerals P, both crystalline and amorphous; and P present as a component of soil organic matter (Barber 1995). P present in soil solution varies widely among soils and climate. In general, the concentration of P needed by different vegetations varies between 0.003 and 0.3 ppm. Generally, tuber crops show very high P response. P is absorbed by plant roots through diffusion and mass flow from the soil and transported to the entire plant for nutrition. Barber (1995) reported that in high organic matter content soil, 50% of the phosphate in soil solution may be in the form of soluble organic

compounds. Plants have developed a mechanism to secrete phosphatases mainly to catalyze hydrolysis of P from organic forms in many soils (Richardson et al. 2001). They may also report to release many organic acids to solubilize P compounds from soil.

2.3 Important Fungi to Mobilize Unavailable P

Numerous fungi belonging to different genera have been reported to mobilize P. Fungi mobilize organic P through the release of phosphatase and phytase enzymes and inorganic P after releasing organic acids that solubilize soil inorganic P compounds into plant-available forms. Fungi have potential also to immobilize available phosphates into cellular material and promote the solubilization of fixed or insoluble mineral forms of P mainly through the production of chelating agents. Organic chelates form complexes with Ca, Fe, or Al and thereby release phosphate in water-soluble forms as follows:

$$\mathrm{CaX_2} \times 3\mathrm{Ca}\,(\mathrm{PO_4})_2 + \text{chelate} = \text{soluble } \mathrm{PO_4}^{=} + \text{calcium chelate complex (where x = OH or F)}$$

and

$$\mathrm{AL(Fe)} \times (\mathrm{H_2O})_3(\mathrm{OH})_2\mathrm{H_2PO_4} + \text{chelate} = \text{soluble } \mathrm{PO_4}^{=} + \mathrm{AL(Fe)}\text{-chelate complex}$$

A list of the most effective fungi for P mobilization and solubilization is presented in Table 2.2.

As total organic P is highly correlated with total organic carbon in most of the soils, therefore, mineralization may be expected to increase with increasing total organic C. Temperature, aeration, and pH are other factors that dictate the quantity of P mineralization/immobilization. Among 30 species of filamentous fungi isolated from Brazilian soil, *Aspergillus caespitosus* produced and secreted the highest level of alkaline phosphatase (Guimaraes et al. 2003). It has been well known that fungi produced low-molecular-weight organic acids (e.g., citric acid, oxalic acid) in the

Table 2.2 Important fungi for native P mobilization

Aspergillus awamori	*Aspergillus terreus*	*Paecilomyces variotii*
Aspergillus candidus	*Aspergillus ustus*	*Penicillium purpurogenum*
Aspergillus flavus	*Chaetomium globosum*	*Penicillium rubrum*
Aspergillus fumigatus	*Curvularia lunata*	*Penicillium simplicissimum*
Aspergillus niger	*Emericella nidulance*	*Phoma* sp.
Aspergillus parasiticus	*Emericella rugulosa*	*Pseudorotium zonatum*
Aspergillus rugulosus	*Gliocladium catenulatum*	*Trichoderma harzianum*

rhizosphere soil which enhances the solubility of mineral P by ligand exchange and complexation of metal ions such as aluminum, iron, and calcium.

Arbuscular mycorrhizal (AM) fungi are well known to present abundance in agricultural soils and proved to enhance P nutrition of plants by scavenging the available P due to the large surface area of their hyphae that make them efficient for more P uptake and transport (Moose 1980). They are also able to release/produce P (Paul and Sundara Rao 1971) that could solubilize the insoluble mineral phosphates from the soil. It has also been noticed that AM can produce/release phosphatase that are efficient enough to mobilize organic P (Tarafdar and Marschner 1994; Tarafdar 1995). The organic acid production by AM fungi would definitely enhance the availability of acid-labile insoluble phosphate. Lapeyrie et al. (1991) also demonstrated that ectomycorrhizal fungi have possessed P-solubilizing activity. It has also been shown that they are incapable of utilizing P from inositol phosphates and have phosphatase activity that could further affect their ability to release P from organic matter (Koide and Schreiner 1992). But the use of AM as phosphate biofertilizers is not widespread due to the inability to culture them in vitro, since they are obligate symbionts.

2.4 P Solubilization

The ability to solubilize P by fungi mainly depends on the nature of the N source used. It is noticed to have greater solubilization in the presence of ammonium salts than when nitrate is used as the N source. This may be due to the extraction of protons to compensate for ammonium uptake, resulting in the lowering of extracellular pH (Roos and luckner 1984). The release of organic acids or protons enhances the ability of fungi to reduce pH of their surroundings and encourage solubilizing the Ca-P complexes. The organic acids secreted by the fungi can either directly dissolve the mineral phosphate as a result of anion exchange of $PO_4^{=}$ by acid anion or chelate both iron and Al ions associated with phosphate (Bardiya and Gaur 1972). The important organic acids such as acetate, lactate, oxalate, tartarate, succinate, citrate, gluconate, ketogluconate, and glycolate produced by the fungi have been found to be very effective for P solubilization. The efficiency of fungal organic acids toward the release of available P has been computed and presented in Table 2.3.

Table 2.3 Release of plant available P (ppm) under different types of soil with the action of 1 mM organic acids produced by fungi

	Soil types		
Organic acids	Alfisol	Aridisol	Inceptisol
Citric	9.1 ± 1.2	15.6 ± 1.8	22.1 ± 1.2
Formic	14.0 ± 1.5	17.4 ± 1.3	30.2 ± 1.9
Lactic	14.3 ± 1.3	20.6 ± 1.5	31.8 ± 2.1
Malic	7.8 ± 0.8	8.0 ± 0.9	10.8 ± 0.7

Table 2.4 Minimum organic acid concentration required to solubilize P

Organic acids	Minimum concentration required to solubilize P (mM)
Citric	0.26
Formic	0.22
Lactic	0.20
Malic	0.50

Subba Rao (1982) has demonstrated that the ability to reduce pH in some cases does not correlate with the amount of mineral phosphate solubilized which indicated that acidification is not the only mechanism of solubilization; the chelating property of the organic acids may also be important that is also reflected in the work of Kucey (1988), which showed that the addition of 0.05 M EDTA to the media had the same solubilizing effect as the inoculation with *Penicillium bilaii* (Kucey 1988). Organic acids, in general, help in cation–anion balance and hence for the net release of H^+ that is likely to occur to compensate for this net efflux of negative charges. It has also been noticed that root respiration can contribute to significant acidification of the rhizosphere. It is in general believed that roots and rhizosphere fungi relying on root exudates respire and thereby produce CO_2 and hence carbonic acid in the rhizosphere. There are many reasons believed to account for the variations in the effectiveness of fungi inoculations on plant growth enhancement and crop yield. They are survival and colonization of inoculated fungi in the rhizosphere, competition with native microorganisms, nature and properties of soils and plant varieties, insufficient nutrient in the rhizosphere to produce enough organic acids to solubilize soil phosphates, and inability of fungi to solubilize soil phosphates. The minimum concentration required to solubilize P by fungal organic acids varies among the type of organic acid produced. Lactic acid was found to be most effective, and malic acid needs more concentration to solubilize per unit P (Table 2.4).

2.5 P Mobilization by Fungal Phosphatases

Major P fractions in most of the soil are in the organic form. To utilize organic P fractions by plants, these P compounds must be hydrolyzed by phosphatases or phytase, which are of plant and microbial origin. Both the enzymes may therefore be very important in the P nutrition of plants (Tarafdar and Claassen 1988). Fungi are very efficient in producing both phosphatases (acid and alkaline). It has been noticed that among the fungi, the genus *Aspergillus* was most efficient in producing phosphatases. Higher fungal buildup and increased root exudation in the rhizosphere are reported to be the result of higher phosphatase activity and more P mobilization. Fungi may cleave C–O–P ester bond of organic P with the help of phosphatases and phytase released by them. Fungal activity may also result in alterations of root exudate composition both qualitatively and quantitatively due to the degradation of exudate compounds and the release of microbial metabolites (Neumann and

Table 2.5 P-mobilizing enzyme release potential by some important fungi

	Enzyme release (EU × 10^{-3} per g fungal mat)					
	Acid phosphatase		Alkaline phosphatase		Phytase	
Fungi	Intra	Extra	Intra	Extra	Intra	Extra
Aspergillus flavus	20.7	5.2	5.0	1.9	33	1246
Chaetomium globosum	14.6	4.0	5.3	1.2	26	954
Curvularia lunata	13.7	3.4	1.1	0.2	19	699
Paecilomyces variotii	37.5	9.4	0.9	0.2	48	1824
Penicillium sp.	8.9	2.2	1.5	0.3	13	507
LSD ($p = 0.05$)	1.81	1.21	0.89	0.18	2.33	8.91

EU enzyme unit, *LSD* least significant difference

Table 2.6 P depletion from different organic P fractions by fungal phosphatases

P fraction	% depletion
Labile fraction	43.9–90.4
Moderately labile fraction	15.7–21.3
Moderately resistant fraction	2.8–16.2
Highly resistant fraction	0.5–2.0

Roemheld 2000). Kucey et al. (1989) concluded that fungal activity is a central factor in the soil organic P cycle and influenced the transformation of inorganic P into the system. The importance of soil fungi in increasing available P and transfer to the plant roots has been suggested by many workers. Tarafdar and Marschner (1995) demonstrated also the role of co-inoculation with different compatible fungal combinations to mobilize more P from the soils for plant nutrition, for example, the mycorrhizal fungi *Glomus mosseae* and *Aspergillus fumigates*, which have known phytase activity (Wyss et al. 1999). Yadav and Tarafdar (2003) indicated that fungal isolates differed in their abilities to hydrolyze different organic P compounds. The efficiency of some fungi in releasing phosphatases and phytase both intra- and extracellularly is presented (Table 2.5).

Tarafdar and Gharu (2006) demonstrated the role of *Chaetomium globosum* to release/produce phosphatase and phytase enzymes, which is efficient in native P mobilization and enhances the production of wheat and pearl millet crop. *Penicillium purpurogenum* was also reported as an excellent P mobilizer under arid agroecosystems (Yadav and Tarafdar 2011). In general, P mobilization by fungal phosphatases was more from the labile fraction followed by moderately labile fraction and least from the highly resistant fraction of the organic P compounds (Table 2.6). It indicates that fungi are less capable in mobilizing P from the relatively resistant pool.

The plant and microbial contribution to mobilize plant-unavailable P compounds has been partitioned. It has been noticed that the microbial contribution was much higher in the initial stages of plant growth than in the later stages (Table 2.7) when plant roots are dominated in P mobilization. However, the microbial contribution was higher than the plant contribution after considering the entire growth period of the plants.

Table 2.7 Partition of plant and microbial contribution for unavailable P mobilization

	Depletion of total unavailable P (mg/kg)					
Plant age (days)	PC	MC	PC	MC	PC	MC
28	3.6 ± 0.3 (20.1)[a]	14.3 ± 1.1 (79.9)	12.8 ± 1.0 (39.0)	20.0 ± 1.2 (61.0)	4.7 ± 0.7 (20.7)	18.0 ± 1.2 (79.3)
35	14.0 ± 1.2 (26.0)	39.8 ± 2.1 (74.0)	18.2 ± 1.1 (40.0)	27.3 ± 1.4 (60.0)	11.9 ± 0.9 (40.3)	17.6 ± 1.4 (59.7)
42	25.9 ± 2.0 (42.0)	35.9 ± 2.7 (58.0)	28.9 ± 1.4 (54.6)	24.0 ± 1.7 (45.4)	19.5 ± 1.2 (46.9)	22.1 ± 1.6 (53.1)
49	33.9 ± 2.5 (47.2)	37.9 ± 2.4 (52.8)	35.2 ± 1.8 (58.6)	24.9 ± 1.7 (41.4)	26.6 ± 1.5 (56.8)	20.2 ± 1.4 (43.2)
56	41.8 ± 3.1 (53.9)	35.7 ± 2.9 (46.1)	45.9 ± 2.5 (64.7)	25.0 ± 1.4 (35.3)	33.4 ± 1.9 (58.2)	24.0 ± 1.2 (41.8)

PC plant contribution, *MC* microbial contribution
[a]Percent total

The decrease in different organic P fractions, in general, was more (41–86%) from water-soluble fractions due to the action of acid and alkaline phosphatases produced by fungi, followed by 50–84% from $NaHCO_3$ fractions, 14–26% from NaOH fractions, and 8–19% from HCl fraction under different vegetations (Gharu and Tarafdar 2016). Between the contribution of acid and alkaline phosphatases produced by the fungi, acid phosphatase was 9–14% more efficient in mobilizing P than alkaline phosphatases. The fungal species are capable in significantly depleting both inorganic and organic P from labile fractions and moderately labile fractions (Yadav and Tarafdar 2003). The depletion from moderately resistant fractions was much less and least with highly resistant fractions. The enzymatic hydrolysis was expected to be complete by 8–12 h. The hydrolysis was initially rapid with the action of fungi followed by gradual decline in hydrolysis. Inoculation of different phosphatase-producing fungi increases dry matter, grain yield, and uptake of various nutrients including phosphorus under different crops and soil types. They may be hydrolyzed and help in translocation of nutrients to the plants. Their activity was found more near the root zone especially in the rhizosphere.

2.6 P Mobilization by Fungal Phytase

Efficient phytase-producing fungi belong to genera *Aspergillus*, *Emericella*, *Gliocladium*, *Penicillium*, and *Trichoderma* such as *Emericella rugulosa*. They can easily hydrolyze the inositol penta- and hexaphosphates (phytates) and their derivatives which are reported for a major component of soil organic P (Anderson 1980). These fungi groups were noted to be most efficient P mobilizer through the production/release of phytase enzymes (Yadav et al. 2010). It is also observed that the release of phytase by fungi was more under P deficient than sufficient P present in the soil (Table 2.8) under different vegetations. In general, 16–55% more phytase activity was expected in P-deficient soil conditions.

The application of phytase into the soil stimulates phytate hydrolysis, and subsequently, the phosphorus transport as orthophosphate to the roots is increased (Beissner and Roemer 1996). The phytin hydrolytic cleavage by phytase controlled the P availability from phytin sources (Findenegg and Neiemans 1993) and ultimately from organic sources. Between extra- and intracellular fungal enzymes, extracellular fungal enzymes were more active than their intracellular counterpart especially in respect to the release of P from phytin sources (Fig. 2.1). The

Table 2.8 Release of phytase by fungi under variable P conditions

Plants	Phytase release (EU $\times 10^{-6}$)		% increase under P-deficient condition
	P deficiency	P sufficiency	
Crops	3.75 ± 0.16	3.24 ± 0.12	16
Grasses	1.67 ± 0.08	1.08 ± 0.05	55
Trees	15.82 ± 1.20	12.63 ± 0.97	25

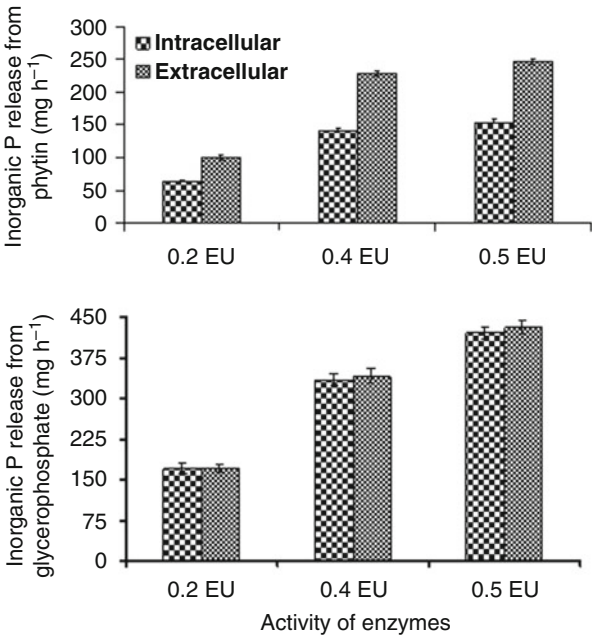

Fig. 2.1 Comparative efficiency of extra- and intracellular fungal enzymes

extracellular phytase released by the organisms was 12.7 times more than their intracellular counterpart. Among the phytase-producing fungi, extracellular phytase activity was more in *Emericella rugulosa*, whereas intracellular phytase activity was higher in *Tricoderma harzianum* (Yadav et al. 2010). *Emericella rugulosa* was found to be the most efficient in hydrolyzing phytin P (98.82 μg/g). The efficiency of fungal phytase to hydrolyze phytin P compounds increases with time up to 24 h of incubation. A significant correlation was observed between the activity of root-associated and root-released extracellular phytase. *Aspergillus fumigatus* phytase has been identified as a phytase for the animal including human nutrition due to their series of favorable properties maximizing phytic acid degradation and for increasing P and amino acid availability. Fungal phytase is regularly used as a supplement in diets for monogastric animals to improve phosphate utilization from phytate, the major storage form of phosphate in plant seeds (Greiner and Konietzny 2006). Experiments also confirmed the favorable stability and catalytic properties of *Aspergillus fumigatus* phytase. In general, phytase-producing fungi after seed inoculation may be able to enhance 18–25% shoot P concentration and 7–10% root P concentration of plants, resulting in 15–23% increase in yield of cereal crops (Fig. 2.1).

Phosphatases and phytase produced by the fungi may release plant-unavailable P mainly from water-soluble fractions and bicarbonate fractions under different cropping systems. The results (Table 2.9) suggested that both organic and inorganic P pools can be utilized by fungal P-mobilizing enzymes from the soils under different vegetations indicating the use of fungal enzymes as effective inoculants for P mobilization. It has also been reported that fungal enzymes can hydrolyze

Table 2.9 Efficiency of fungal phosphatases to release P (%) from different soil P fractions under variable vegetation

	Fallow		Crop		Grasses		Trees	
P fractions	Po	Pi	Po	Pi	Po	Pi	Po	Pi
WS-P	64.7	72.9	72.2	68.1	68.1	72.0	53.3	64.9
Bicarab-P	70.1	78.0	70.9	67.2	68.0	75.9	69.4	71.3
NaOH-P	22.8	27.9	21.1	25.4	16.4	15.6	16.2	15.0
HCl-P	15.7	18.5	12.6	9.2	11.9	7.8	12.6	14.5
LSD ($p = 0.05$)	2.8	3.4	3.1	2.4	2.0	3.1	1.8	2.7

Po organic P, *Pi* inorganic P, *LSD* least significant difference

other phosphorylated amino acids like O-phosphothreonine, O-phosphotyrosine, and O-phosphoserine (Guimaraes et al. 2003). Plants utilize organic P after hydrolysis by fungal phosphatases, but inorganic P seems to be more important and preferentially used by plants; organic P may be essential in high P-fixing soils for the nutrition (Tarafdar and Claassen 2005). The release of plant-available P from different P fractions under different vegetations is presented in Table 2.9.

Depletion of P fractions in the rhizosphere varies with the plant species and soil types. The P depletion by different plants in the rhizosphere has been related to differences in root morphology; root density; root surface area; root hair length and density; root-induced chemical, biochemical, and biological changes; and root–soil interactions (Foehse et al. 1988; Haussling and Marschner 1989). The differences in the ability of different fungi depend on their quality of enzyme released both extra- and intracellularly although they might be releasing a similar quantity of enzymes.

2.7 P Mobilization by AM Fungi

Mycorrhizal symbiosis between plant roots and soil fungi is generally noticed in ecosystems (Yang et al. 2018). The presence of AM fungi is widespread in soils, and they form symbiotic as well as mutualistic associations with many plant species. Their colonization with plant roots often increases plant growth by improving P uptake, particularly on P-deficient soils (Smith and Read 1997). Due to their long aerial mycelium (Fig. 2.2), AM fungi can transport P from a long distance where plant roots cannot reach. They can also release some organic acids to solubilize P as well as phosphatases to mobilize P from the unavailable native P sources. Root infection with AM fungi may enhance the efficiency of nutrient absorption and, in turn, enhance growth of mycorrhizal-infected plants, particularly at low availability of phosphorus in the soil. AM infection has also influenced the root morphology depending on the density of mycorrhizal association (Fig. 2.2).

The significant effect of mycorrhizal fungi was observed to the relatively immobile nutrients. Regardless of the cropping system and the P concentration in soil, AM fungi have been reported to improve dry biomass and crop yield besides increasing

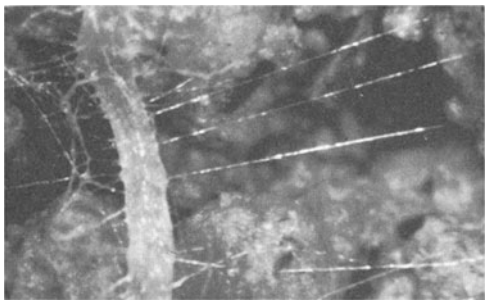

Fig. 2.2 Extraradical hyphae of AM fungi to transport P from distant places

Table 2.10 Effect of AM fungi on important arid legumes

Inoculation	Cluster bean	Moth bean	Mung bean
A. Acid phosphatase (n Kat per 100 g soil)			
Control	8.0	9.8	8.2
Glomus mosseae	9.8***	12.1***	10.4***
Glomus fasciculatum	9.5***	11.0*	9.7***
B. Alkaline phosphatase (n Kat per 100 g soil)			
Control	13.0	17.5	11.0
Glomus mosseae	15.5***	20.0**	13.5*
Glomus fasciculatum	14.8**	19.8**	13.6*
C. P concentration (mg/g)			
Control	1.7	1.5	1.4
Inoculated	2.1***	1.9**	1.9***
D. Grain yield (q/ha)			
Control	5.4	4.2	4.8
Glomus mosseae	7.0***	5.1***	6.0***
Glomus fasciculatum	6.2**	4.9***	5.8***
E. Shoot dry mass (q/ha)			
Control	23.0	18.2	21.5
Glomus mosseae	31.7***	23.5***	27.9***
Glomus fasciculatum	29.9***	21.8*	25.8**

*Significant at 5% level; **Significant at 1% level; ***Significant at 0.1% level

the survival ability of plants against drought through water transport from the deep. They help in more nodulation under legumes as well as the root surface area of the plants to capture and transport more nutrients. Consequently, symbiotic N_2-fixation in legumes, a process being dependent on P supply, is improved by AM fungi. The effect of AM fungi on arid legumes is presented in Table 2.10.

The percentage of root length infected by AM fungi was often reduced by high P application in soil (Mosse 1973). In addition to the production of phosphatases and release of organic acids, AM fungi may help in stabilization of soil aggregates. Tisdall et al. (1997) demonstrated that fungal hyphae bring mineral particles and

organic materials together to form stable microaggregate and demonstrated to bind microaggregate into macroaggregates. The enhanced growth of plants infected by AM fungi results primarily from improved uptake of soil immobile nutrients especially phosphate through the mobilization, or extra phosphate reaches the root through the fungal hyphae that tap the soluble P in soil beyond the phosphate depletion zone near the root surface. Besides P, they may increase the uptake of other nutrients like Zn, Cu, and N. The AM fungus is believed to be obligatorily dependent on the plant, that is, the plant often benefits from the fungus, and the balance between the two is much influenced by soil fertility, especially phosphate levels. The AM fungal system must be regarded as consisting of three components, plant, fungal endophyte, and soil, involving a three-way interaction among them. Cantrell and Linderman (2001) reported that AM can also help in drought resistance to plants and can alleviate deleterious effects of saline soils on crop yield.

2.8 Some Important Fungal Inoculants for P Mobilization

P-mobilizing/P-solubilizing fungal inoculants are mainly used as seed inoculation. In general, 1 g of fungal mat was crushed and mixed with 50 mL of extracellular fungal aliquot; thereafter, approximately 150 g of absorbent material was added, properly mixed, and air-dried. The important sticking materials used are guar gum/carboxyl methyl cellulose/guar. French chalk powder, peat, lignite, or charcoal is used as the absorbent material for inoculum preparation. The brief procedure for fungal inoculum production for seed inoculation is sketched in Fig. 2.3. The amount of inoculum required depends on the size of the seeds.

$$\begin{gathered} 50 \text{ g seed} + 5 - 10 \text{ mL of sticking solution } (1\%) \text{ and mix thoroughly} \\ + \\ 25 - 35 \text{ g of inoculants} \end{gathered}$$

They are mixed thoroughly and air-dried.

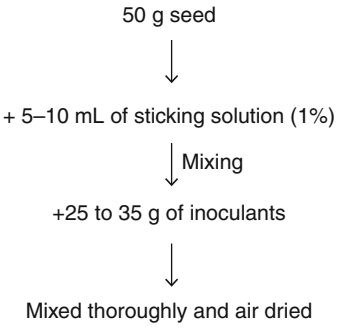

Fig. 2.3 Inoculum preparation

2.8.1 Chaetomium globosum

It is an efficient phosphatase- and phytase-producing fungus with the potential to release organic acids. It has the potential to decrease the soil pH from 7.4 to 5.6 in the rhizosphere within 4 weeks after inoculation. In general, at least one unit decrease in soil pH was expected due to release of organic acids by *Chaetomium globosum*. The important organic acids released by these fungi are lactic acid up to 0.08 mM, citric acid 0.06 mM, malic acid 0.04 mM, and formic acid 0.02 mM. They also release a huge amount of phosphatase and phytase to mobilize organic P fractions from the soil. After seed inoculation, the population buildup was noticed between 7.5 and 16 times of the inoculated population within 4 weeks. Pure spores of CFU of 10^7–10^9 cells/mL are generally used as inoculums. The moisture by weight of the inoculums was generally maintained between 30% and 40% (w/w), and the effect was expected to be about 45–60 kg SSP (single superphosphate) equivalent P mobilization under different crops. On average, 16–25% increase in crop yield was expected after the inoculation. The effect of seed inoculation of *Chaetomium globosum* on different arid crops is presented in Table 2.11.

The maximum effect of inoculation on different soil enzyme activities (acid phosphatase, alkaline phosphatase, phytase, and dehydrogenase) was observed between 5 and 8 weeks of plant age. A significant improvement in plant biomass, root length, plant P concentration, seed and straw yield, and seed P content resulted from inoculation (Tarafdar and Gharu 2006). *Chaetomium globosum* also showed higher competitive ability under harsh arid conditions than other native microorganisms as well as thrived under any adverse condition. Under field conditions, inoculation of *Chaetomium globosum* resulted in on average 53% more acid phosphatase activity over control after 5 weeks, 72% more alkaline phosphatase activity over control after 6 weeks, 48% more phytase activity over control after 7 weeks, and 110% more dehydrogenase activity over control after 8 weeks of crop growth. Seed inoculation of plants showed a gradual increase in the mobilization of mineral P, organic P, and phytin P fractions throughout the crop growth period. A significant

Table 2.11 Effect of seed inoculation by *Chaetomium globosum* in Aridisol (average of 4 years), yield (kg/ha)

	Pearl millet (HHB 67)		Cluster bean (RGC 936)		Moth bean (RMO 257)		Mung bean (K851)	
Treatment	Grain	Stover	Grain	Stover	Grain	Stover	Grain	Stover
Control	1131	2963	699	1258	520	728	760	1292
P40	1312	3689	798	1484	614	896	867	1387
P60	1348	3715	854	1503	639	984	899	1483
Chaetomium globosum	1319	3699	839	1510	621	925	891	1479
LSD ($p = 0.05$)	12.31	16.82	13.95	15.27	7.91	11.23	8.21	14.72
% increase over control	16.6	24.8	20.0	20.0	19.4	27.1	17.2	14.5

P40 SSP as 40 kg P per ha, *P60* SSP as 60 kg P per ha, *LSD* least significant difference, *SSP* single superphosphate

improvement in plant P concentration (20%) and seed P content (25%) was also observed under different inoculated crops.

2.8.2 Penicillium purpurogenum

It is an important P-mobilizing organism that can be effectively used for seed inoculation. Plants inoculated with the fungi *Penicillium purpurogenum* showed significant improvement in phosphatase (acid and alkaline), phytase, and dehydrogenase activities in soil compared to uninoculated fields (Yadav and Tarafdar 2011). Their effect on the depletion of organic P was much higher than that of mineral and phytin P. In general, a significant improvement in plant biomass (30%), root length (21%), P uptake (6%), seed (19%) and straw yield (30%), and P concentration of shoot (15%), root (6%), and seed (33%) resulted from the inoculation of *Penicillium purpurogenum*. The said fungi can well thrive under arid ecosystems as well as under very harsh environment. With inoculation, their contribution on P mobilization exceeded the plant contribution in respect to the mobilization of P from the native sources. They are very much compatible with the rhizosphere environment of most of the plants tested. For example, the combined effect (plant and microorganisms) resulted in significant improvement in plant biomass, P concentration, and yield of pearl millet, which indicated that the organism should be considered as an efficient native P mobilizer and possible inoculation tool for cereal production, especially under rain-fed conditions and phosphate-deficient soils like those in arid areas. The inoculum culture should have at least 2×10^6 colony-forming units (CFU) per g/mL of inoculum culture for *Penicillium purpurogenum* and inoculated with 100 g/kg seed in the slurry of carrier-based culture prepared in sterilized jiggery (20% gur) solution and dried under shade prior to sowing. During the preparation of inoculum, the culture broth was blended in a homogenizer and diluted with sterilized, distilled water. The inoculation effect on pearl millet is presented in Table 2.12.

2.8.3 Emericella rugulosa

Emericella rugulosa is one of the other efficient P mobilizers that produce enough phosphatases and phytase that mobilize native P and enhance the production of

Table 2.12 Seed inoculation of pearl millet with *Penicillium purpurogenum*

Treatment	Yield (kg/ha)		P concentration (mg/g)		Seed P content (%)
	Seed	Straw	Shoot	Root	
− Inoculation	1578 ± 41.5	2878 ± 91.1	4.73 ± 0.15	3.59 ± 0.21	0.96 ± 0.15
+ Inoculation	1944 ± 33.9	3802 ± 45.5	5.90 ± 0.53	3.83 ± 0.26	1.30 ± 0.14
% increase	23.2	32.1	24.7	6.7	35.4

many crops. The inoculation with *Emericella rugulosa* was carried out in the slurry of carrier-based culture, and the population generally used was 10^8 CFU. Seed inoculation with the fungi generally improved 20% acid phosphatase secretion and 45% alkaline phosphatase activity. The phytase activity, in general, increases by 46% after inoculation of *Emericella rugulosa* which also influences the dehydrogenase activity by 98% (Yadav and Tarafdar 2007). A gradual increase in depletion of different forms of unavailable P with the inoculation was observed. Increase in dry matter varies between 21% and 35% after inoculation of the fungi. The crop yield may increase up to 23%. In general, more shoot (20%) and root (5%) P concentration was expected compared to the uninoculated plants. A gradual increase in depletion of different forms of unavailable P with the inoculation of *Emericella rugulosa* with plant age was also observed (Yadav and Tarafdar 2007). The fungal contribution varies between 51% and 82% for mineral P, 38% and 65% for organic P, and 44% and 82% for phytin P. Increase in dry matter varies between 21% and 52% after inoculation of fungi under different plant growth stages. The increase in inoculated plant root length varies between 19% and 26%.

Plants acquire phosphorus as phosphate anions from the soil solution. It is probably one of the least available plant nutrients found in the rhizosphere. In particular, plant growth-promoting fungi have been reported to be key elements for plant establishment under nutrient-imbalance conditions. Use of those fungi in agriculture can favor a reduction in agro-chemical use and support more crop production. The phosphatase and phytase release by different fungi can be further enhanced by spraying 10 ppm Zn nanoparticles or 30 ppm Fe nanoparticles to the culture. The additional enhancement of release due to application of nanoparticles on phosphatases was observed between 46% and 56% under different phosphatase-releasing fungi and between 170% and 253% for various phytase-releasing fungi.

2.9 Phosphate Uptake Mechanism

The activity of microorganisms especially fungi present in the rhizosphere dictates the available P status in the soil for plant nutrition (Hinsinger 1998) and strategies of plant for taking up P. The most important process is the decrease in the concentration of phosphate ion in the soil solution, which occurs within the rhizosphere as a direct consequence of the removal of P by the root uptake. The process of depletion of rhizosphere P has been reported by different workers under various soils and plants (Huebel and Beck 1993; Hisinger and Gilkes 1997). This depletion helps in the replenishment of P from the solid phase in the crop-growing period, and P is influenced by the physical-chemical conditions of the soil. The fungal contribution was noticed to be much higher than the plant contribution (Yadav and Tarafdar 2007) to the hydrolysis of different native unavailable P fractions. In addition to the cleavage of the C–O–P ester bond by fungal phosphatases and phytase, the fungi may also produce appreciable quantity of organic acids, which may contribute also in the release of plant-available inorganic P from the native sources.

In low-phosphate soils, the slow rate of diffusion of phosphate results in a zone of depletion of phosphate ions in solution around the roots of plants. Transfer of phosphate to the site of uptake into the root symplasm limits phosphate uptake in such soils. In general, the transfer involves movement across the depletion zone as well as through the root apoplasm. The apoplasm is made up of cell walls of epidermal and cortical cells, together with the associated intracellular spaces. Although the pores in the open lattice of these cell walls permit movement of nutrients around cells, they increase the path length across which phosphate ions have to diffuse. The structural components and net negative charges of the cell walls also influence the effective concentrations of phosphate in the apoplasm. This concentration may be further modified by organic compounds excreted around cell walls and the presence of fungi that use such compounds as carbon sources. A membrane on the inner surface of the cell wall, the plasmalemma, separates the apoplasm from the symplasm. Uptake of nutrients into the root symplasm occurs through transporter proteins embedded in this membrane. The transport process is driven by the potential across the membrane maintained by the action of a H^+-ATPase, the "proton pump," which extrudes protons to the outer surface of the membrane. The expression of genes encoding high-affinity root phosphate transporters is regulated by the phosphorus status of the plant. Under phosphate stress, the expression of genes encoding these phosphate transporters is unregulated. This results in a greater number of transporter proteins in the plasmalemma and enhanced phosphate uptake rates, if phosphate is available at the membrane surface. Uptake occurs around the root tip into epidermal cells with their associated root hairs and into cells in the outer layers of the root cortex. Further back along the root axis, phosphate can also be taken up by transfer from mycorrhizal fungi to root cortical cells.

AM fungi with their symbiotic associations with the root system of many plants play a very important role in the acquisition of phosphate by the plant (Harrison 1999). These fungi colonies have the cortical cells from which they extend a network of hyphae several centimeters out into the surrounding soil, thereby expanding the effective soil volume that the plant can exploit. The hyphae gather nutrients from the soil solution and transfer them back to the cortical cells of the host plant. The fungi develop specialized structures known as arbuscles within infected cortical cells. Materials are exchanged between the symbionts through these arbuscles. The acquisition of phosphate through AM associations involves transport of phosphate from the soil solution across the membrane of the fungal hyphae, movement of that phosphate along the hyphae to the arbuscles, unloading the phosphate from the fungal arbuscles at the arbuscle–cortical cell interface, and uptake of that phosphate by the plant cortical cells. Harrison and Van Buuren (1995) isolated a gene encoding a high-affinity phosphate transporter from the AM fungus *Glomus versiforme*.

2.10 Future Directions

We have to identify the phosphate transporters of plant origin which are responsible for uptake from the interface into the cortical cells to understand the entire mechanism. More attention is also needed on the phosphate concentration at the arbuscle–cortical cell interfaces that are still unknown. Efforts are also needed to find out the suitable culture for multiplication of AM fungi. More experiments are needed on the balance between influx and efflux in the transgenic plants. Research should aim at phosphate nutrition in cropping systems by the P mobilizer. Moreover, suitable molecular technology is needed to introduce appropriate genes and regulatory systems in the key components of the cropping systems. Intensive work is needed to find out the compatible fungal combinations to maximum native P mobilization. P use efficiency and role of nano-induced fungal enzymes to mobilize more P for nutrition need more experimentation. Further experiments are needed to quantify the different forms of organic and inorganic phosphorus mobilized by different phosphatase- and phytase-producing fungi and effectiveness of their extracellular and intracellular enzymes. It is also important to identify more P-mobilizing fungi for use as inoculums for seeds of different crops as well as in the nursery to develop horticultural plants. Methods should be developed to assess the potential bioavailability of organically bound soil phosphorus. P limitation of soil fungi under different ecosystems, soils, and crops needs to be further studied. Assessment is needed on rhizosphere processes that determine the P acquisition efficiency. Further studies are also needed for a complete understanding of the mechanisms of P mobilization, solubilization, and assimilation in microbes. Attention is to be paid on genetic engineering in developing better and effective P mobilizers as well as identification of microbial proteins that are responsible for P mobilization.

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Chapter 3
Potential Applications of Algae-Based Bio-fertilizer

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Abstract To meet the growing demand for food, the production and application of synthetic fertilizers, pesticides, and other chemicals have intensified, which consequently pollute the environment and pose a serious threat to all living beings. Furthermore, agricultural land is losing its fertility due to intensive agricultural practices and climate changes. Various microorganisms such as bacteria, algae, fungi, etc. are receiving much attention as environmental-friendly alternatives to synthetic chemicals because of their ability to improve the soil fertility, fix atmospheric nitrogen for plant availability, produce plant growth hormones and biocides, etc. This chapter will explore the potential role of microalgae and cyanobacteria as bio-fertilizers.

Keywords Microalgae · Cyanobacteria · Blue-green algae · Soil fertility · Nitrogen fixation

3.1 Introduction

World population is expected to reach 9 billion by the midcentury, and it poses a significant challenge to existing agriculture system (Food and Agriculture Organization 1996). The world must produce more food and feed to meet the demand of the growing population. From the mid of the last century, the yield of crops increased significantly—thanks to the development of disease-resistant and high-yielding crops and intensive use of synthetic fertilizers (Singh et al. 2011). Atmospheric nitrogen is converted to ammonia, a precursor of synthetic nitrogen fertilizer, using

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Haber-Bosch process which contributes to 1.8–3% of annual global energy usage (IPTS/EC 2007; Valera-Medina et al. 2018). Unlike nitrogen, phosphorus source is limited, and according to some scientists, the world is currently facing "peak phosphorus" phenomenon (Cordell et al. 2009; Lee et al. 2018). While a fraction of the applied fertilizer is consumed by the plant, a large amount of it is lost due to leaching, volatilization, and soil erosion (Mikha and Rice 2004; Grant et al. 2012). Leaching of excess nutrients in the receiving water bodies leads to eutrophication and subsequent death of aquatic animals (Wang et al. 2018). In addition to fertilizers, various pesticides, herbicides, fungicides, etc., are applied in the field to eliminate unwanted invasion; these chemicals could also remove the useful microbiomes of the soil (Santísima-Trinidad et al. 2018). Excessive and improper applications of chemicals are also linked to loss of biodiversity and soil fertility (Bossa et al. 2012). Under the current scenario, it is crucial that innovative approaches be developed for further increase in crop yield and minimize energy input and environmental pollution (Tilman et al. 2002; Foley et al. 2011). Certain living organisms (e.g., bacteria, algae, fungi, etc.) and different metabolites extracted from their biomass have shown to influence the microbial activity and nutrient characteristics in the soil leading to the enhanced growth of plant and crop yield (Read and Perez-Moreno 2003; Haas et al. 2018; Win et al. 2018). These living microorganisms and metabolites are termed as "bio-fertilizers," and these could be used as one of the environmental-friendly alternatives of synthetic fertilizers. This chapter will only focus on the potential use of algae (both microalgae and cyanobacteria) as bio-fertilizer and the associated challenges.

Algae are a large group of photosynthetic eukaryotic (green microalgae, diatoms) and prokaryotic (cyanobacteria) microorganisms. It was estimated that approximately 30,000 species of algae exist in nature (Guiry 2012). Under favorable growth conditions, some of these strains could multiply their cell numbers several times a day. Although algal cells primarily comprise protein, lipid, and carbohydrate, a number of other secondary metabolites (pigments, growth hormones, vitamins, antimicrobial compounds, etc.) and micronutrients (Fe, Cu, Zn, etc.) are also found inside the cells; however, the content of each of these metabolites could vary among strains and cultivation conditions (Rizwan et al. 2018). Use of algae as bio-fertilizer has shown multiple advantages over synthetic fertilizer. Some of the cyanobacteria species can fix the atmospheric nitrogen within their cells (Singh et al. 2018). Most of the earlier studies were focused on the use of these cyanobacteria on the paddy field to make atmospheric nitrogen available to the plant (Ladha et al. 2016; Ma et al. 2019). However, in recent times, the biomass of other cyanobacteria and microalgae strains is also being investigated for improved soil quality and plant growth. The following sections will explore the potential applications of algal bio-fertilizers, indirect benefits of using algal fertilizer, and challenges and strategies of producing algal fertilizers.

3.2 The Potential Application of Algal Bio-fertilizers

3.2.1 Reduction in Synthetic Nitrogen Fertilizer

A number of cyanobacteria (e.g., *Anabaena*, *Tolypothrix*, *Nostoc*, etc.) can fix atmospheric nitrogen within their cells, mostly as heterocyst (Saikia and Bordoloi 1994; Fewer et al. 2002; Kumar et al. 2010); taking advantage of this phenomenon, such cyanobacteria are widely used in the paddy fields in many Asian countries like China, India, Vietnam, Japan, etc. (Lumpkin and Plucknett 1982; Saadatnia and Riahi 2009; Sahu et al. 2012). Fixed nitrogen from the heterocyst may get liberated as ammonia, free amino acids, vitamins, polypeptides, etc., in the surrounding environment by the microbial degradation of the dead cells which would make the nitrogen available to the higher plants; similarly, some cyanobacteria could secrete the biologically fixed nitrogen (Subramanian et al. 1994). It was estimated that these cyanobacteria could fix as much as 22.3–53.1 kg N/ha which might save 25–50% of chemical nitrogen fertilizer (Issa et al. 2014). Additionally, the application of cyanobacteria in the field provided similar crop yield and quality that were achieved by chemical fertilizer alone. Recent reports suggested that the application of these nitrogen-fixing cyanobacteria could also be extended to other crops and vegetables (Osman et al. 2010; Swarnalakshmi et al. 2013; Gheda and Ahmed 2015; Bidyarani et al. 2016).

3.2.2 Increase in Seed Germination Rate

To achieve desired growth and yield of crops, appropriate care must be taken in the seed germination stage to produce healthy seedlings. Both algal biomass and extracts of algal biomass were shown to increase the seed germination rate in addition to improved root and shoot development for the seedlings. As early as in 1967, the aqueous extract of *Phormidium foveolarum* (BGA) was found to have beneficial effects on rice seed germination; the hormones in the algal extract promoted the root and shoot of the seedlings (Shukla and Gupta 1967). Similarly, the extract of *Phormidium foveolarum* showed beneficial effects on the maize seed germination (Kushwaha and Gupta 1970). Both the inoculum of *Nostoc muscorum* and its extract were beneficial in increasing the seed germination rate for cotton, wheat, sorghum, maize, and lentil (Adam 1999; Ibraheem 2007). The application of *Chlorella* sp. suspension enhanced the germination rate of wheat, barley, and maize seeds (Uysal et al. 2015; Odgerel and Tserendulam 2017). Supercritical fluid extracts of *Spirulina* biomass were found to have a beneficial effect on the seed germination of cress and winter wheat (Dmytryk et al. 2014; Michalak et al. 2016). The application of *Acutodesmus dimorphus* biomass and its aqueous extract on the Roma tomato seeds allowed a 2-day faster seed germination compared to control experiment (Garcia-Gonzalez and Sommerfeld 2016). Garcia-Gonzalez and Sommerfeld further

noticed that microalgae treated seeds had greater lateral roots which could improve the ability of the plants in uptaking water and nutrients (Garcia-Gonzalez and Sommerfeld 2016). El Arroussi et al. (2016) studied the effect of *Dunaliella salina* hydrolysate on wheat seed germination in a saline soil; the exopolysaccharides had stimulated the seed germination and growth of seedlings (El Arroussi et al. 2016). Intracellular polysaccharides from two microalgae (i.e., *Dunaliella salina,* and *Phaeodactylum tricornutum*) were found to enhance the germination rate of bell pepper seeds in saline conditions (Guzman-Murillo et al. 2013) (Table 3.1).

3.2.3 Increase in Crop Yield

3.2.3.1 Enhancement of Soil Quality

Long-term usage of machines for tillage in preparing agriculture land alters soil structure and reduces organic matter in soil (Mikha and Rice 2004; Gupta Choudhury et al. 2014). The growth of algae on the soil will primarily increase the organic content of the soil by fixing the atmospheric carbon dioxide through photosynthesis. In addition, some cyanobacteria could fix the atmospheric inorganic nitrogen into organic nitrogen (Fay 1992; Bergman et al. 1997). Under specific growth conditions, some microalgae and cyanobacteria produce and secrete extracellular polymeric substances (or EPS) (Pereira et al. 2009; Barclay and Lewin 1985; Angelis et al. 2012; Delattre et al. 2016). EPS represents a group of high-molecular-weight biopolymers that are mostly comprised of monosaccharides; however, EPS could also comprise of noncarbohydrate compounds (e.g., proteins, lipids, nucleic acids, etc.) (Singh 2014). When the growth conditions are not favorable, algae produce these compounds to protect their cells from the stressed conditions (Chi et al. 2007; Delattre et al. 2016). Deposition of EPS in the soil is one of the mechanisms of increasing the soil organic content (Thomas and Dougill 2007). The organic compounds derived from the death and decay of the algal cells will also eventually increase the organic content of the soil (Han et al. 2014). Overall, the inoculation of algae on the soil could be an important source of organic carbon (Shields and Durrell 1964; Ibraheem 2007; Yilmaz and Sönmez 2017; Chamizo et al. 2018).

Maintaining soil aggregate is one of the essential parameters for soil fertility. Improvement of soil aggregation leads to an increase in water-holding capacity of the soil (Bailey et al. 1973; Lehmann et al. 2017). Algal EPS was also identified as a major component for soil stabilization (Burns and Davies 1986; Rossi et al. 2017). The growth of algae on the soil surface significantly increased the soil polysaccharides which improved the soil aggregation and soil structure while reducing soil erosion (Bailey et al. 1973; Roychoudhury et al. 1983; Rao and Burns 1990; Weiss et al. 2012). As *Nostoc* sp. was inoculated into poorly aggregate soil and saline-sodic soil, the filamentous cells and the secreted EPS together increased the aggregate stability of the soil which was attributed to the combined effect of coating,

Table 3.1 Application of various algal strains as bio-fertilizers

Algal strain	Mode of action as bio-fertilizer	Reference
Phormidium foveolarum	Promotes seed germination, enhanced root and shoot growth	Kushwaha and Gupta (1970), Shukla and Gupta (1967)
Nostoc muscorum	Enhanced seed germination rate in cotton, maize, wheat, lentils	Adam (1999)
Chlorella	Enhanced germination rate of wheat, maize, barley	Odgerel and Tserendulam (2017), Uysal et al. (2015)
Spirulina	Promoted seed germination in cress and winter wheat	Michalak et al. (2016)
Acutodesmus dimorphus	Faster seed germination in Roma tomato	Garcia-Gonzalez and Sommerfeld (2016)
Dunaliella salina	Promoted seed germination in wheat	El Arroussi et al. (2016)
Dunaliella salina, and *Phaeodactylum tricornutum*	Enhanced germination rate in bell pepper seeds	Guzman-Murillo et al. (2013)
Nostoc	Improved stability and mineral content of saline soil	Malam Issa et al. (2007), Maqubela et al. (2009), Weiss et al. (2012)
Botryococcus, *Chlamydomonas*, and *Chlorella*	Improved soil stability	Chi et al. (2007), Fay (1992), Weiss et al. (2012)
Chroococcidiopsis and *Anabaena*	Enhanced shoot length, spike length, lateral root, grain weight in wheat plant	Hussain and Hasnain (2011)
Scenedesmus obliquus	Increased growth rate in *Rhizobium japonicum*	Fingerhut et al. (1984)
Haematococcus pluvialis	Increased root growth and secondary metabolite in *Beta vulgaris* and *Tagetes patula*	Rao et al. (2001)
Spirulina platensis	Enhanced secondary metabolite production in *Beta vulgaris*	Rao et al. (2001)
Calothrix elenkinii	Improved microbial community in roots of rice plants	Natarajan et al. (2012)
Chlorella vulgaris	Biocidal effect and promoted lettuce yield	Faheed and Fattah (2008)
Spirulina platensis	Increased pepper and beet yields	Dias et al. (2016)
Spirulina	Improved postharvest shelf life of eggplant	Dias et al. (2016)
Chlorella and *Spirulina*	Increased potato, pea, and wheat yield and quality	Ronga et al. (2019)
Scenedesmus dimorphis	Increased plant and flower growth in tomato	Sommerfeld (2014)
Dunaliella salina	Improved germination and seed growth in wheat plants	El Arroussi et al. (2016)
Chlorella, *Scenedesmus*, and *Spirulina platensis*	Improved growth in leafy vegetables, wheat, and tomato	Das et al. (2018c), Renuka et al. (2017), Wuang et al. (2016)
Chlorococcum humicolum	Inhibited growth of *Botrytis cinerea* in strawberry and *Erysiphe polygoni* in tomato, turnips, and saprophytes	Kulik (1995)

enmeshment, binding, and gluing of aggregates and minerals (de Caire et al. 1997; Malam Issa et al. 2007; Maqubela et al. 2009). It was further demonstrated that algal EPS could fortify the soil porosity and increase the penetration resistance of soil by reducing the damaging impact of water addition (Falchini et al. 1996; Chamizo et al. 2018). Even inoculation of green microalgae (e.g., *Botryococcus*, *Chlamydomonas*, *Chlorella*, etc.) on the field improved the soil stability by increasing the EPS content of the uppermost strata (Barclay and Lewin 1985; Weiss et al. 2012; Yilmaz and Sönmez 2017). Algal crust formation phenomenon could be utilized as an alternative ecological option in combating desertification in arid, semi-arid, and dry subhumid areas (Park et al. 2017). As the algae increase the organic matter in the soil, these compounds could act as carbon and energy source for heterotrophic microorganism community in the soil. Studies have shown that inoculation of alga increased the total microbial community in the soil column (Padmaperuma et al. 2018).

Typically, gypsum is added in the soil to improve the water permeability or hydraulic conductivity in the soil when electrolyte concentrations in the soil get reduced (Oster 1982). Soil cyanobacteria, often, together with indigenous bacteria, forms micro-networks using filaments and EPS; this lead to improved soil structures with increased porosity and water permeability (Chamizo et al. 2012; Sadeghi et al. 2017). It was reported that the addition of 10 kg/ha blue-green algae in the alkaline soil could reduce gypsum addition as much as 1 ton/ha (Kaushik and Krishna Murti 1981). EPS in the soil could also play an important role in the retention of moisture (Chamizo et al. 2013).

Phosphorus is the second most important element, after nitrogen, for the plant and even algae growth. The average phosphorus content in the soil is approximately 0.05%; unfortunately, only a small fraction (approximately 0.1%) of this phosphorus is available for plant uptake (Zhu et al. 2011). However, there are several soil microorganisms (e.g., fungi, bacteria, cyanobacteria) which showed the ability to solubilize inorganic phosphorus and mineralize insoluble organic phosphorus, thereby making phosphorus available for plant uptake (Cameron and Julian 1988; Yandigeri et al. 2011; Long et al. 2018). Similarly, as the iron concentration becomes limiting, some cyanobacteria and green algae could produce and release low-molecular-weight iron-specific chelators, also known as siderophores, which make iron available to microbes and plants (Wilhelm and Trick 1994; Benderliev 1999). Apart from iron, algae are also known to enrich other microelements (e.g., Cu, Mn, Zn, Co, etc.) in plant parts (Lange 1976; Das et al. 1991).

3.2.3.2 Source of Phytohormones

In different groups of microalgae and cyanobacteria, all the eight different phytohormones (e.g., auxins, cytokinins, abscisic acid, gibberellins, jasmonic acid, salicylic acid, ethylene, and brassinosteroids) were found (Lu and Xu 2015; Romanenko et al. 2015). Some of the algae strains produce these hormones as intracellular metabolites, while the others secrete these hormones directly in the surrounding environment (Abdel-Raouf 2012). These phytohormones could serve as

growth-promoting substances in agriculture or lead to activation of certain cascades in plant metabolism that eventually lead to improved plant growth and crop quality (Zhao et al. 2005). These phytohormones could also improve plant tolerance in various biotic and abiotic stress conditions (Maršálek et al. 1992). Rice plants inoculated with cyanobacterial strains showed the presence of indole acetic acid and indole butyric acid (Li et al. 2018).

Phytohormones like auxins and cytokinin, from *Chroococcidiopsis* sp. and *Anabaena* sp., significantly enhanced shoot length, spike length, lateral root, and grain weight of inoculating wheat plants (Hussain and Hasnain 2011). Hormones produced by cyanobacteria and microalgae could act as elicitors. Some cyanobacteria, in a symbiotic relationship with host plants, release arabinogalactan proteins that play a vital role in regulating overall plant growth and development (Bergman et al. 1996; Singh 2014). Cyanobacterial extracts and the inoculation of cyanobacterial species on rice fields were found to produce root-accelerating hormone known as gibberellic acid (Dong et al. 2016). Bioactive compounds released by cyanobacteria could increase the phytohormonal level in plants that regulate enzymatic activities and metabolism of plants (Han et al. 2018). Phytohormones are also known to promote plant-microbe interactions, thereby indirectly enhancing root colonization by other microbial communities (Di et al. 2016). The extract of *Scenedesmus obliquus* increased the growth of slow-growing *Rhizobium japonicum* (Fingerhut et al. 1984). Pea plants inoculated with cyanobacteria were found to have increased protein content in pea due to certain induced metabolic processes caused by the presence of gibberellins (Osman et al. 2010). The application of *Haematococcus pluvialis* biomass extracts in the cultivation of *Beta vulgaris* and *Tagetes patula* led to an increase in their hairy roots and accumulation of desired secondary metabolite (betalains and thiophenes); however, the extract of *Spirulina platensis* was only effective for *Beta vulgaris* (Rao et al. 2001). Similarly, the extracts of algae had shown beneficial effects on somatic embryogenesis of *Daucus carota* and pigment production in *Carthamus tinctorius* (Wake et al. 1991; Hanagata et al. 1994).

3.2.3.3 Plant Tissue Colonization

Cyanobacteria and microalgae and some other microorganisms have been known to colonize various parts of plant and areas surrounding their roots, i.e., rhizosphere (Uzoh and Babalola 2018). Sometimes, the extent of colonization is such that plant genes are lesser than the total microbial genes present in rhizosphere (Mendes et al. 2013). Cyanobacteria and microalgal colonization was found to have a profound effect on seed germination, plant growth and productivity, disease control, etc. (García-Salamanca et al. 2013; Yang et al. 2017). Plants rely on various microorganisms to perform certain vital and specific functions. Plants tend to deposit their organically fixed carbon into the surrounding rhizosphere, thereby feeding the surrounding microorganisms; so it plays an important role as a symbiotic partner (Adams et al. 2013).

There are multiple mechanisms by which cyanobacteria and microalgae colonize vascular and non-vascular parts of plants and rhizosphere zone. Certain cyanobacteria gain entry into plant tissues via stomatal openings and subsequently invade intercellular spaces, stomatal zones, and parenchymal cells (Li et al. 2014; Garcia-Gonzalez and Sommerfeld 2016). Certain cyanobacteria could also colonize in the epidermis and cortical cells of wheat crop roots (El-Zemrany 2017). A cyanobacterium, *Calothrix elenkinii*, was found to colonize the root and shoot tissues of rice plant and improved the microbial activity in the colonized parts (Natarajan et al. 2012). Similarly, certain cyanobacteria species could colonize chickpea plant roots, in nodule forms, and improve rhizospheric microbial flora which led to improved soil fertility and increased plant yields (Bidyarani et al. 2016; Prasanna et al. 2017).

As the cyanobacteria and microalgae colonize plants, they release certain elicitor metabolites such as peptides, vitamins, phytohormones, and polysaccharides; these elicitor compounds lead to certain signal transductions and gene responses that lead to phytochemical changes in plant (Singh 2014). These phytochemical changes in plant leads to production of increased ascorbic acid, anthocyanins, phenolic compounds in mangoes and apples; increased flavonoid compounds in strawberries; increased sterols in potatoes; increased beta-carotene and lycopene contents in tomatoes; high levels of limonene, terpene, and caryophyllene in carrots; and capsaicin and anthocyanin in capsicum (Kulik 1995; Rudell et al. 2002; Pandhair and Gosal 2009).

3.2.4 Improving the Quality of Fruits and Vegetables

Microalgal- and cyanobacterial-based bio-fertilizers can improve plant yield and quality of certain vegetable and food crops. Generally, the techniques used to inoculate bio-fertilizers on fruits and vegetable crops are in the form of a foliar spray or dry powder (Latha et al. 2013; Nagy and Pintér 2014). There was an increase in lettuce yield when *Chlorella vulgaris* dry powder was applied on soil with lettuce; while some compounds of the biomass protected the plant against pathogens, some other micronutrients and growth hormones increased the lettuce yield (Faheed and Fattah 2008). Foliar applications of *Spirulina platensis* on beet and pepper crops resulted in increased yields; these were found to be at par when compared with beet and pepper crop yields obtained using commercial NPK fertilizers (Dias et al. 2016). *Spirulina*-based bio-fertilizers have been found to increase the postharvest quality of eggplant; the pulp firmness of the eggplant was enhanced for a longer period of time even at increased temperature conditions, thereby allowing an extended postharvest shelf life of eggplants (Dias et al. 2016). A foliar mixture containing *Chlorella* sp. and *Spirulina* sp., enriched with nitrogen, phosphorous, magnesium, zinc, and potassium, increased potato, pea, and wheat yield and quality (Ronga et al. 2019). When *Spirulina* sp. was applied directly to the soil with sunflower, chili, soybean, green gram, and groundnut, there were positive

effects on plant growth and product yield which were attributed to the *Spirulina* sp. growth hormone (i.e., cytokinin) (Michalak et al. 2016).

Application of whole-cell microalgae biomass as a bio-fertilizer for fruits and vegetables production had beneficial effects on faster seed germination rate, improved crop quality, and reduced time in crop maturity. A number of tomato and organic fruit producers spray *Chlorella* sp. live microalgae suspension which allows the delivery of complex polysaccharide compounds and microelements directly through plant stoma leading to improved aromatic and natural smell (Ronga et al. 2019). Various types of algal extracts are commercially available, which could improve the fruit yield and quality (El-Sharony et al. 2015).

3.2.5 Reclamation of Degraded Land

Alkalinity and salinity both influence the fertility of the soil. In general, the alkaline soil has high pH, high potential for exchange of sodium ions, low carbonates, poor hydraulic conductivity, and low aeration. On the contrary, the saline soil has high salt content which reduces the water- and nutrient-absorbing capacity of the plant roots from the soil. All these factors make both alkaline and saline soils highly infertile. Conventional practices use sulfur, gypsum, and excessive irrigation to improve the condition of these degraded lands (Day et al. 2018). However, these methods are either expensive or not environmentally friendly (Seenivasan et al. 2016).

Research and some field applications have shown that algae could be a solution to reclaiming degraded lands. Cyanobacteria and certain microalgae species could thrive in highly alkaline and saline soils where these organisms form a thick layer in soil using EPS; retain N, P, and organic carbon; and improve permeability, aeration, hydraulic conductivity, electrical conductivity, and osmoregulation; all these factors make algae potential candidates for reclamation of lands affected by high alkalinity and salinity (Rai 2015). However, entrapment of sodium ions by the algal EPS could be a temporary solution, and these ions will be released back to the surrounding environment after the death and decay of the algal cells (Cuddy et al. 2013). Some of the algae strains, in a symbiotic relationship with bacteria, could degrade the oil and petroleum compounds (Abed 2010; Das et al. 2018a). Therefore, such algal strains have the potential to remediate the oil-contaminated site while providing other benefits as bio-fertilizer (Suresh Kumar et al. 2015; Xiao and Zheng 2016; Srivastava et al. 2018).

Algae are extremely efficient in the removal of heavy metals from the contaminated water through cellular uptake and adsorption (Wilde and Benemann 1993; Mehta and Gaur 2005). Similarly, in metal contaminated sites, algae were efficient in reducing the heavy metal uptake by the plants (Allard and Casadevall 1990; Bender et al. 1995; Chen et al. 2015). Both microalgae and cyanobacteria are also known to produce exopolysaccharides that could bind the soil together, increase the soil organic content, and improve the moisture absorption capacity of the desert soil.

In recent times, several studies have shown that use of microalgae, cyanobacteria, and even consortia of microalgae and bacteria on the desert soil restored and stabilized the soil and improved the seed germination and plant growth (Trejo et al. 2012; Xu et al. 2013; Park et al. 2017; Chamizo et al. 2018; Mugnai et al. 2018).

3.3 Method of Algal Bio-fertilizer Application

Proper application of microalgal bio-fertilizer is a crucial step for maximizing the benefits. In the past, the most common use of algal bio-fertilizer was the inoculation of live culture in the field. However, with time, more advanced techniques of algal bio-fertilizer applications (e.g., spraying of specific algal extract, carrier-based inoculation, biofilms, and consortia) were developed. The mode of algal bio-fertilizer application will mostly depend on the plant type and soil condition.

3.3.1 Inoculation of Live Cultures

Live algae could be inoculated in the field either as a monoculture or as polycultures of multiple organisms including algae, bacteria, yeast, etc. Application of live cultures is advantageous as the live cells multiply on the field which doesn't require separate algal cultivation process. A vast majority of earlier works studied the effect of monoculture on the soil quality and plant growth parameters (Priya et al. 2015; Uysal et al. 2015; Odgerel and Tserendulam 2017). The ability of algae to fix atmospheric nitrogen and produce and secrete plant growth-promoting substances, pest control, etc. will vary among strains. Therefore, some studies used algal consortia in the field to gain multiple benefits which otherwise couldn't be achieved using monocultures (Osman et al. 2010; Babu et al. 2015; Xue et al. 2017; Chittapun et al. 2018). Further, the application of algae-bacteria consortia was also effective in soil improvement and plant growth (Manjunath et al. 2011, 2016; Subashchandrabose et al. 2011; Rana et al. 2015). Live cultures of algae and algal consortia could also be applied on the field with the help of a carrier medium such as animal waste, paddy or wheat straw, compost materials, fly ash, etc. While these carrier materials have beneficial effects on the soil, there are other contaminants of concerns in these materials such as heavy metals, pathogens, pharmaceutical compounds, etc. One of the roles of the algae in the carrier medium was to control these contaminants. Several recent studies revealed that algae were effective in reducing the metal availability to plant, degrading pharmaceutical compounds, and reducing pathogens of the carrier materials (Rai et al. 2000; Tripathi et al. 2008; Young et al. 2016; Yu et al. 2017; Pan and Chu 2017; Kaur and Goyal 2018). The application of algal biofilm on the field is another emerging method of bio-fertilization; in the biofilm, algae serve as the matrix, and other micro-organisms (e.g., bacteria, and fungi) are selected to cater specific functions (Prasanna et al. 2011; Bidyarani et al. 2016; Kanchan et al. 2018).

3.3.2 Spraying of Algal Extract

Although some of the algae release plant growth-promoting substances in the surrounding environment, extracting these beneficial compounds from other algae would require additional processes. Therefore, these strains are grown separately, and specific metabolites are extracted from the harvested biomass. Spraying *Scenedesmus dimorphus* microalgal extracts (on tomato plants) showed increased plant growth, higher photosynthetic efficiency, and enhanced flower growth (Sommerfeld 2014). *Dunaliella salina* extracts improved germination and seed growth in wheat plants (El Arroussi et al. 2016). Furthermore, it was shown that spraying algal extracts on the leaves of plants tend to improve water utilization potential of plants (Shukla 1967).

3.4 Indirect Benefits of Using Algal Bio-fertilizers

3.4.1 Reduction in Greenhouse Gas Emission

It was estimated that approximately 50% of microalgal biomass is comprised of carbon, and production of 1 kg microalgae would require 1.73 kg of CO_2 (Jiang et al. 2013; Verma and Srivastava 2018). Therefore, large-scale microalgae cultivation to produce bio-fertilizer would indirectly act as long-term carbon sequestration (Upendar et al. 2018). However, it must be noted that depending on the cultivation and harvesting methods, production of microalgae biomass could be very energy intensive and thereby diminish the advantages of greenhouse gas (GHG) reduction (Medeiros et al. 2015). The nitrogen content in microalgae could vary between 2 and 10%, whereas it is 44% in urea (Markou et al. 2014). Unlike synthetic fertilizer, microalgae biomass could act as slow-release bio-fertilizer, and therefore the required amount of biomass would be lesser than the synthetic fertilizer. While some of the microalgae and cyanobacteria require synthetic nitrogen fertilizers, some cyanobacteria could fix atmospheric nitrogen, and cultivation of such cyanobacteria could provide additional GHG reduction potential. Production, packaging, transportation, and application of typical synthetic fertilizers consume a lot of energy and thereby contribute to 47.7% GHG emission related to crop production (Hillier et al. 2009; Wang et al. 2017). Therefore, live algal culture inoculation in the field could substantially reduce the GHG emission.

3.4.2 Biocidal Applications

The application of synthetic chemicals to control insects, pest, fungi, and bacteria in the field is associated with adverse environmental effects and human health; therefore, there is a growing demand of bio-based alternative products. Algae and cyanobacteria were proposed as promising and safe biocide agents (Nassar et al. 1999; Schrader et al. 2002; Gol'din 2012). Some species of cyanobacteria have the ability to produce certain compounds that show antifungal, insecticidal, nematocidal, cytotoxicity, and herbicidal properties (Biondi et al. 2004). Amides, indoles, lipopeptides, and fatty acids are some of these bioactive compounds that could kill or suppress various unwanted microorganisms and microflora/fauna. These bioactive compounds inhibit physiological and metabolic activities in the targeted pathogens. For example, studies indicate that cyanobacterial extracts of *Chlorococcum humicolum* have inhibited the growth of pathogens like *Botrytis cinerea* in strawberry and *Erysiphe polygoni* in tomato seedlings, turnips, and saprophytes (Kulik 1995). Several cyanobacteria, isolated from paddy field, were effective in preventing fungal growth in soil (Kim 2006); similarly, cyanobacterial strains could also prevent fungal growth in vegetables and flowers (Manjunath et al. 2010; Prasanna et al. 2013). A study by Victor and Reuben (2000) showed that the inoculation of cyanobacteria in the rice field could reduce the mosquito number (Victor and Reuben 2000). Extract from cyanobacteria also showed mosquito larvicidal activity (Singh et al. 2003). Certain cyanobacterial formulations were effective in preventing root rot disease in cotton and improving the rhizosphere (Babu et al. 2015). Microalgae possess antibiotic properties; algal extracts containing tochopherols, polyphenols, pigments, and oils also demonstrated antimicrobial properties (Dewi et al. 2018).

Extracts from microalgae and cyanobacteria increase plant immunity by enhancing plant defense enzyme activities (Florin Oancea et al. 2013). Inoculation of algae and application of dry algae powder were found to effectively reduce the gall formation and nematode infestation (Paracer 1987; Hamouda and El-Ansary 2017). Extracts of cyanobacterial toxins were effective in combating leaf-roller larvae and moth (Sathiyamoorthy and Shanmugasundaram 1996; Jimenez et al. 2009). In addition to exhibiting biocidal properties, some cyanobacteria were able to degrade organophosphorus pesticides and other chlorinated organic (Subramanian et al. 1994; Kuritz 1998; Ibrahim et al. 2014). A major problem for organic grapevine growers is the infestation of their crops with fungi; copper-based pesticides are commonly used to prevent fungi growth. However, there is a drawback in using copper-based antifungal agents as these tend to accumulate in soil and kill other beneficial microorganisms present in the soil (Michaud et al. 2008; Hussain et al. 2009). Recent studies showed that microalgal extract had a beneficial effect in inhibiting fungal growth (e.g., mildew, botrytis, ectoparasites, etc.) while enhancing the plant growth, thereby making it a substitute for conventional copper-based antifungal agent (Bileva 2013; ProEcoWine 2018).

3.5 Challenges in Developing Algae-Based Bio-fertilizer

Despite the immense potential of algae biomass as bio-fertilizer, there are still some challenges that must be addressed for wider application of algal bio-fertilizer. There are some algae strains, especially cyanobacteria and diatoms, which could produce various types of toxins (e.g., cyanotoxins) under specific environmental conditions which could be toxic to humans, animals, soil microbes, and plants (Katırcıoğlu et al. 2004). Even worse, there is evidence that these cyanotoxins could be accumulated in the food crops (Corbel et al. 2014). Therefore, before applying any algal strain on the field, it is critical to evaluate its toxicity potential. Another major drawback is that when live algal cultures are inoculated in the soil, these could be consumed by grazers such as helminths, protozoa, small crustaceans, etc. To tackle this situation, a combination of plant extract from neem or tobacco could be used as a carrier for microalgal and cyanobacterial fertilizers (Jha and Prasad 2005).

The production of algae biomass in a cost and energy efficient way is very crucial. Unlike the nitrogen-fixing cyanobacteria, which are inoculated on the field, other microalgae and cyanobacteria must be produced separately which would require additional land, water, nutrients, and energy (Markou et al. 2014). Fortunately, algae can be grown in non-fertile marginal land using saline, brackish, and wastewater (Das et al. 2016, 2018a). Furthermore, algae are extremely efficient in utilizing the supplied nutrients, and any leftover nutrients in the algae culture media could be recycled back in the next batch of cultivation. Harvesting of microalgae still remains a major obstacle for producing microalgae-based low-cost products (Barros et al. 2015). There are few microalgae and cyanobacteria which form flocs and precipitate spontaneously in the absence of mixing and thus eliminate the need of energy-intensive preliminary biomass harvesting (Das et al. 2018b). For the other microalgae and cyanobacteria, appropriate harvesting methods should be developed so that the biomass doesn't get contaminated with unwanted compounds and the quality of the biomass remains intact. While some cyanobacteria were found to lock the sodium in the soil in reducing the soil salinity, repetitive use of the marine algae biomass could increase the salinity content of the soil.

To overcome the cost of the algal bio-fertilizer, the algal biorefinery approach could be very beneficial. Algae are known to produce a range of high-value primary and secondary metabolites which include polyunsaturated fatty acids (PUFA), phycobiliproteins, and carotenoids (beta-carotene, lutein, astaxanthin, etc.). Upon extraction of these metabolites, the leftover biomass still could be used as bio-fertilizer, as shown in Fig. 3.1.

Hydrothermal liquefaction (HTL) is considered as a promising technology for producing biocrude oil from algal biomass (Biller and Ross 2011); as a byproduct of the process, solid biochar can be obtained which could also potentially be used as bio-fertilizer. Lipid-extracted biomass could also serve as a bio-fertilizer leading to increased crop yields like maize (Maurya et al. 2016). The left-over material of anaerobically digested algal biomass, still rich in nitrogen and other nutrients, could be used in soil improvement (Solé-Bundó et al. 2017). Cultivation of microalgae in

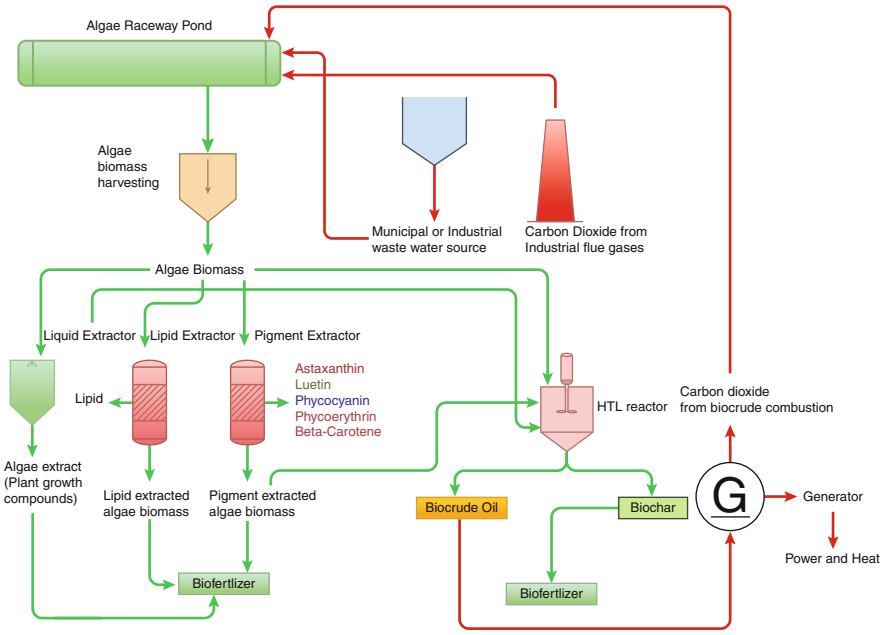

Fig. 3.1 Integrated algae biorefinery for bio-fertilizer production

different wastewaters, including municipal and industrial wastewaters, could concentrate various nutrients (e.g., N, P, trace metals, etc.) within the biomass; the produced biomass in the wastewater could be a cheap source of bio-fertilizer. Wastewater grown microalgae biomass (e.g., *Chlorella* sp., *Scenedesmus* sp., *Spirulina* platensis) was found to improve the growth of different plants (wheat, leafy vegetables, tomato, etc.) (Wuang et al. 2016; Renuka et al. 2017; Das et al. 2018c).

3.6 Conclusion

Despite some challenges, microalgae and cyanobacteria have shown tremendous potential as bio-fertilizer, plant growth promoter, and even as biocides. While live cells of algae are the used to take advantage of their ability to fix atmospheric carbon dioxide and nitrogen, wastewater-grown algal biomass could be another source of bio-fertilizer. From the biorefinery perspective, the effects of algal extracts on seed germination, plant growth, crop quality, and plant defense are very promising. Therefore, it can be expected that both the research and application of algal bio-fertilizer will broaden in the coming years.

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Chapter 4
Ectomycorrhizal Fungi: Role as Biofertilizers in Forestry

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Abstract Ectomycorrhizal fungi (ECMF) play a fundamental role in the nutrient cycle in terrestrial ecosystems, especially in forest ecosystems. In this chapter, the value of ECMF species is reviewed from a global framework, not only to increase the production of edible fruit bodies and biomass of plants but also for the regular practices of reforestation and restoration of ecosystems, with implicit applications in biofertilization, bioremediation, and control of soil pathogens. The valuation of the ECMF in forest management must be considered fundamental for innovation and sustainable development. Ecological functions and bioactive compounds of the ECMF of interest to mankind are briefly reviewed. The direct implications of the ECMFs in forestry are described. To do so, its role as a biotechnological tool in forest nursery production is briefly analyzed, as well as the role of MHB bacteria (mycorrhizal helper bacteria). Subsequently, the direct role as biofertilizers of the ECMF in forest management is discussed: reforestation, plantation management, and ecosystem restoration.

Keywords Nutrient cycle · Ecosystem restoration · Reforestation · Sustainable development

4.1 Introduction

Certain groups of fungi establish a symbiotic relationship with the roots of plants, called mycorrhizae. Frank established two large subdivisions of mycorrhizae, ecto- and endomycorrhizae (Smith and Read 2008). Ectomycorrhizal fungi (ECMF) form

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mantle and Hartig network of intercellular hyphae in the roots of forest species. The arbuscular mycorrhizal fungi (AM) form arbuscules, vesicles that are more variable than that of the ECMF, since it forms a symbiosis with trees and herbaceous plants. Endomycorrhizae are classified as arbuscular mycorrhizae, ericoid mycorrhizae, arbutoid mycorrhizae, monotropoid mycorrhizae, ectendomycorrhizae, or orchid mycorrhizae. Each of these categories is characterized by the invasion of plant root cells by fungal hyphae, but differs in the nature of intracellular hyphal development (Peterson et al. 2004; Sharma 2017).

Ectomycorrhizal fungi are predominantly *Basidiomycetes* and some *Ascomycetes*. In these symbiotic structures, the Hartig network is the interface for the metabolic exchange between the fungus and the root. The mycorrhizal mantle is connected to the filaments of fungi that extend into the soil (extraradical mycelium), directly involved in the mobilization, absorption, and translocation of soil nutrients and water to the roots (Suz et al. 2012). More than 7000 species of fungi form ectomycorrhizae (Rinaldi et al. 2008), many of them with important commercial trees such as poplar, birch, oak, pine, and spruce (Wiensczyk et al. 2002). The reproductive structures (fruiting bodies) of the macromycetes are known as mushrooms when they grow in the soil and, like truffles, when they grow underground.

The community of mycorrhizal fungi can be determinant in the structure of the plant community (Fitter 2005), therefore, the identification of the mycobiont partner and its functional structure (Agerer 2001) are fundamental to understand the ecological importance of this symbiotic relationship. ECMF diversity studies were initially based on studies of fruiting bodies and, more recently, on the direct identification of ectomycorrhizae (Horton and Bruns 2001).

Most of the cultivated species of edible fungi are saprophytes, and only some of them are ECMF (Savoie and Largeteau 2011). The tickets (*Boletus edulis*), Chanterelles (*Cantharellus* spp.), the matsutake mushroom (*Tricholoma matsutake*), and the truffle (many species of the *Tuber* genus) are some ECM fungi for which the crop has been studied (e.g., Chang and Hayes 1978; Chevalier 1998; Bencivenga 1998). The black truffle or Périgord, *Tuber melanosporum*, is widely grown, while other species of ECM mushrooms have not yet been cultivated, including fungi porcini (*Boletus edulis* S.) and the high-priced Italian fungus, white truffles (*T. magnatum*).

4.2 Evaluating ECMF

Forest ecosystems and mycelial networks of ectomycorrhizal fungi play an important role in biogeochemical cycles, biodiversity, climatic stability, and economic growth (Smith and Read 2008). Ectomycorrhizal fungi not only promote the growth and health of host plants but also form vast metabolic networks that may be of critical value to ecosystem functions (Leake et al. 2004; Courty et al. 2010).

Ectomycorrhizal fungi are also important drivers for sustainable innovation in different fields of research (Azul et al. 2014), such as the food industry,

biotechnology, biomedicine, and agroforestry (Donnini et al. 2013). These are desirable areas of innovation, given the threats to native forests around the world from poor management, soil degradation, pollution, water scarcity, fire, and the spread of invasive species and diseases (FAO 2010). The relationships between the various native edible ECM fungi have been, until relatively recently, insufficiently considered in the strategies of forest management (Dahlberg et al. 2010), and the role of ECMF has been underestimated in bio-industrial innovation. Some authors have presented several examples of representative models of the valuation of the ECMF from a holistic conception (Suz et al. 2012; Azul et al. 2014).

Some of the intrinsic values of the ECMF to human activity are the food (gastronomy, local, and international markets); the value of the landscape; the popular culture; the ecological tourism, as indicators of environmental quality; and the multifunctionality.

So far, different bioactive compounds have been identified from ECM fungi with different biological activities, applications, or properties: low molecular weight organic compounds, which may be used in the food industry to mimic mushroom flavors (Mizuno and Kwai 1992), which may have anticancer properties (Wang et al. 2003) or antioxidant activity (Reis et al. 2011); polysaccharides, which may be included in diabetic diets or to present immunosuppressive and anticancer activity (Hu et al. 1994); fatty acids and other lipids, which may have antioxidant, anti-inflammatory, anticancer (Reis et al. 2011), or immunosuppressive activity (Kreisel et al. 1990); enzymes, which may have application in the paper industry, textile industry, and detergent production (Campbell and Bedford 1992); or enzymes which may have application in environment-contaminant degradation (Pointing and Vrijmoed 2000), paint decoloration (Casieri et al. 2010), food industry (Gupta et al. 2003), cosmetic industry (Liese et al. 2000), etc.; terpenoids, with anticancer activity; and, finally, phenolic compounds, which define organoleptic properties fungi (Ribeiro et al. 2006).

4.3 Ecological Functions of ECMF

Some of the traditionally known functions of the ECMF in the ecosystem are:

- Increase in the water and nutrient supply, extending the volume of land accessible to the plants. Different fungal species (drought-sensitive hydrophilic or drought-tolerant hydrophobic) can have different effects on hydraulic redistribution patterns (Prieto et al. 2016).
- Increase in the plant's nutrient supply, assimilating nutrients in the ways that would not normally be available to plants.
- The mechanisms of improvement in the absorption of P would be: extension of extramatrical hyphae and Pi transfer (inorganic), Pi transporters in the fungus/soil interface; mobilization of organic P (labile), emission of phosphatases; and

mobilization of insoluble mineral Pi, emission of low molecular weight organic acids.
- The mechanisms of improvement in N nitrogen absorption would be intervention in the mineral N cycle ($NH4^+$, $NO3^-$) and assimilation of organic N (emitting proteases, chitinases, others).
- Colonization of the root by ECMF can provide protection against soil pathogens.
- The non-nutritive benefits to plants due to changes in water relations, the level of phytohormones, the assimilation of carbon, etc., have already been verified.
- Carbon is transferred through the fungal mycelium of ECMF that connects different species of plants. This can reduce competition among plants and contribute to the stability and diversity of ecosystems.
- Epigeous and hypogeal sporocarps of ECMF are important food sources for placental and marsupial mammals. The mycorrhizal roots, the mycelium, and the fruiting bodies of the fungi are important as food sources and habitats for invertebrates.
- Mycorrhizae influence the microbial populations of the soil and the exudates in the mycorrhizosphere and hyphosphere.
- The hyphal network produced by ECM fungi significantly alters and improves the structure of the soil.
- Mycorrhizal fungi contribute to the storage of carbon in the soil by altering the quantity and quality of organic matter in the soil.
- Enhancing plant tolerance to (biotic and abiotic) stresses.

Recent advances in the knowledge of nutrient translocation processes in the fungus-plant and fungus-soil interaction are especially interesting, in particular, the priority role of transporters of P, N, and C (Bonfante and Genre 2010). The inorganic P and mineral or organic forms of N, such as $NH4^+$, $NO3^-$, and amino acids (AA), are absorbed by specialized transporters located in the fungal membrane in the extraradical mycelium. NH^{3+}/NH^{4+} and inorganic P (from polyphosphates) are imported from the symbiotic interface to the cells of the plant through selective transporters. Transporters of hexoses import carbon of plant origin into the fungus, while the transporter proteins that participate in the export of nutrients from the plant or the fungus have not yet been identified. The nutritional strategies seem to be different between symbiotic and pathogenic fungi, for example, in the translocation of C. Even different transport strategies have been found between ECMF symbionts belong to Ascomycota and Basidiomycota. The understanding of the different systems of transporters or nutrient channels involved both at the level of the extraradical mycelium and at the level of the symbiotic interface will clarify in the future the processes of nutrition in the plant-fungus and fungus-soil interaction.

4.4 ECMF Genomic Studies

So far, genome sequencing of two ECMF (ectomycorrhizae), the *Laccaria bicolor* and *Tuber melanosporum* (black truffle), helps in the identification of factors that regulate the development of mycorrhiza and its function in the plant cell (Bonfante

and Genre 2010). The study of symbiotic and transcriptomic genomes will provide in the future, among others, the following lines of knowledge:

- A better understanding of the mesocosm of the tree (i.e., the interactions of the host plant with its courtship of endophytes, symbiotics, and pathogenic microorganisms).
- A basis for the study of the crosstalk of encoded proteins between symbiotic partners that involve mycorrhizal effectors.
- A molecular definition of the mechanisms that lead to the initiation of the carpophore and its development.
- The metabolic pathways that control the transport and assimilation of nutrients in the symbiosis and in the body of fructification.
- Bioinformatic exploration of important symbiotic gene networks and major transcriptional factors—the mycorrhizal genetic landscape.
- Comparative transcriptomics with other economically important saprobionts, and with pathogenic fungi (Martin and Bonito 2012).

4.5 ECMF Selection Criteria for Sustainable Development

Some of the most common criteria considered for the selection of a most valued species or strain of ECMF (some of them implicit in others) are the abiotic criteria like climatic conditions, such as temperature, insolation, and humidity and improvement of soil properties, such as texture and permeability, abiotic soil stress mitigation, soil contamination mitigation, soil metal mobilization, or nutrient cycling. There may also be criteria regarding the host, such as the plant/fungus specificity, the improvement of plant health, or the increase in the biomass of the plant. The criteria regarding the fungus include abundance, effectiveness, propagules' competitiveness, fungus growth rate, or edibility. The other criteria may be the conservation of native biodiversity, the functioning of the ecosystem, human health, food, nutraceutical value, etc. (Suz et al. 2012; Azul et al. 2014).

4.6 Applications: ECMF and Forestry

Since the late 1950s, mycorrhizal fungi were utilized as biofertilizers to promote plant growth, because of their ability to increase the plant uptake of P, N, mineral nutrients, and water (Feldmann et al. 2009; Koide and Mosse 2004; Miransari 2011). Much of our understanding of the functions of ECMF has come from research directed toward practical application in forestry (Fig. 4.1). Although successful inoculation of tree seedlings (already planted) in field has been known, nursery inoculation is more common. Seedlings inoculated in nursery can establish a healthy ECMF system before outplanting.

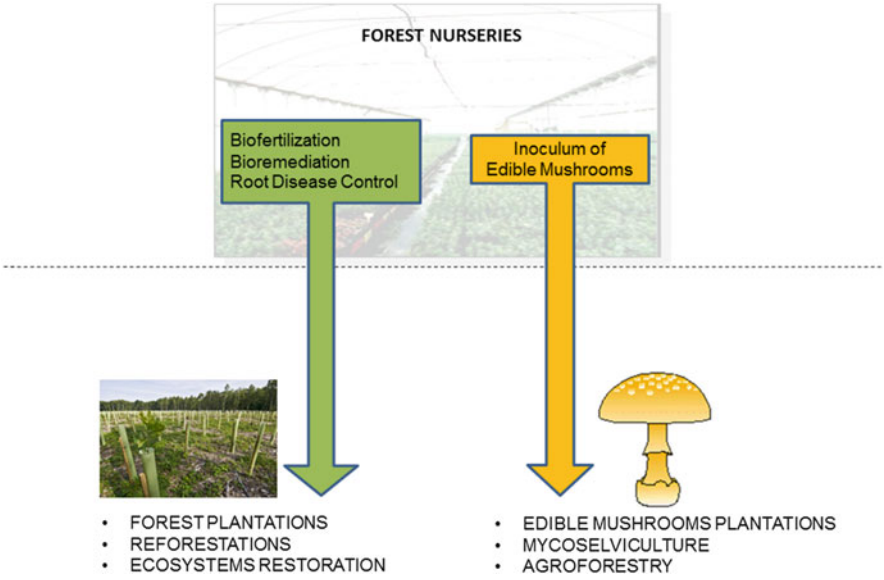

Fig. 4.1 Main objectives of the inoculation of ectomycorrhizal fungi in forest nurseries and their corresponding forest applications

4.7 ECMF in Forest Nurseries

The challenge in the controlled synthesis of the ectomycorrhizal symbiosis is to produce quality mycorrhizal plant, only colonized by the desired fungus. Accurate identification of the inoculum used and avoiding contamination during the growth of the inoculated plants are essential parts of the production process to avoid the introduction of unwanted species and to avoid the mixing of their genetic material with indigenous species (Murat and Martin 2008).

The appropriate selection of suitable plant-host species is essential for the success of mycorrhization (Olivier 2000). Relatively fast-growing fungi are generally preferred for inoculation because of their short incubation period. Unfortunately, many otherwise desirable ECMF grow slowly. According to Marx (1980), fresh cultures are preferred to cultures repeatedly transferred and stored for several years. He further suggested passing important fungus cultures through a host inoculation and mycorrhiza formation followed by re-isolation, every few years, to maintain mycorrhiza-forming capacity. Moreover, fungi which produce large hyphal stands of rhizomorphs in culture of soil may be superior in soil exploration and mineral uptake to those which lack rhizomorphic growth. On the other hand, the fruiting of the ECMF species is not based solely on the mycorrhizal state of the seedlings. After planting, in addition to the presence of indigenous competitors, the biotic and physicochemical characteristics of the soil also influence the persistence and spread of the cultivated fungus (Hortal et al. 2009).

The type of ECMF material used for inoculation can affect the success of a mycorrhizal inoculation program. In addition to remaining viable during storage and transport, the inoculant must also maintain its infectivity for several months after its introduction (Rossi et al. 2007).

There are three main sources of fungal inoculum:

(a) The use of the soil or humus collected from the area in which the mycorrhizal seedlings are going to be planted: Its main disadvantage is the lack of control of the species of ECMF present in the soil or of microorganisms and harmful germs. It is widely used in developing countries, although it is currently discarded in mycorrhization programs. Also, planting mycorrhizal "nurse" seedlings or incorporating chopped roots of ECMF hosts into nursery beds as a source of fungi for neighboring young seedlings has been successful (Sim and Eom 2006).

(b) The use of spores of fruit bodies collected in the field: The main advantages are that the spores do not require the extension of the aseptic culture and that the spore inoculum is not heavy (Marx and Cordell 1989). Most of the recent research has been with *P. tinctorius*; however, inoculation with *Rhizopogon* species also appears promising. Abundant *Rhizopogon* mycorrhizae formed on seedlings produced from the coated seed of *P. radiata* with basidiospores of *Rhizopogon luteolus* (Sharma 2017). However, it has three main drawbacks: (A) significant quantities of fruiting bodies are required and may not be available each year; (B) the success of the inoculation is highly dependent on the viability of the spores; and (C) the lack of genetic definition. Freeze drying and storage at a low temperature in the dark is helpful to maintain its viability. The spores can be mixed with physical supports before the soil inoculation, suspended in water and soaked in the soil, sprinkled, sprayed or pelleted and emitted to the ground, encapsulated or coated on the seeds and they can be embedded in hydrocolloid chips (Marx and Cordell 1989).

(c) Mycelial inoculum: It is the use of hyphae as an inoculum in a solid or liquid medium or substrate. Fungal hyphae are cultivated mainly from sterile parts of fruiting bodies, less frequently from mycorrhiza due to their low (approx. 5–20%) success rate (Molina and Palmer 1982) and rarely from sclerotia (Trappe 1969) or sexual spores (Fries and Birraux 1980). It is considered the most appropriate method since it allows the selection of particular strains of a fungus previously tested for its ability to promote the growth of plants (Marx 1980). Many species do grow well in culture, e.g., most species of *Suillus*, *Hebeloma*, *Laccaria*, *Amanita*, *Rhizopogon*, and *Pisolithus*. Liquid substrates have the advantage over solids because they are easily mixed, and they produce more uniform conditions for crop growth, but the risk of bacterial contamination and costs are higher (Rossi et al. 2007). On the other hand, the main advantages of the solid medium (Cannel and Moo-Young 1980) are the reduction of bacterial contamination due to the lower water content, the low costs of the equipment, and the simplified design of the bioreactors. The main drawback of the use of mycelial inocula is that several species of ECMF are difficult to grow under

laboratory conditions, or growth is very slow (due to the absence of their symbiont), and it is not always easy to produce large amount of inoculum viable for large-scale nursery inoculation programs. Some advances have been made using mycelium encapsulated in "beads" of calcium alginate (Le Tacon et al. 1983), but they have to be refrigerated. Inoculant beads can remain viable for several months under refrigeration, although the results vary between fungal species. For several species, the mycelial inoculum has been tested with trees of economic interest. This technique has great potential for the inoculation of seedlings in reforestation programs. For example, Rossi et al. (2007) designed a bioreactor with the capacity to produce inoculum for 300,000 seedlings, enough to reforest 200 ha. Based on a global demand of 3.0 billion cubic meters of wood, an estimated 4.3 tons of mycelium would be needed to inoculate 12 billion seedlings (5 g of dry mycelium per plant, Rossi et al. 2007). An advantage of alginate gel is the possibility of preparing a multimicrobial inoculant.

4.7.1 Mycorrhizal Helper Bacteria

The concept of "mycorrhizal helper bacteria" (MHB) was introduced in a "Tansley Review"—Helper Bacteria: a new dimension of mycorrhizal symbiosis (Garbaye 1994)—which has led to new research in the plant-fungus model system, as for the meaning of these bacteria that promote the formation of mycorrhizae and cause many physiological effects of mutualistic interaction.

In general, the ability of some microorganisms to influence the formation and functioning of the symbiosis is known, through activities of various kinds such as the activation of infective propagules of the fungus in presymbiotic stages (Azcón-Aguilar and Barea 1996), facilitating the formation of entry points in the root (Linderman 1988), and increase the growth rate (Carpenter-Boggs et al. 1995). The MHB improve mycorrhiza formation, although the same MHB can benefit mycorrhization for certain fungi and be negative for others (Garbaye and Duponnois 1992). The above reflects the fungal specificity by isolate, which exemplifies the genetic distance between isolates of different origin.

Among the mechanisms presented by the MHB are:

(a) Promotion of the establishment of the symbiosis by stimulation of the mycelial growth. The germination of spores and mycelial growth are improved by the production of growth factors (Keller et al. 2006).
(b) Increased contact and colonization root-fungi surfaces: increasing of lateral root number by the production of phytohormones (Bending et al. 2002) and the improvement of radical colonization by induction of flavonoid production (Xie et al. 1995).
(c) Reduction of the impact of adverse environmental factors on the mycelium of the mycorrhizal fungus. Bacteria can detoxify soils, restoring their conductivity,

similarly freeing them from contamination generated by heavy metals (Brulé et al. 2001) and reducing the concentrations of phenolic antagonist compounds produced by the same mycorrhizal fungi (Duponnois and Garbaye 1990). The rhizospheric microorganisms also have an effect on the growth of the plants, reaching a synergistic effect, where the presence of the micro-fungus and the other microorganism produce an increase in the growth, vigor, and protection of the plant (Domínguez et al. 2012). These effects are based on activities such as the acquisition of nutrients, inhibition of the growth of pathogenic fungi (Budi et al. 1999), and improvement of the root ramification (Gamalero et al. 2004).

In recent years, a potential capacity of bacteria associated with ectomycorrhizae to fix atmospheric nitrogen has been suggested (Frey-Klett et al. 2007). Several studies suggest a real possibility that the bacteria present in mycorrhizal tissues contribute to the nutritional needs of both the fungus (ascocarp development) and consequently of the plants, by providing them with available nitrogen derived from atmospheric nitrogen (N_2).

MHB belong to a wide range of genera (*Burkholderia*, *Paenibacillus*, Poole et al. 2001; *Pseudomonas*, *Bacillus*, Duponnois and Garbaye 1991; *Streptomyces*, Maier et al. 2004).

However, the molecular mechanisms by which MHB induce the growth of ECMF are not well described. Recently, changes in expression of genes involved in the development of certain ECMF have been studied at the molecular level in confrontations with MHB (Schrey et al. 2005; Riedlinger et al. 2006; Deveau et al. 2007; Zhou et al. 2014). Research in mycorrhizae should, therefore, strive towards an improved understanding of the functional and molecular mechanisms involved in interactions in the mycorrhizosphere, in order to develop ad hoc biotechnology that allows the application of optimized combinations of microorganisms as effective inoculators within sustainable systems of plant production (Artursson et al. 2006).

4.7.2 Polymicrobial Formulations

Polymicrobial formulations containing a diverse mixture of beneficial rhizosphere microorganisms with multiple functionalities is attractive because combining different classes of soil organisms can take advantage of multiple plant growth-promoting mechanisms and could be applied to multiple crops (Avis et al. 2008; Gravel et al. 2007; Hayat et al. 2010; Malusa et al. 2012; Vestberg et al. 2004). A key concept in constructing effective polymicrobial multifunctional formulations is the selection and use of a right combination of rhizosphere bacteria and fungi that are mutually compatible, have complementary functionalities, effectively colonize the rhizosphere of the crop(s) of interest, and bring about a synergistic promotion of growth and yield of crop(s) (Avis et al. 2008; Azcón-Aguilar et al. 2009; Barea et al. 2005; Hata et al. 2010). It is to be expected that well-designed

multifunctional formulations such as the one described would be a welcome addition to the fast-growing inoculant enterprises worldwide. Such an inoculant is also expected to be eco-friendly and suitable for organic farming and other integrated production systems, where synthetic fertilizer inputs are not allowed or restricted by law. However, construction of such complex formulations is technically demanding (Reddy and Saravanan 2013).

Ectomycorrhizal fungi exhibit synergistic interactions with other plant-beneficial organisms such as symbiotic N_2-fixers. For example, ectomycorrhizal symbiosis enhanced the efficiency of inoculation of two *Bradyrhizobium* strains on the growth of legumes (Andre et al. 2005). It is also of interest that similar synergies were seen when AMF (*Glomus mosseae*), ECM fungus (*Pisolithus tinctorius*), and *Bradyrhizobium* sp. were used together to inoculate *Acacia nilotica*; enhancement of N_2 fixation, growth, and dry biomass were observed when all three organisms were present (Saravanan and Natarajan 1996, 2000).

Also, using plant growth-promoting microorganism (PGPM) strains that form stable and effective biofilms could be a strategy for producing commercially viable inoculant formulations (Malusa et al. 2012; Seneviratne et al. 2008). A majority of plant-associated bacteria found on roots and in the soil are found to form biofilms (Ude et al. 2006). Bacterial, fungal, and bacteria/fungal biofilms were suggested as possible inoculants. This is a novel and interesting idea, but to what extent this approach would be practiced remains to be seen (Reddy and Saravanan 2013).

4.8 Application of ECMF in Forest Management

The inoculation of ECMF can be done not only with the objective of producing edible carpophores but also because of its considerable value in forest management (Fig. 4.1); in particular, they have had great importance in reforestation programs where it was expected that the quality and economic productivity of the plantations would increase (Garbaye 1990). The success of the plantations with mycorrhized seedlings from the nursery depends on their ability to quickly access the nutrients and water available within the soil matrix (Duñabeitia et al. 2004).

In mycorrhizal plantations (productive or conservation forest reforestations), a consequence of the recognition of the advantages of fungal diversity in ecosystems will be an increase in the refusal to introduce potentially dominant species in mixed communities. On the other hand, unfortunately, it seems that many of those fungi selected for optimal colonization in the nursery have been poor competitors in the field, especially when the planting sites contained indigenous populations of mycorrhizal fungi. There are several possible explanations for the inoculation failure (from the nursery) to produce beneficial effects in the planting sites. Probably, among the most important of these is the inability of inoculum introduced to persist in the roots of the plant after the transfer of the nursery to the field. The soil conditions experienced in the nursery and with the plant growing in a container are very different from those of most of the planting sites; in addition, the raising, storage,

and transport of seedlings can reduce the vigor of fine roots and their fungal associates. Species such as *Pisolithus tinctorius* (15 sub spp), in circumstances such as degraded environments, with absence or scarcity of autochthonous mycorrhizal populations, have achieved the greatest success in inoculation programs (McAfee and Fortin 1986).

In the case of an artificially mycorrhized plant with edible ECM fungi of interest, such as *Tuber melanosporum* (black truffle), the establishment of plots has always had the main objective of producing fruiting bodies, leaving in the background the contribution of ecological functions of the symbiosis (in the plant, soil, and, in general, the ecosystem, Domínguez et al. 2006). The example of mycorrhizal plantations for truffle production has been generally successful (Olivier et al. 1996), obtaining productions from 6 to 7 years of implantation.

In restoration of ecosystems, the biofertilization, bioremediation, or the control of soil pathogens are prominent roles of the mycorrhizal forest plants. Degraded ecosystems are the result of a wide range of characteristics and factors related to unfavorable land management or industrial activities. Environmental degradation of the soil is increasing worldwide at an alarming rate due to erosion, acidity, salinization, compaction, the depletion of organic matter, and water scarcity. In a healthy ecosystem, there is a balanced microbiota of the soil, in such a way that the potential of pathogenic and mycorrhizal fungi coexists in apparent harmony. Ectomycorrhizal fungi can survive in extreme habitats with high or low temperature (Tibbett and Cairney 2007; Geml et al. 2011), salt and metal concentration (Colpaert et al. 2011), drought (Azul et al. 2010), and other circumstances related to the degradation of the ecosystem. The importance of ECM fungi in the balance of the ecosystem can be enormous, since they can be used to increase the tolerance of plants against abiotic stresses, especially their capacity to fix heavy metals or to degrade a wide variety of persistent organic compounds; to interact with soil bacteria; to attack fungi, bacteria, and pathogenic nematodes; and to improve the vegetative growth and the nutritional status of its symbiont plant. In addition, the extraradical mycelium of the ECM fungi provides a direct pathway for the translocation of photosynthesized carbon to microsites in the soil and a large surface area for interaction with other microorganisms (Sun et al. 1999; Suz et al. 2012). Very little is known about how the tolerance of fungi to metals affects the transfer of metal to the host plant. The ability to accumulate metals depends not only on the inter- and intraspecific variation of the sensitivity of mycorrhizal fungi to metal but also on environmental factors (Suz et al. 2012). Meharg and Cairney (2000) revised potential ways in which ectomycorrhizal fungi might support rhizosphere remediation of persistent organic pollutants (POPs). Recently, the importance of low molecular weight organic acids and metal-chelating agents (such as siderophores) from ECMF in the fixation of metal ions and their transmission or not to the root of the host plant has been described (Machuca 2011).

4.9 Conclusions

Research on ectomycorrhizae should focus on better understanding the functional and molecular mechanisms involved in interactions in the mycorrhizosphere. It should aim to find the appropriate technology for the commercial techniques of multiplication and large-scale inoculation of the mycorrhizal inoculum and the application of optimized combinations of plant-microorganisms, adopted under well-defined environmental and soil conditions. The role of ECMF as biofertilizers in reforestation and environmental restoration has been fundamental up to now, and its importance in the balance of the ecosystem can be enormous, increasing the tolerance of plants against biotic and abiotic stress.

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Chapter 5
Perspectives on the Role of Arbuscular Mycorrhizal Fungi in the In Vivo Vegetative Plant Propagation

Ravichandran Koshila Ravi and Thangavelu Muthukumar

Abstract Vegetative propagation is an important method for increasing the productivity of economically important agricultural and horticultural plants. Apart from the application of phytohormones, beneficial microorganisms such as arbuscular mycorrhizal (AM) fungi being natural biofertilizers are also widely used in the field of horticultural production systems. The mutualistic association between the AM fungi and plant are not only known for their efficient water and nutrient uptake, less vulnerability to pathogens, and ability to withstand or tolerate abiotic and biotic stresses but are also involved in the production of plant hormones and adventitious root formation in asexual propagation. The inoculation of AM fungi to the rooting substrate could result in similar responses on the cuttings to those obtained through the application of exogenous plant growth regulators. In addition, the combined use of AM fungi along with plant hormones leads to increased root initiation and development of plant parts. The early inoculation of AM fungi onto the rooting medium enhances the plant growth rate of vegetatively propagated plant species after forming a symbiotic relationship with the plant. Moreover, a series of sequential signaling events are known to occur between AM fungi and the host plant during the development of roots. The present chapter focuses on the role of AM fungi in various types of vegetative propagation including cutting, layering, and grafting, the interaction between the plant hormones, and the AM symbiosis. The mechanism involved in the production of plant hormones through AM fungi and thereby the physiological changes occurring in the plant metabolism during propagation is also discussed.

Keywords Cuttings · Plant hormones · Grafting · Adventitious roots · Mycorrhizal colonization

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5.1 Introduction

Agriculture is the major source of food supply and places important pressure on the environment and the natural resources. Horticulture being the major part of agriculture includes the production of vegetables, ornamentals, fruits, and medicinal plants (Sonah et al. 2011). There has been a significant increase in the productivity as well as the quality of the agricultural crops obtained through several new farming technologies (Edgerton 2009). Nevertheless, there is less progress in the domestication of tree species due to long generation times, irregular production of flowers and fruits, and high prevalence of outbreeding leading to loss of genetic gain in successive generations (Leakey et al. 1994). In addition, farmers often cannot afford high-quality tree transplants, or sometimes seeds may not be available, and some plants or tree species have very low germination rates. In order to overcome these limitations, vegetative propagation method was introduced for rapid production, better quality of horticultural crops and tree species thereby greatly enhancing their yield (Davies et al. 1994; Bisognin 2011).

Plant propagation are of two types, sexual propagation and asexual propagation, of which asexual propagation is considered as an important propagation method in which vegetative parts of plants such as stems, roots, leaves, or other special vegetative structures when detached from the mother plant and placed under suitable conditions develop into novel individuals that are genetically similar to the parent plant. Vegetative propagation is also of great relevance in rapid replication of a plant species under threat with a goal to sustain certain desired characteristics (Hartmann et al. 2002). The propagation of plants involving vegetative parts is advantageous over sexual methods, as the vegetative parts are much larger when compared to seeds and consist of more reserve energy. This enables rapid, constant early growth and facilitates the young plants called clones to establish successfully in spite of extreme competition for light, water, and minerals from already existing vegetation. Therefore, vegetatively propagating perennials can flourish over a wide range of dense plant communities. For example, some grassland weeds like creeping buttercup and stinging nettle invade vigorously through vegetative methods (Forbes and Watson 1992).

The vegetative organs of plants in the wild always prefer to propagate in an environment that is favorable for its growth. Mostly, it circumvents waterlogged or dry soil and heavily compacted area. Hence it is generally site-selective in nature. In contrast, seed dispersal is often a random process in sexual propagation. As the new individual plants or offsprings are produced through purely mitotic cell divisions in vegetative propagation, they are genetically similar to the parent plant, and genetic recombination does not take place (Forbes and Watson 1992). Therefore, the successful plants with genetically identical characteristics suitable to its environment propagate to develop well-adapted offsprings for many generations. Plant propagation through vegetative means is beneficial to agriculturists and horticulturists as they could raise crops and ornamentals that do not produce viable seeds. For instance, one of the initial and major developments in the agricultural system was

the production of important crop species such as grapes and figs through the insertion of the base of their woody stems into the ground to develop the adventitious roots and thus regenerating into new plants (Steffens and Rasmussen 2016). Several crop species like strawberries, potatoes, onion, etc. are well developed under natural condition through vegetative propagation method (Megersa 2017).

Besides several advantages, vegetative propagation is not easy or cheap when compared to propagation through seeds. Further, no hybrid or a new variety of plants could be raised by this propagation method (Mckey et al. 2010). The multiplication of vegetative organs could lead to overcrowding of individuals around the parent plant and invariably results in competition for resources like water and nutrients. In natural conditions, vegetatively propagated plants allow only short-range spread. In addition, as there is no genetic variation, plants can lose their vigor easily (Mckey et al. 2010). For example, if a plant is vulnerable to any specific pathogen or disease, all its offsprings produced by the mother plant are also equally vulnerable thus leading to the destruction of the whole plant population in a very short period of time.

The most common method of vegetative propagation includes cutting that is obtained by stem, leaf, or root, layering, grafting or through specialized organs such as tuber, rhizome, or bulbs (Megersa 2017). Of these, propagation by cuttings is the easiest, cheapest, and suitable method for a wide range of herbaceous and woody plant species. When the plant material is scarce or in order to raise a particular plant species rapidly, leaf cuttings or leaf bud cuttings are of great significance. Further, stem cuttings are placed into the growing substrate so as to produce rooting and other vegetative parts and thus developing into a new intact plant. Some of the plants do not root easily by cutting. Such type of plants can be propagated through layering where the propagated plant part is rooted when still remain attached to the mother plant and the sap flow does not get disturbed (Preece 2003). Moreover, forest tree species and other tropical fruits can be propagated through grafting technique in which two parts of the living plant, scion and rootstock, are grafted together that unite and develop into a new plant (Pina and Errea 2005). These different types of propagation techniques have both advantages and disadvantages of their own.

The vegetative propagation of plants through above-mentioned methods could be improved by the application of plant growth regulators for quick and early regeneration of plant parts (Păcurar et al. 2014; Adekola et al. 2012). Apart from plant growth regulators, some of the beneficial soil microorganisms also play a vital role in upraising plants through vegetative propagation techniques (Du Jardin 2015). Among several soil microbes, arbuscular mycorrhizal (AM) fungi act as an eco-friendly biostimulant that has a significant role in horticulture crops (Rouphael et al. 2015). Apart from numerous positive effects, AM fungi also play a vital role in the formation of adventitious roots when supplemented to the rooting substrate in most of the plant species (Scagel 2004a, b; Fatemeh and Zaynab 2014), thus contributing to the vegetative propagation of plants. Therefore, in the present chapter, we outline the importance and effect of AM fungal application on the regeneration and development of plant species through different methods of vegetative propagation (cutting, grafting, and layering). The interactions between plant

hormones and AM fungal symbiosis and the mechanism through which AM fungi enhance the growth of clones raised by vegetative propagation techniques is also discussed.

5.2 Arbuscular Mycorrhizal Fungi

Mycorrhizal symbiosis is a mutualistic association between the soil fungi and plant roots. About 80% of the land plant roots forms a symbiotic association with the AM fungi which supports the host plant by providing essential nutrients in exchange for carbohydrates provided by the host plant (Smith and Read 2008). The AM fungal symbiosis is not limited to space within the roots, as the AM fungi produce extraradical mycelium that explores the soil surrounding plant roots. Arbuscular mycorrhizal fungi are characterized by the presence of two important structures: arbuscules and vesicles (Fig. 5.1). The AM fungal hyphae colonize the cortical cells of roots forming a highly branched structure within the cells called arbuscules that function as a site for nutrient exchange (Berruti et al. 2015). The fungal hyphae originating from roots extend into the adjacent soil where they scavenge nutrients especially phosphorus (P) and transfer it to the host plants (Smith and Read 2008). Vesicles are the storage organ developed by the AM fungi in the form of terminal or intercalary hyphal swellings in the root cortical regions consisting of cytoplasm and lipids (Biermann and Linderman 1983). They are inter- or intracellular and are generally initiated after the formation of arbuscules, however, continue to develop even after the formation of arbuscules has ceased. Spores of AM fungi consist of lipids and are covered by multilayered cell wall allowing them to be viable for long duration and thereby are important propagules for initiating new colonization (Brundrett 1991).

Although AM fungal spore can germinate in the absence of the host plant, they fail to form a wide mycelial network and cannot complete their lifecycle without forming an association with the plant host (Porcel et al. 2012). In low fertile soils, AM fungi enhance the crop productivity by improving the uptake of immobile nutrients other than P such as zinc (Zn) and copper (Cu). Mycorrhizal fungi absorb nitrogen (N) from ammonia and transport to the host and enhance the crop productivity in soils of low potassium (K), calcium (Ca), and magnesium (Mg) content (Liu et al. 2002). There is an increasing body of literature exhibiting the beneficial aspects of AM fungi that include improved plant growth, increased acquisition of nutrients and water, tolerance to salinity, drought and metal toxicity, resistance against root pathogens, and maintaining of the soil structure and fertility (Harrier and Watson 2004; Rillig and Mummey 2006; Smith and Smith 2012; Yang et al. 2015).

Further, AM fungi are the important component of rhizosphere soil microbial community and have a positive effect on both soil and plant under natural ecosystem. They promote modifications in the chemical and biological properties of plants under stressed conditions. In addition, AM fungi are widely used as bioinoculants in most of the agricultural crops, thus in turn contributing to sustainable agricultural

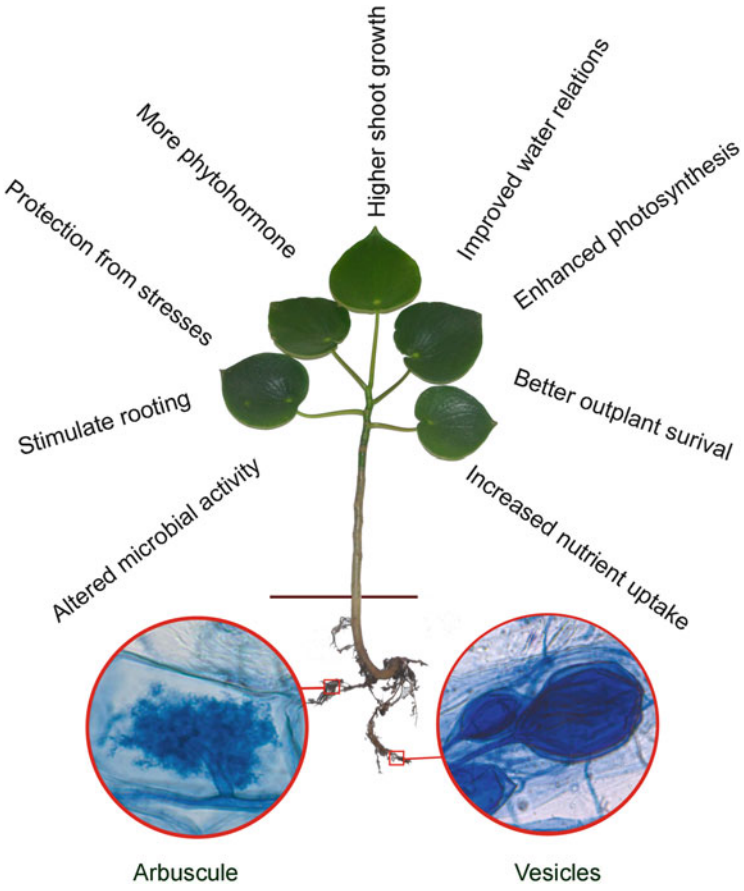

Fig. 5.1 Various plant benefits in response to arbuscular mycorrhizal (AM) symbiosis in vegetatively propagated clones. The important AM fungal structures, arbuscules, and vesicles are also shown within red circles

practices (Berruti et al. 2015). Apart from these positive effects, AM fungi are of great significance in the field of plant propagation as they stimulate the development of root system, enhance photosynthesis, produce more plant hormones, protect the plants from various stresses, and help in the successful establishment of young plants under natural conditions with improved output survival (Fig. 5.1).

5.3 Effect of AM Fungi on Cuttings

Arbuscular mycorrhizal fungi help in plant's adaptation by promoting the survival and establishment of rooted cuttings (Fatemeh and Zaynab 2014). The inoculation of AM fungi into the rooting medium during propagation by cuttings enhances the

rooting ability in different plants (Linderman and Call 1977; Singh 2002; Scagel 2004a, b). The response to AM fungal inoculation by the different plant cultivars propagated through cuttings is presented in Table 5.1. However, the efficiency of the AM symbiosis differs depending upon the AM fungal species and the ability of plant species to form roots (Scagel 2004b). For example, inoculation of *Prunus maritima* Marshall cuttings (hardwood and softwood) with three different AM fungal species, *Funneliformis mosseae* (= *Glomus mosseae*), *Claroideoglomus etunicatum* (= *Glomus etunicatum*), and *Glomus diaphanum*, in sterilized soil induced increased adventitious root growth. Of these, *F. mosseae* was more efficient in adventitious root production (Zai et al. 2007). Nevertheless, the method followed for plant propagation through cuttings does not permit mycorrhizal formation naturally as the rooting medium or substrate is generally sterilized to avoid interference of pathogens or soilless substrates that lack AM fungi are used (Essahibi et al. 2017) (Table 5.1). The quality of cutting, rooting medium, and the environmental condition are important factors for successful rooting of the cuttings. An ideal root medium allows good aeration, avoid water logging, and maintain moisture content and improved and higher root development (Washa et al. 2012).

The application of AM fungi into the rooting medium in the greenhouses could be helpful for the growth of propagating plants in outdoor conditions after transplantation. The early inoculation of cuttings with AM fungi during the formation of adventitious roots benefits the plant growth (Scagel et al. 2003). The response of olive cuttings to inoculation with two AM fungal species *Rhizophagus irregularis* (= *Glomus intraradices*) and *F. mosseae* in the nursery and under field conditions exhibited increased plant growth and yield. Further, pre-inoculation of AM fungi into the field enhanced the plant growth response through the early establishment of symbiosis in clones raised in sterilized substrates (Estaun et al. 2003). Nevertheless, the effect of pre-inoculation treatment reduces over time as the seedlings get colonized with the indigenous AM fungi in the field (Siqueira et al. 1998; Estaun et al. 2003).

Successful establishment of clonal plants in an environment depends on the ability of the clones to produce a large volume of roots, superior root length and clonal vigor (Washa et al. 2012). The mycorrhizal fungal inoculation improves the root growth characteristics of plant species propagated by cuttings. Moreover, Wimalarathne et al. (2014) reported greater root architecture such as root biomass, root length, root volume, and root mean diameter in *Piper nigrum* L. rooted cuttings inoculated with different quantities of *F. mosseae* inoculums in a sterilized rooting medium comprising of top soil, cattle manure, and river sand. Similarly, both runner and orthotropic shoots of *P. nigrum* inoculated with mycorrhizal fungi [*Rhizophagus fasciculatus* (= *Glomus fasiculatum*), *Gigaspora margarita*, and *Acaulospora laevis*] induced higher root growth characteristics when compared to the uninoculated and indole butyric acid (IBA)-treated *P. nigrum* cuttings (Thanuja et al. 2002). Plants of *Origanum vulgare* L., *Origanum onites* L., *Mentha piperita* L., *Mentha spicata* L., and *Mentha viridis* L. raised by stem cuttings when transferred to sterile rooting medium containing *C. etunicatum* propagules had increased the plant growth, nutrients, and production of essential oil (Karagiannidis

Table 5.1 Response of plant species vegetatively propagated through cuttings to the presence of arbuscular mycorrhizal (AM) fungi

Plant species	Plant part	Rooting medium	AM fungal species	Observations	References
Actinidia deliciosa (A. Chev.) C.F.Liang & A. R.Ferguson	Stem (Hardwood cuttings)	Steam-sterilized peat-perlite mixture	*Funneliformis mosseae*	Greater mycorrhizal colonization; variations in the morphology of fungal development within the roots	Calvet et al. (1989)
Arctostaphylos uva-ursi (L.) Spreng.	Stem (softwood cuttings)	Sterilized peat moss/coarse sand	*Rhizophagus intraradices*	Increased rooting	Nelson (1987)
Ceratonia siliqua L.	Stem	Sterilized peat	*F. mosseae*, *Rhizophagus fasciculatus*, *R. intraradices*	Increased rooting; improved growth and physiology	Essahibi et al. (2017)
Chrysanthemum morifolium Ramat.	Stem	Sterilized soil	*Glomus* sp.	Increased plant height, leaf area, root length, and fresh and dry weight of shoots, and roots; improved flowering quality and micro- and macronutrient uptake	Sohn et al. (2003)
Coleus aromaticus Benth.	Stem	Sterilized soil	*R. fasciculatus*	Increased leaf numbers and branches; total biomass, N and P contents	Earanna et al. (2001)
Cyclamen persicum Mill. var. Rosa mit Auge	Stem	Peat-based medium	*F. mosseae*, *R. intraradices*, *Funneliformis geosporum*, *Funneliformis claroideum*	Decreased plant mortality percentage, increased plant height, produced more leaves and flowers	Dubsky et al. (2002)
Dalbergia melanoxylon Guill. & Perr.	Stem treated with auxin IBA	Steam-sterilized soil/sand	*Glomus versiforme*	Increased rooting and root parameters of middle and basal cutting positions	Ezekiel Amri (2015)
D. melanoxylon	Stem/rooted stem	Sterilized soil	Indigenous mycorrhizal species	Improved rooting	Washa et al. (2012)
Euphorbia pulcherrima Willd. ex Klotzsch	Stem	Peat-based medium	*F. claroideum*, *F. mosseae*, *R. intraradices*, *F. geosporum*	Decreased plant mortality, increased plant height, number of leaves and flowers	Dubsky et al. (2002)

(continued)

Table 5.1 (continued)

Plant species	Plant part	Rooting medium	AM fungal species	Observations	References
E. pulcherrima	Stem	Sterilized vermiculate	*Gigaspora margarita*	Stimulated rooting, increased number and weight of roots; amolerated transplant shock under high temperature and low moisture	Barrows and Roncadori (1977)
E. pulcherrima	Stem	Perlite, peat-based substrate	*R. intraradices*	Promoted adventitious roots formation; accumulated more carbohydrate in leaves and stems	Druege et al. (2006)
Malus pumila Miller	Rootstock	Mixture of carbonized rice husk and coconut fiber	*Glomus* sp.	Enhanced plant growth and survival	Sbrana et al. (1994)
Mentha piperita L.	Rooted stem	Autoclaved perlite	*Claroideoglomus etunicatum*	Higher production of essential oil, improved plant growth and nutrient uptake	Karagiannidis et al. (2012)
M. piperita	Stem	Mixture of carbonized rice husk and coconut fiber	*Rhizophagus clarum*, *C. etunicatum*, *Acaulospora scrobiculata*	Increased aerial biomass and root wet matter of plant (*C. etunicatum*)	Silveira et al. (2006)
Mentha spicata L.	Rooted stem	Autoclaved perlite	*C. etunicatum*	Increased plant growth, nutrient content and essential oil production	Karagiannidis et al. (2012)
Mentha viridis L.	Rooted stem	Autoclaved perlite	*C. etunicatum*	Improved plant growth, nutrient uptake in low fertile soil, and higher essential oil production	Karagiannidis et al. (2012)
Olea europaea L.	Stem	Sterilized soil (sand/clay loam)	*R. intraradices*, *F. mosseae*, *Septoglomus viscosum*	Improved growth; protection against root-knot nematodes (*Meloidogyne incognita* and *Meloidogyne javanica*)	Castillo et al. (2006)

O. europaea	Rooted stem	Sterilized vermiculate/sand	*R. intraradices*	Enhanced plant growth and improved levels of its major polyphenols	Malik et al. (2017)
O. europaea	Rooted stem (semi-woody)	Sterilized vermiculate and perlite	*F. mosseae*, *R. intraradices*, *F. claroideum*	Stimulated plant growth during both early and nursery development stages	Porras Piedra et al. (2005)
Olea europaea sub sp. *Laperrinei* Batt. & Trab	Stem	Autoclaved sand/peat	*Glomus* sp.	Enhanced plant growth of the cuttings	Sidhoum and Fortas (2013)
Origanum onites L.	Rooted stem	Autoclaved perlite	*C. etunicatum*	Enhanced plant growth, production of essential oils, root colonization and nutrient uptake	Karagiannidis et al. (2012)
Origanum vulgare L.	Rooted stem	Sterilized perlite	*C. etunicatum*	Increased plant growth, nutrient content and essential oil production	Karagiannidis et al. (2012)
Pedilanthus tithymaloides L.	Stem	Sterilized soil	*R. fasciculatus*, *F. mosseae*, *G. macrocarpum*, *R. intraradices*, *C. etunicatum*, *Acaulospora laevis*, *G. margarita*	Improved plant growth, biomass and P uptake of cuttings inoculated with *R. fasiculatus* followed by *A. laevis*	Kadam et al. (2011)
Pelargonium zonale L.	Rooted stem	Peat/biochar	*R. intraradices*, *F. mosseae*	Improved plant growth, lower electrolyte leakage, increased relative water content and chlorophyll content	Conversa et al. (2015)
Piper nigrum L.	Rooted Stem	Sterilized top soil, cow dung, coir dust and sand	*F. mosseae*	Increased root length, shoot dry weight	Mala et al. (2010)
P. nigrum	Rooted stem	Sterilized top soil, cattle manure, and river sand	*F. mosseae*	Improved shoot and root development	Wimalarathne et al. (2014)
P. nigrum	Stem	Sterilized sand	*R. fasciculatus*, *G. margarita*, *A. laevis*	Enhanced rooting and root growth and P content	Thanuja et al. (2002)

(continued)

Table 5.1 (continued)

Plant species	Plant part	Rooting medium	AM fungal species	Observations	References
Podocarpus cunninghamii Colenso	Rooted stem	Heated sand bed	*A. laevis* (indigenous AMF species), *Glomus* sp. (exotic AM species)	Improved the nutrient uptake and showed positive plant growth response (*A. laevis*)	Williams et al. (2013)
Prunus cerasifera L.	Rootstock	Mixture of carbonized rice husk and coconut fiber	*Glomus* sp.	Induced earlier growth of cuttings	Sbrana et al. (1994)
P. cerasifera	Rooted microplants	Quartz sand	*F. mosseae*, *R. intraradices*	Improved plant growth parameters, P content in shoots, branching of roots, enhanced soluble proteins in roots and mycorrhizal colonization	Berta et al. (1994)
Prunus maritima Marshall	Stem	Sterilized vermiculate/sand	*F. mosseae, Rhizophagus diaphanus*, *C. etunicatum*	Enhanced rooting and growth and uptake of macronutrients (*F. mosseae* and *C. etunicatum*)	Zai et al. (2007)
Rosa hybrida L. cv. Grand Gala	Rootstock	Mixture of perlite-coconut fiber	*F. mosseae*, *R. intraradices*	Improved plant biomass, leaf nutrients, and flower quality	Garmendia and Mangas (2012)
Rosa L. (Miniature roses)	Rooted stem	Sterilized peat/perlite (8:2)	*R. intraradices*	Enhanced root biomass and number of adventitious roots and increased stem protein content	Scagel (2004a)
Rosmarinus officinalis L.	Stem (Hardwood, semi-hardwood, soft wood cuttings)	Sterilized sand	*R. intraradices*, *F. mosseae*	Increased rooting percentage, root numbers and total root length in softwood cuttings (*R. intraradices*)	Fatemeh and Zaynab (2014)
Salix purpurea L.	Rooted stem	Sterilized sand/vermiculate	*Rhizophagus irregularis*	Promoted plant growth under Cu stress, modulated physiological and metabolic responses	Almeida-Rodríguez et al. (2015)
Sciadopitys verticillata Sieb & Zucc.	Stem	Sphagnum-based potting mix	*R. intraradices*	Increased survival, callus development, and rooting percentage	Douds et al. (1995)

Strobilanthes ciliates Nees.	Stem	Sterilized soil/ farm yard manure	*Rhizophagus aggregatus*	Increased aboveground plant growth parameters and root colonization	Asha Thomas and Rajeshkumar (2014)
Taxus × *media* Rehder	Stem	Sterilized coarse perlite/peat moss/sand	*R. intraradices*	Stimulated root initiation, increased number of primary roots, root dry weight and growth of adventitious roots	Scagel et al. (2003)
Theobroma cacao L.	Stem	Sterilized sand	*Scutellospora*, *Glomus* sp.	Increased plant growth, N content and root colonization rates	Chulan and Martin (1992)
Vitis champini L.	Rootstock	Sterilized soil	*R. fasciculatus*	Increased plant growth and dry matter under salinity	Belew et al. (2010)
Vitis rupestris Scheele	Rootstock	Sterilized soil	*R. fasciculatus*	Increased plant growth and dry matter under salinity	Belew et al. (2010)
V. riparia × *V. rupestris* × *V. viniferax*, *V. candicans* × *V. labruska*	Rootstock	Sterilized soil	*R. fasciculatus*	Increased plant growth and dry matter under salinity	Belew et al. (2010)
Vaccinium meridionale Swartz	Stem (softwood cuttings)	Autoclaved perlite	Mixture of *Glomus, Entrophospora*, *Scutellospora*, *Acaulospora* genera	Increased viability of cuttings	Ávila Díaz-Granados et al. (2009)
Viburnum dentatum L.	Stem	Sterilized perlite/ vermiculate	*R. fasciculatus*	Increased root development and growth	Verkade and Hamilton (1987)
V. champini	Rootstock	Sterilized soil	*R. intraradices*	Promoted plant growth, higher K and Mg concentration and K/Na ratio in leaf tissue under salinity	Khalil (2013)
Vitis vinifera L.	Rootstocks	Sterilized sand/ vermiculate	*R. aggregatus*	Higher root development, greater mycorrhizal colonization and plant performance	Aguín et al. (2004)

et al. 2012). In addition, the uses of AM fungal soil inoculums have been reported to enhance the survival and establishment of *Khaya anthotheca* (Welw.) C. DC. cuttings and also in the restoration of plants in the degraded lands (Dugbley et al. 2015). The colonization of roots by AM fungi promotes the growth rate and nutrient uptake in clones propagated through cuttings (Sohn et al. 2003; Karagiannidis et al. 2012).

The application of indigenous AM fungi is more useful than using exotic AM fungal species for raising plants by cuttings. It has been suggested that the combination of both indigenous and exotic AM fungal species could lead to negative response on plant growth (Klironomos 2003). In support of this statement, Williams et al. (2013) found that addition of indigenous AM fungal species (*A. laevis*) to a slow-growing tree species, *Podocarpus cunninghamii* Colenso rooted cuttings, in pasteurized soil exhibited early and positive growth responses than application of exotic or commercially produced AM fungi (*Glomus* spp.). Different types of cuttings including softwood, semi-hardwood, and hardwood cuttings and also root cuttings of *Dalbergia melanoxylon* Guill. & Perr. tree raised under soil-containing AM fungi exhibited greater rooting traits thereby increasing the plant growth (Washa et al. 2012).

The adventitious root formation in cuttings is a vital process in plants that are widely propagated through vegetative methods. The formation of adventitious root in the tissues of the shoot is a complex developmental process that includes induction, differentiation, dedifferentiation, and growth of roots (Hartmann et al. 2002). It mostly depends on nutrients like carbon (C) and N and is specifically controlled by the interaction of plant hormones (Druege et al. 2004; Kevers et al. 1997). A root-colonizing endophytic fungus, *Piriformospora indica* when inoculated in root substrate with the cuttings of *Pelargonium* and Poinsettia increased the number and length of the adventitious root thereby promoting the formation of adventitious root at the higher rate of seven at the low fungal root colonization rates (Druege et al. 2006). Likewise, the inoculation of hormone-treated miniature rose cuttings with *Rhizophagus intraradices* (= *Glomus intraradices*) enhanced the root biomass and adventitious root formation before the root colonization, which suggests that AM fungi-plant signaling processes could have occurred earlier to rooting (Scagel 2004a).

5.4 Influence of AM Fungi on Grafting

Grafting is one of the major methods of vegetative plant propagation that has a crucial role in the development of horticultural crops which involves the production of new plants by inserting the shoot part (scion) onto the rootstock that forms the root system of the scion and generates into a new plant (Lee 1994). The rootstock influences the formation and accomplishment of the union graft. The rapid development of prominent root system is essential for the successful development of the plant, so the rootstock strongly relies on the effective root formation (Yetisir and Sari

2003). As the root system has a pronounced effect on root functions, it is important to know the influence of AM fungi on the performance of rootstock. It is observed that the initial or early inoculation of AM fungi is beneficial for the development of rootstock (Kumar et al. 2008).

Arbuscular mycorrhizal fungi influence the root morphogenesis through metabolites of AM fungi and hormones that are independent of the external supply of nutrients (Hooker et al. 1992). The effect of AM fungal species inoculation on plants through grafting method is presented in Table 5.2. In a study, Kumar et al. (2008) observed that AM fungal inoculation (*G. margarita* and *R. fasciculatus*) increased the rootstock vigor and vegetative and root parameters of mango thus contributing to successful grafting. Likewise, the rootstock of *Syzygium cuminii* L. treated with *R. fasciculatus* and *R. intraradices* when subjected to softwood grafting exhibited higher percentage of graft success and survival when compared to the uninoculated grafted *S. cuminii* (Neeraja Gandhi et al. 2010). The production of growth hormones such as auxins, gibberellins, and vitamins by AM fungi could contribute to the growth enhancement of rootstock. Furthermore, greater root geometry and increased nutrient supply mediated by AM fungi lead to the extramatrical hyphal growth that in turn improves the plant growth. The higher percentage of AM fungal root colonization enlarges the surface area for absorption and nutrient uptake in the rootstocks.

Inoculation of the AM fungal species (*A. laevis* and *C. etunicatum*) isolated from the rhizosphere soil of cashew plants from different sites improved the growth performance and the vigor of the cashew rootstock developed through grafting process. The AM fungal inoculation benefitted the grafted plants to withstand the transplant shock and to thrive well under field conditions (Lakshmipathy et al. 2004). Further, some studies have revealed an increased salinity tolerance in response to mycorrhizal inoculation of grafted plants through extension of the mycorrhizal hyphae into the substrate for higher uptake of nutrients and enhancing the root architecture parameters thereby improving the growth performance and fruit yield of grafted plants (Oztekin et al. 2013). The AM fungal root colonization varies among different grafted plant species. For example, Schreiner (2003) investigated the root colonization by AM fungi of ten different rootstocks of grapevines (*Vitis vinifera* L.) and reported only small variations in the mycorrhizal colonization of the rootstock genotype, where root length density of fine roots and AM colonization of fine roots were correlated to vigor and yield of scion. Further, AM fungal mycorrhizal colonization was related to the growth performance of the scion on varied rootstocks (Schreiner 2003).

The scion's quality and yield are gaining more interest in horticulture when compared to the rootstock which is meant for absorption. Some studies have reported that genotypes of scion exert a higher effect on AM fungal communities when compared to rootstock raised in varied types of soil (Song et al. 2015). For instance, Shu et al. (2017) conducted an experiment to find out the influence of Avocado (*Persea americana* Mill.) scions on AM fungi and development of root hairs in rootstocks and observed that scions did not have any impact on AM fungi, but scion influenced both the AM absorption and root directed pathways

Table 5.2 Influence of arbuscular mycorrhizal (AM) fungi on grafted plants

Plant species	Scion	Rootstock	AM fungal species	Observations	References
Anacardium occidentale L.	Ullal-3 (Cashew variety)	Ullal-1	*Acaulospora laevis*, *Claroideoglomus etunicatum*	*A. laevis* increased the plant height, stem girth, and grafting success of the graft; enhanced both shoot and root P and mycorrhizal colonization rates	Lakshmipathy et al. (2004)
Citrullus lanatus (Thunb.) Matsum & Nakai	Minirossa (*C. lanatus*)	RS841 (*Cucurbita moschata* Duchesne × *Cucurbita maxima* Duchesne	*Glomus* spp.	Increased plant growth, fruit yield, and characteristics; greater grafting vigor and productivity	Miceli et al. (2016)
Citrus lemonia L.	Seedless lemon	*C. lemonia*	*Funneliformis mosseae*, *A. laevis*	Enhanced the grafting success, grafting survival, thicker stem, and greater sprout length	Barman et al. (2006)
Cucumis sativus L.	Ekron F1 (*C. sativus*)	Nimbus F1 (*Cucumber maxima* × *Cucumber moschata*)	*Glomus* spp.	Improved growth rate after transplantation and increased yield	Babaj et al. (2014)
Poncirus trifoliata L.	*P. trifoliata.*	Kumquat *Fortunella hindsii* (L.) Swingle	*Fuscutata heterogama, Gigaspora margarita, C. etunicatum, Acaulospora* sp.	Increased plant growth parameters	Back et al. (2016)
P. trifoliata.	*P. trifoliata*	citrange 'Fepagro C37 Reck' (*P. trifoliata.* × *Citrus sinensis* (L.) Osbeck.]	*F. heterogama*, *G. margarita*, *C. etunicatum*, *Acaulospora* sp.	Increased plant height, stem diameter, leaf area, root and shoot dry biomass	Back et al. (2016)
Solanum lycopersicum L.	191 (Gokce) F1	Maxifort and Beaufort hybrid	*Gloumus* spp.	Enhanced fruit yield, root fresh and dry weights and increased salinity tolerance	Oztekin et al. (2013)
S. lycopersicum	Milas (*S. lycopersicum*)	Efialto (*Fusarium oxysporum* wilt resistant)	*Rhizophagus intraradices*	Increased plant height and dry biomass; reduced disease incidence	Bolandnazar et al. (2014)
Syzygium cuminii L.	*S. cuminii*	*S. cuminii*	*Rhizophagus fasciculatus*, *R. intraradices*, *Sclerocystis dussii*	Higher graft take, sprout height, leaf number and stionic ratio	Neeraja Gandhi et al. (2010)
Vitis vinifera L.	*V. vinifera*	Richter 110	*R. intraradices*	Improved leaf number, fresh weight, dry weight, plant development and increased root colonization rate	Camprubi et al. (2008)

systematically. It is believed that the plant hormones and secondary metabolites that are produced by the leaves and shoots and then transferred to the roots are crucial for the development of root hair and AM fungal colonization (Micallef et al. 2009; Shu et al. 2017).

Several studies have highlighted the role of AM fungi in plant protection against phytopathogens. Mora-Romero et al. (2015) conducted a grafting experiment using two varied pathogens, *Sclerotinias slerotiorum* (Lib.) de Bary (fungal pathogen) infected common bean (*Phaseolus vulgaris* L.) and tomato (*Solanum lycopersicum* L.) plant infected with the bacterial pathogen (*Xanthomonas campestris pv. vesicatoria*) and raised the presence and absence of AM fungi. The results of the study showed that for both the plant pathogens, the scions originated from non-mycorrhizal plants had the capacity to exhibit disease protection induced by mycorrhizal fungi through their grafting to rootstocks inoculated with mycorrhizal fungus (*R. irregularis*) (Mora-Romero et al. 2015). Bolandnazar et al. (2014) also reported a decrease in the incidence of *Fusarium* wilt disease in tomato plants through grafting onto resistant rootstocks and mycorrhizal inoculation.

The influence of AM fungi varies according to different plant species subjected to grafting technique and the quality of scion and rootstocks. Grafting of mini watermelon (*Melothria scabra* Naudin) onto mycorrhiza inoculated hybrid variety (*Cucurbita moschata* Duchesne × *Cucurbita maxima* Duchesne) rootstocks increased the vigor, production, and quality of mini watermelon fruits. In addition, the vitamin C content in fruit was enhanced due to the increased nutrient uptake, well-developed root system in rootstocks, and production of endogenous hormones on mycorrhization (Miceli et al. 2016). The production of rootstocks of citrus species (citrange 'Fepagro C37 Reck', 'Kumquat') with AM fungal species such as *C. etunicatum*; *Fuscutata heterogama* (= *Scutellospora heterogama*); *G. margarita*; and *Acaulospora* sp. resulted in increased plant growth performance and percentage of AM fungal colonization in citrange 'Fepagro C37 Reck' when compared to the other citrus rootstock which reveals that the effect of AM fungi on vegetative development relies on rootstock species (Back et al. 2016). Moreover, different methods of grafting have also been carried out to determine the successful grafting process. For instance, cucumbers raised using different types of grafting including self-grafted, splice grafted, and root pruned splice graft and inoculated with *Glomus* spp. exhibited higher plant growth and yield. Of these three methods, root pruned splice grafted cucumber produced more yield and superior plant growth response on inoculation with indigenous AM species under greenhouse conditions (Babaj et al. 2014).

In addition to improving plant quality and performance, grafting technique has received great reputation as an important research tool, especially in studies pertaining to the signaling mechanisms between root and shoot (Gaion et al. 2018). In their classical study, Gianinazzi-Pearson and Gianinazzi (1992) showed that intergeneric grafting of lupin scions onto pea root stocks greatly reduced root colonization by *F. mosseae* and *R. intraradices* and totally prevented the development of arbuscules in the root cortical cells. Based on the results, the authors suggested the possible involvement of mobile factors originating in shoots

preventing the establishment of mycorrhizal symbiosis in lupines (Gianinazzi-Pearson and Gianinazzi 1992). Foo et al. (2015) based on the intergeneric grafting experiment between lupin and pea showed that AM symbiosis and nodulation are regulated independently of each other probably due to the long-distance signaling. Further, the low strigolactone content in lupin scions grafted pea roots was suggested a possible cause for the suppression of AM symbiosis in lupin-pea graft combination.

In a greenhouse experiment, Kumar et al. (2015) investigated the influence of grafting and *R. intraradices* inoculation on the biochemical, physiological, and metabolite changes as well as gene expression analysis of tomato under two different levels of cadmium (Cd) stress. In this study, there are two graft combinations: self-grafted (*S. lycopersicum* cv. Ikram and *S. lycopersicum* cv. Ikram) and grafted onto interspecific hybrid rootstock Maxifort (*S. lycopersicum* × *S. habrochaites*). The presence of AM fungus was not able to ameliorate the effect of Cd stress and significantly increased the accumulation of Cd in the tomato shoots which subsequently decreased the growth and yield. However, plants of Ikram/Maxifort graft combination accumulated more proline, had higher antioxidant enzyme activity, and reduced lipid peroxidation. Moreover, Ikram-/Maxifort-grafted plants had higher accumulation of P, K, Ca, iron (Fe), manganese (Mn), and Zn and metabolites like fructans, inulins, and phytochelatin PC2 than Ikram/Ikram combination. The increased nutritional status of Ikram-/Maxifort-grafted plants was attributed to the upregulation of LeNRAMP3 gene in leaves (Kumar et al. 2015).

5.5 Mycorrhizal Fungi and Layering

Layering is one of the techniques in vegetative propagation in which a branch of the plant produces roots before it is detached from the mother plant. The successful propagation via layering depends on many factors such as moisture availability, season, the position of branching, and quality of rooting substrate and wrapping material (Mishra et al. 2017). Layering is of different types such as simple layering, compound layering, tip layering, and air layering. The combined inoculation of AM fungal species, *Scutellospora* and *Glomus*, in *Theobroma cacao* L. obtained through air layering showed an increase in dry biomass, stem diameter, and P concentration in shoots (Chulan and Martin 1992). Arbuscular mycorrhizal fungi increased the growth of Lychee (*Litchi chinensis* Sonn.) tree propagated by air layering in a soil-free substrate. In addition, AM fungi (indigenous Glomalean fungi) enhanced the copper (Cu) and Fe uptake in the Lychee (Janos et al. 2001). Moreover, the application of AM fungi along with vermicompost and *Azotobacter* as the rooting media improved the root and shoot characteristics and also the survival percentage of air layers of Lychee (Mishra et al. 2017). Furthermore, Sharma et al. (2009) also reported an enhanced total number of roots in *Litchi* air layers combined inoculated with *R. fasciculatus* and *Azotobacter* sp. The betterment in root architecture of air-layered *Litchi* trees was due to enhanced carbohydrates and metabolic activities by the rooting substrate (Mishra et al. 2017). Only very few studies have been carried

out through layering propagation using AM fungal species when compared to other types of vegetative propagation. The precise mechanism of AM fungi in propagation through layering is still obscure.

5.6 Interaction Between Plant Hormones and AM Fungi

The relationship between the host plant root and AM fungi involves a constant exchange of signals that lead to proper symbiosis development (Gianinazzi-Pearson 1996). Arbuscular mycorrhizal fungi regulate the hormonal balance of the plant by producing growth regulators under stressed conditions (Nadeem et al. 2014). The plant hormones regulate a number of events during the developmental stage of plants and constitute signaling molecules to regulate the establishment of a symbiosis. For example, auxins regulate the shoot and root architecture of plants and also stimulate the early events thereby helping in the formation of lateral roots on the host plant (Kaldorf and Ludwig-Müller 2000). Further, abscisic acid and jasmonates are involved in the formation of arbuscules (Herrera-Medina et al. 2007). However, in the formation of spore and vesicles, no hormones have been specified so far. Thus, these alterations in the fungus development may be induced by autonomous signals of the fungi itself. In addition, phytohormones take part in the temporary defense responses that are essential for establishing a homeostasis between AM fungi and the host plant (Garcia-Garrido and Ocampo 2002). Moreover, they might also stimulate resistance against pathogens to protect the host plant (Pozo et al. 2002).

The application of AM fungal species on cuttings treated with auxins exhibited controversial results. For instance, inoculation of AM fungi and auxin on stem cuttings of *D. melanoxylon* improved the rooting ability in terms of rooting percentage and root parameters (Ezekiel Amri 2015). An increase in the levels of auxins after inoculation of AM fungi in maize and soybean plants has been observed by Kaldorf and Ludwig-Müller (2000); Meixner et al. (2005). Production of indole-3-acetic acid by *R. irregularis* was reported by Ludwig-Müller et al. (1997). Jasmonic acid is known to establish symbiotic association between plant and AM fungus by modifying the endogenous jasmonic acid through repeated wounding of the plant (Landgraf et al. 2012). One of the hormones responsible for inducing AM spore germination is strigolactones, and it acts as a signaling molecule in rhizosphere to form AM symbiosis (García-Garrido et al. 2009).

The production of abscisic acid by the AM fungal hyphae of *R. irregularis* was revealed by Esch et al. (1994). This could give rise to early signal to enhance the production of indole-3-butyric acid to increase the lateral root numbers in the young roots and thus constituting a path for the fungal entry (Kaldorf and Ludwig-Müller 2000) as the production of indole-3-butyric acid was stimulated by abscisic acid (Ludwig-Müller et al. 1995). This might be a good example which indicates that hormonal signal formed by the symbiont can affect synthesis of hormones in plants. Deficiency of abscisic acid leads to increased level of ethylene that adversely regulates mycorrhizal fungal colonization. Moreover, abscisic acid deficiency

seems to downregulate the formation of arbuscules directly (Martín-Rodríguez et al. 2011).

5.7 Mechanism of AM Fungi in Plant Propagation

The primary mechanism accountable for plant growth is the improvement in the uptake of nutrients especially P induced by AM fungi. The production of plant hormones through these mutualistic fungi may also contribute to plant metabolic processes. Both the physiological and morphological alterations that microbial plant hormones could stimulate in the plant may help in the AM fungal symbiosis establishment and its activity, thereby resulting in the increased acquisition of nutrients by the host plants. In addition, gibberellins enhance the leaf area and lateral root formation, cytokinins play an important role in the fundamental processes of plant growth such as enhancement of photosynthetic rate, and auxins regulate the formation of roots and improve cell wall elasticity (Barea and Azcón-Aguilar 1982). Moreover, increased levels of cytokinin are reported with the association of plant roots with AM fungi thereby maintaining the chlorophyll levels and influencing the iron transport (Khade and Rodrigues 2009). The AM fungal colonization enhances the internal cytokinin levels in the colonized tissue and increases the fluxes of cytokinin to other plant parts, independent of the nutrient status of the host plant (Hirsch et al. 1997).

A series of sequential signaling events take place during various stages of plant-AM fungi interactions; however, there is no accurate information available about these signaling molecules (Roussel et al. 2001). The functioning of these molecules is examined in root-AM fungi interactions, but not between the stem and AM fungi (Scagel 2004a). In the propagation of plants obtained through cuttings, AM fungi benefit the plants when inoculation is done during the formation of the adventitious root (Fatemeh and Zaynab 2014). Moreover, the presence of precolonization signal among propagules of AM fungi and cutting is alike to those prevailing in the existence of host plant roots (Scagel 2001). This signal is activated in the cuttings of basal ends due to the release of carbon dioxide or other metabolites that was able to stimulate AM fungi propagule (Tamasloukht et al. 2003). The exudates released by the AM fungi might cause alterations in the metabolism of cuttings, thus increasing initiation of the adventitious root, thus improving the rooting ability on the cuttings on inoculation with AM fungi (Scagel 2004a). Furthermore, AM fungi induce new root formation after colonizing the root by enhancing the phenolic compound accumulation that is involved in tolerance against soilborne pathogens and also increases the water and nutrient uptake through the extraradical mycelia (Larose et al. 2002).

Arbuscular mycorrhizal symbiosis improves the ability of roots to uptake soil elements that are of low mobility through their mycelial network, thus enhancing plant growth. Inoculation of AM fungi in the soilless rooting substrate decrease the mortality percentage during transplantation and enhance the productivity of several

ornamental plants through vegetative propagation (Scagel 2004a). Mostly, another mechanism behind the rooting of cuttings is ascribed to the alterations in the N, amino acid, protein, and carbohydrate metabolism occurring during the development of adventitious roots. For example, miniature roses inoculated with AM fungi showed changes in the protein and amino acid contents in the cuttings (Scagel 2004a).

The beneficial aspect of AM fungi is more noticeable in the adaptation of rooted cuttings. As already mentioned, AM fungi improved the survival of the clones through the hardening stage and protected them from transplantation shocks (Yadav et al. 2013). Arbuscular mycorrhizal fungi improve the nutrient contents and stomatal conductivity of rooted cuttings. Mycorrhization positively influences the plant's gas exchange through enhancing the stomatal conductance (Sánchez-Blanco et al. 2004), subsequently supplying a large amount of carbon dioxide assimilation to the plant and hence increasing photosynthetic process in cuttings (Essahibi et al. 2017). Arbuscular mycorrhizal fungi increase the production of secondary metabolites (Sangwan et al. 2001). The increased synthesis of secondary metabolites in AM-inoculated plants could be ascribed to the stimulation of the aromatic biosynthesis pathway. The age and developmental stages of the plant are also important during secondary metabolite production. The AM symbiosis results in increased secondary metabolism due to the higher content of chlorophyll, amino acids, and proteins (Tejavathi et al. 2011).

5.8 Conclusion

The application of AM fungi in raising horticulturally important crops and tree plantations through vegetative propagation techniques is of great importance. The mycorrhizal inoculation increased the viability, rooting ability, survival, and overall plant growth of the vegetatively propagated plants. It has been suggested that production of hormones by AM fungi is responsible for the stimulation of plant growth in addition to the formation of adventitious roots and improved nutrient uptake. A number of signaling events take place during the interaction between the host plant and AM fungi during root formation on cuttings (Scagel 2004a, b). Although hormone production has been recognized as the potential mechanism responsible for plant growth promotion, the exact mechanism still remains unclear. Further, the role of AM fungi in plant propagation through layering is not explored largely as for plants obtained through cuttings and grafting methods. Therefore, studies related to AM fungi and layering method could be useful in understanding their effects on plants. The use of indigenous or native AM fungal species might be considered to be beneficial than inoculation with exotic AM species, thereby improving the growth performance of plants under field conditions. Though mycorrhizal fungi enhance the plant growth through plant propagation methods, the combined application of plant hormones and other beneficial microbes such as plant growth-promoting rhizobacteria can increase the rooting of cuttings more

efficiently. The application of beneficial microbes like AM fungi over chemical treatments could reduce the propagation costs in the nursery and defend against soil pathogens.

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Chapter 6
Silicon (Si)- and Zinc (Zn)-Solubilizing Microorganisms: Role in Sustainable Agriculture

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Abstract Across the world today, loss of the health of the soil is a key constraint causing reduced soil productivity and fertility, and also influencing crop yield, all major threats to food security. Intensive use of land by farmers, without undertaking appropriate nutrient management practices, results in the removal of more nutrients from the soil, which is connected to the decline in the productivity of crops. Plants need various nutrients in different ratios for their growth and development. The plants obtain these essential nutrients from soil, water, and air. Some of these nutrients are required in large amounts, whereas others are necessary in only small quantities for vegetative and reproductive growth of crop plants. As per recent speculation, reduced yield is mainly associated with reduction in the appropriate supply of nitrogen (N) by the soil, although total available N remains unaffected. In rice, silicon-solubilizing microorganisms have been noticed recently as more important for their role in the solubilization and mobilization of silicate minerals, rendering K (potassium) silicate and making potassium and silicon easily available to crop plants. Major causes of zinc deficiency in India are intensifying cultivation, unbalanced supply of nutrients, generally without zinc (Zn), and the predominance of lands with low organic matter content, calcareous nature, and high pH. Alternately, numerous microorganisms, especially those allied with roots, may increase the growth and productivity of plants. In the recent few years the use of

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Zn-solubilizing bacteria (ZSBs) as bio-fertilizers has acquired momentum, and bacteria are significant in improving soil nutrient content and sustaining crop production. ZSBs have been proven to have great ability to enhance Zn availability in the rhizosphere and to improve Zn supply to crop plants. Many genetically modified strains (GMSs) may be able to mobilize/solubilize more plant nutrients from the root zone. Development of GMSs with improved solubilization/mobilization of nutrients through genetic engineering and DNA technology is necessary to maintain an environmentally friendly and sustainable agriculture production system. Plant breeding strategies also appear to be a more reliable and cost-effective technique to enhance Zn content in plants. This chapter is mainly focused on silicon and zinc microorganisms, their role in the uptake mechanisms and solubilization activities in plants relative to nutrient dynamics, and the potential to apply this knowledge in managing a sustainable and eco-friendly agriculture system.

Keywords Enzymatic activities · Mechanisms · Significance · Silicon-solubilizing bacteria · Sustainable agriculture · Zinc-solubilizing bacteria

6.1 Introduction

The use of bio-fertilizers is a critical factor in integrated nutrient management (INM). Bio-fertilizers are a renewable source of nutrients, environmentally safe in comparison to synthetic fertilizers, and also low in cost. Among the sources of plant nutrients, growth-promoting rhizobacteria (PGPR) offer a possible way to increase production and quality of grains without affecting the environment. Several research studies have shown that bio-fertilizers are a good substitute for synthetic fertilizers to improve the growth of plants as well as crop yields, reducing the use of hazardous agro-chemicals. These microorganisms colonize root surfaces and internal plant tissues. PGPRs improve plant growth by N-fixation, supply of inorganic phosphorus (P), solubilization of silicon and zinc, siderophore production, phytohormone synthesis, and reducing pathogen effects (Lugtenberg and Kamilova 2009). For higher plants, silicon (Si) is not considered an essential nutrient but has been found beneficial for many plant species, particularly tropical poaceous plants such as rice; it is also required for the healthy growth and development of plants (Liang et al. 2007). As other essential plant elements, Si has a key function that is mechanical rather than physiological. These characteristics of silicon function show why the effects are easily found in plants that accumulated silicon to a small extent and why a silicon effect is more explicit in biotic or abiotic stress. Silicon makes thicker and stronger plant cell walls as well as increasing the size of the vascular system (Meena et al. 2014a). This thick plant cell wall makes the plant stronger in all aspects, and the enlarged vascular system allows more water and nutrient intake, resulting in larger, healthy plants producing higher yields. Silicon-solubilizing bacteria (SSBs) are bio-fertilizers that are based on selected strains of

bacteria of the genus *Bacillus* found to be naturally beneficial. These bacteria can be utilized as effective soil bio-inoculants that solubilize silicon, provide the potential to tolerate biotic and abiotic stress, and enhance plant resistance to diseases from attacks by insects and other pests. It is used in organic agriculture along with bio-fertilizer inocula such as nitrogen-fixing bacteria; phosphate-solubilizing bacteria (PSBs), potash-mobilizing bacteria (PMBs), zinc-solubilizing bacteria (ZSBs), sulfur-solubilizing bacteria (SSBs), iron-solubilizing bacteria (FSBs), manganese-solubilizing microbe (MSMs), and vesicular-arbuscular mycorrhiza (VAM). Such bio-fertilizers are also safe to use with plant extracts (botanical) and bio-pesticides, and an effective component in IPM/INM programmes, thus leading to significant reduction in use of synthetic/chemical fertilizers, which not only create residues in the soil but also cause resistance and resurgence problems in the environment.

In the changing global scenario, the role of Si becomes more important for a higher yield with sustained productivity. Silicon-solubilizing bacteria (SSBs) could be significant in solubilizing not only the insoluble forms of silicon but also potassium and phosphates, therefore enhancing soil fertility and enhancing crop productivity (Maleva et al. 2017). Phosphorus (P) and potassium (K) are key elements for growth and development of plants, and P and K fertilizers are commonly applied in soluble form to obtain optimum yields. This strategy is especially important for reclamation of infertile or degraded soils that are not suitable for sustainable agriculture. Various researchers have reported the effect of SSBs on nutrient uptake from the soil, and their positive influence on photosynthesis and the growth of some crops (Han et al. 2006). The addition of SSBs-enriched bio-fertilizer to a clay substrate significantly increased the thickness of the mesophyllic layer, the number of mesophyll cells, plastid material volume, photosynthetic rate, and photosynthetic pigment content in the leaves of *Brassica juncea* (Fig. 6.1) (Maleva et al.

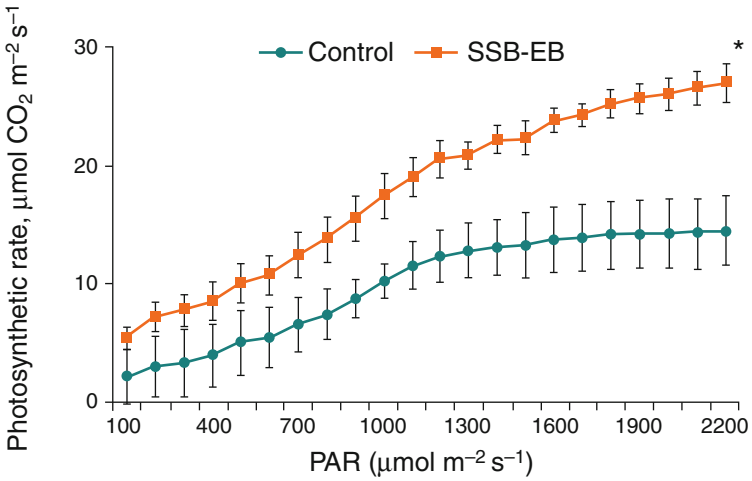

Fig. 6.1 Photosynthetic rate in the leaves of *Brassica juncea* affected by silicon-solubilizing bacteria (SSB)-enriched bio-fertilizer (Maleva et al. 2017)

2017), providing enhanced CO_2 uptake by Indian mustard. Consequently, we can conclude that bio-fertilizer based on SSBs improved the photosynthetic activities of *B. juncea*. Changes in the studied parameters of mustard plants grown with added silicon-solubilizing bacteria (SSB)-enriched bio-fertilizer (EB) can result from increasing the available forms of macro-nutrient content in the substrate by solubilization of clay silicates, as confirmed by enlargement of the total P and K concentration in the leaves of *B. juncea* (Maleva et al. 2017). Pedda et al. (2016) found that maximum grain yield (3622 kg/ha) was obtained with the application of SSB + FYM followed by FYM (farmyard manure) and SSB alone.

Uses of Zn partly cater to plant needs as 96–99% of supplied Zn is converted into various insoluble forms, depending on soil types and physicochemical reactions in the 7 days of application (Saravanan et al. 2004). Soil microorganisms are potential options that could serve Zn needs by solubilization of the complex Zn available in the soil. Many soil microbes, such as *Pseudomonas* spp. and *Bacillus* spp., are observed to solubilize Zn. Microorganisms solubilizing the metal form by chelated ligands, and oxido-reductive and proton systems, are present on the surface of cells and membranes (Crane et al. 1985; Wakatsuki 1995). These bacteria also showed different beneficial traits for plants, such as the formation of vitamins, antifungal substances, phytohormones, antibiotics, hydrogen cyanide, and siderophores (Rodriguez and Fraga 2004).

Similarly, Zn deficiency is a common issue in plants as well as human beings. Its shortage in plants checks nitrogen metabolism and photosynthesis, decreases flowering and fruit setting, reduces the synthesis of phytohormones and carbohydrates, and delays crop maturity, resulting in reduced crop yield and seed quality. Chaudhary et al. (2007) observed that Zn deficiency is the key determination of paddy production in many parts of the country. Almost 50% or more of the world's soils that are under a cereal-based cropping system have lower available Zn, which causes reduced yield and quality of seeds and grains (Welch and Graham 2004). Zn is required for all living forms including plants, humans, and microorganisms (Kumawat et al. 2013a, b; Kumar and Bohra 2014). All humans and macro- and microorganisms need Zn in small quantities throughout life to complete their physiological activities (Kumar et al. 2018), and Zn is also an important micronutrient for the life cycles of plants (Kumar et al. 2015a).

The main aim of bio-fortification is to produce plants having augmented content of bio-available nutrients in the consumable portions (Kumar et al. 2017). Cereals and other staple plants are the main food for the larger part of the world's population but these may have shortage in micronutrients, from a nutritive outlook, having less Zn and other required plant nutrients (Kumar et al. 2015b, c). Under the process of bio-fortification the major drawback is the root or shoots barriers and the process of grain filling (Kumar et al. 2016a). Research has shown different possible ways to combat these situations. The distribution of Zn can be mainly controlled by heavy metal transport of P-ATPase and the metal tolerance protein family (Kumawat et al. 2012, 2015; Kumar et al. 2016b, c). For a better understanding of Zn transport, mechanisms are needed to enhance grain quality and to reduce the deposit of hazardous metals (Kumawat et al. 2017). Most soils are either Zn deficient or the Zn content is in a fixed form not available to plants; thus, in these soils, a Zn

deficiency appears. Zn deficiency is more frequently found in paddy fields, soils having a higher level of P and Si, and highly weathered acid and coarse textured, neutral, sandy, and calcareous soils (Kumar and Meena 2016). Zn deficiency may be related to the properties of the soil, as in calcareous soils. If Zn is present in soils at less than 10^{-11} to 10^{-9} M, plant growth may be affected (Saravanan et al. 2007). In 70% of the soils in the Pakistan, Zn deficiency has been reported (Shaikh and Saraf 2017), and Zn deficiency has been found in 50% of the cultivated lands in China. Available Zn is mainly found in the form of sphalerite (ZnS); low-Zn-containing minerals include zinkosite ($ZnSO_4$), zincite (ZnO), hopeite [$Zn_3(PO_4)_2.4H_2O$], franklinite ($ZnFe_2O_4$), and smithsonite ($ZnCO_3$).

6.2 Significance of Bio-inoculants in Sustainable Agriculture

Bio-inoculants are the most important factor of sustainable agriculture, having living microorganisms with the capacity to solubilize/mobilize important plant nutrients from unavailable to readily available forms by microbial paths. Bio-fertilizers have comes to stay in Indian farming in the past three decades regarding low cost, significance to crop production, and health of the soil as well as their eco-friendly nature. Use of bio-fertilizers is a key component for integrated nutrient management (INM) as these are renewable sources of nutrients to supplement synthetic fertilizers for a sustainable farming system. Bio-fertilizers include nitrogen-fixing microbes (NFM) (*Rhizobium*, *Bradyrhizobium*, *Azotobacter*, *Azospirillum*), phosphorus-solubilizing microbes (PSM) (*Aspergillus*, *Bacillus*, *Pseudomonas*), phosphate-mobilizing microorganisms (mycorrhizae) (PGPR), and potassium-solubilizing microorganisms (KSM), ZSBs, and SSBs. For their metabolism, growth, and development, plants require different types of nutrients. Microbes have significant roles in solubilization of nutrients required by the plants. Among the essential plant nutrients, the micronutrient Zn is a most important plant nutrient that is essential for healthy development and better reproduction for all the plants. *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* are facultative thermophilic iron oxidizers that solubilize Zn from sulfide sphalerite (Hutchins et al. 1986). Zn is a plant nutrient source when it is in low concentration, but at higher doses it may be toxic to plants as well as human beings. The solubilizing of Zn might have extended the growth of bacteria at higher doses. Unless media tolerate higher doses of Zn, its solubilization will not be continued. A few fungi groups have the capacity to solubilize Zn; among them, *Aspergillus niger* was reported to grow in 1000 mg Zn, so this fungi is used to quantify Zn in soils having low Zn (2.0 mg/kg Zn) (Bullen and Kemila 1997).

Microorganisms present in the root zone of different plants produce or release auxins as secondary products/metabolites because of higher proving of substrates exuding from the roots in comparison to non-rhizospheric soil. Bacteria of the genera

Pseudomonas, *Azospirillum*, *Rhizobium*, *Xanthomonas*, *Enterobacter (cloacae)*, *Alkaligenes (faecalis)*, and *Acetobacter diazotrophicus*, and a few fungi and algae, are able to produce auxins, which exert a pronounced effect on plant growth and development (Patten and Glick 1996). Indole acetic acid (IAA) is also an important physiologically active auxin. Several microorganisms produce L-tryptophan metabolism. IAA is also produced by ZSBs that may be also having some effect on growth of different plant species (Rajkumar and Freitas 2008).

6.3 Plant Nutrients

Crop plants require different nutrients in different quantities for their growth and development. Plants obtain these essential nutrients from soil, water, and air. Some of these nutrients are required in large amounts, whereas for others small quantities are adequate for the vegetal and reproductive growth stages of the crop plant. Seventeen nutrients are essential to healthy growth and development of plants. The macronutrients are nitrogen (N), phosphorus (P), potassium (K), sulfur (S), calcium (Ca), magnesium (Mg), carbon (C), hydrogen (H), and oxygen (O); the micronutrients are copper (Cu), iron (Fe), boron (B), manganese (Mn), molybdenum (Mo), chlorine (Cl), zinc (Zn), and nickel (Ni). These plant nutrients are generally divided into three major categories. In the first category are the three macronutrients, carbon (C), hydrogen (H), and oxygen (O), which can taken up from water, air, or both by the plants. These nutrients do not need to be provided by the soil; therefore, synthetic fertilizer is not needed. The remaining 14 essential plant nutrient categories are soil-originated macronutrients and soil-originated micronutrients. The soil-originated macronutrients are N, P, K, S, Ca, and Mg; and the soil-originated micronutrients are B, Cl, Cu, Fe, Mn, Mo, Zn, and Ni.

Micronutrients are generally needed in relatively small amounts (100 mg/kg dry weight) by plants, but have significant roles in cellular and metabolic activities such as energy metabolism, gene regulation, signal transduction, and hormone perception (Tripathi et al. 2015). Many micronutrients are major ingredients for essential amino acids and enzyme complexes in crop plants and microbes. The low levels of the S-containing amino acids, methionine and cysteine, in major food crops limit Zn bioavailability; thus, it would be worthwhile to increase the level of these amino acids in these food crops to overcome the negative effect of anti-nutritives on Zn bioavailability (Johaning and O'Dell 1989). If deficiency of one or more micronutrients can affect viral and bacterial pathogens, chlorosis, necrotic disease, increased vulnerability to fungi can stunt plant growth, affecting the productivity and health of plants. These micronutrients are mostly limited in availability in the soil from many causes such as low availability or deficiency, soil type, flooded/dry situations, drainage, soil texture, soil pH, moisture availability, and weather conditions (Imran and Gurmani 2011).

6.4 Silicon (Si)

In the universe, by mass silicon is the eighth most important key element; however, it is very rarely found as a pure free element in nature, having a wide range of distribution in the form of silicon dioxide or silicates. The most prevalent element in the Earth's crust is oxygen; the second most prevalent element is Si, included in more than 25% of the Earth's crust. In fact, the concentration of Si in soil is equal to macronutrients such as K and Ca and is also well in excess of P levels. Silicon is a tetravalent cation (Si^{4+}) with atomic number 14, oxidation states of +2, +4, and −4, and molecular weight 28.09. It does not react with acids, except hydrofluoric acid. On the periodic chart, silicon is surrounded by near-neighbours B, C, N, O, P, and S. It is interesting to notice that all these neighbours are found to be essential elements whereas Si is identified as necessary only for plants (Gascho 1978).

Most Si is commercially used in Portland cement to make concrete, ceramics such as porcelain, traditional quartz-based glass, and synthetic polymers. In the modern era, a large amount of Si is utilized in steel refining, aluminum casting, chemical industry, semiconductor electronics, and integrated circuits for computers on which modern technology is greatly dependent. Silicon is an essential element in biology. In trace quantities, it is needed by animals, but various sea sponges and microorganisms such as diatoms and radiolarians secrete a skeletal structure composed of Si. Silicon is often deposited in plant tissues in all parts of most of the crops and plants in the universe. Silicon is a functional nutrient although it is not considered as an essential nutrient in crops; therefore, a systematic survey of Si status in soils and its relationship with soil properties, response of applied Si on growth characters, yields, juice quality, nutrient uptake, disease, pest resistance, etc., would be of practical importance.

6.4.1 Significance of Si in Plants

Silicon is mainly available to plants in the form of monosilicic acid [$Si(OH)_4$], which is absorbed by the plant roots from soil water. The element is then deposited as amorphous silica throughout the plant, mainly in the cell walls. Si is identified as a major constituent of soils. Si alleviates abiotic stresses such as radiation, lodging, drought, freezing, high temperatures, and ultraviolet, and composite stresses such as nutrient imbalance, metal toxicity, and salt tolerance (Epstein 1994). It aids in drought resistance by maintaining the photosynthetic rate, erectness of leaves, water balance, and structure of the xylem vessels in higher transpiration rates, mainly the result of higher temperatures and moisture deficiency (Hattori et al. 2005). The role of Si in plants is multifunctional. It aids the strength and thickness of cell walls, keeps plants upright, and positions the leaves for good light interception. Many plants such as rice, sugarcane, and tomato actually require Si as an essential element, although in many species Si has been shown to offer such growth benefits as

increased absorption and translocation of several macro- and micronutrients (Meena et al. 2014b, c). The concentration of Si in plant species ranges from 0.1% to 10% (Epstein 1994). SSBs secrete many organic acid compounds as a part of its metabolism that has a double role in Si weathering. SSBs release H^+ ions to the medium and stimulate hydrolysis and organic acids including keto-acids, oxalic acid, citric acid, and hydroxyl carbolic acids that bond with cations and are made easily available to the plant. Joseph et al. (2015) observed a few identified bacteria that can by solubilization or mobilization change insoluble minerals (silicates, phosphates, potash) into readily available forms by releasing many organic compounds such as 2-ketogluconic acid, polysaccharides, and alkalis. Barker et al. (1998) found that many microbes are made available to silicates by developing organic ligands, hydroxyl anions, protons, extracellular polysaccharides, and enzymes. Seven crops are Si accumulators among plant species that accumulated more than 1.0% Si on the basis of dry matter (Hodson et al. 2005). Worldwide, 210–224 Mt Si/year is removed by crops (Savant et al. 1997). Narayanaswamy and Prakash (2009) reported that total Si removed by paddy plants grown in Inceptisol soils ranged from 205 to 611 kg/ha.

6.4.2 *Dynamics and Occurrence of Si in Soils*

Using plant ash to improve the fertility status of degraded soils was suggested by the Roman Empire poet and scientist Virgil (Vergilius). Chinese scientists applied parts of paddy straw to the soils. In the China Kingdom, there were few fertilizers that could be classified as Si fertilizers, and plant ash was named '*Burning Manures*.' Jons Jacob Berzelius discovered Si as an element in 1824, and he was the first person to study the interaction of silicon and organic matter in nature (Mathew et al. 2004). Silicon is the second most important element in the Earth's layers, almost exclusively present in the form of silicon dioxide (SiO_2) in association with the wide arrays of Si-bearing minerals in crystalline, poorly crystalline, and amorphous phases (Sommer 1926). An average of 28% Si by weight ranging from 0.52% to 47% was found in the pedosphere of the Earth's crust. Minerals of Si are commonly found in carbonaceous rock such as carbonites and limestones, whereas rocks such as orthoquartzite and basalt have a high content of Si (23–47%) (Wedepohl 1995; Monger and Kelly 2002). Silicon content ranges from 200 to 300 Si g/kg in clay soils and 450 Si g/kg in sandy soil (Kovda 1973; Matichenkov and Calvert 2002). Silicon in soils varies from 1.0% to 45% on a dry weight basis (Sommer et al. 2006). Silicon is the key fertilizer for growing crops, enhancing soil resistance to environmental stress (Liang et al. 2005). Weathering of silica minerals is the end source of dissolving Si (monosilicic acid, H_4SiO_4), which contributes to continental soils by linked biogeochemical processes (Basile-Doelsch et al. 2005). Silicon releases to the soil from weathering of silicate-containing minerals are rather slow and are controlled by precipitation and neo-formation of authigenic Si components, uptake and assimilation by plants and microorganisms, preservation of stable Si forms in the profile, and addition to external atmospheric input (Fig. 6.2) (Cornelis et al. 2011).

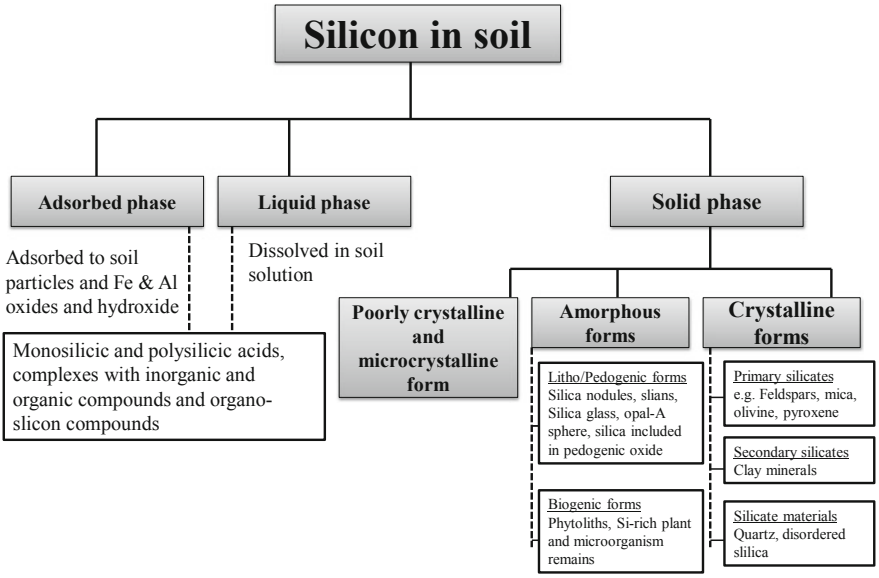

Fig. 6.2 Different fractions of Si in soils (Tubana and Heckman 2015)

These are linked processes, and the largest inter-pool Si transfer takes place between biomass (biogenic silica and microorganisms) and soil solution (at rates ranging from 1.7 to 5.6 $\times$ 10^{12} Si kg/year. In the oceans, the largest inter-pool Si transfer is between biogenic silica from diatoms and dissolved Si at 6.7 $\times$ 10^{12} Si kg/year (Tréguer et al. 1995; Matichencov and Bocharnikova 2001). It is assumed that the average quantity of Si is transformed into biogenic silicas at 2.5 $\times$ 10^{12} Si kg/year (Laruelle et al. 2009).

6.4.3 Si-Solubilizing Bacteria (SSBs)

Many microorganisms are present in soil, but few are capable of solubilizing silicon. *Proteus mirabilis*, *Bacillus caldolyticus*, *Pseudomonas*, and *Bacillus mucilaginosus* var. *siliceous* were observed to be most suitable to solubilize Si from natural silicates (Meena et al. 2014a, b, c). These SSBs are capable of decomposing silicates, mainly Al_2SiO_5. These microbes secrete many organic substances during their growth period that can assist in weathering, also freeing K from K-containing minerals. Solubilizing of silica minerals by microorganisms is considered as a good source of Si to be provided for vegetation. These microbes enhance the growth characteristics, chlorophyll value, 1000-grain weight, filled grains, and biological yield of paddy crop (Avakyan et al. 1986). Use of SSBs in soil gave greater yields of potato, wheat, maize, and tomatoes and increased the microbial population in the maize rhizosphere (Fig. 6.3) (Aleksandrov 1958).

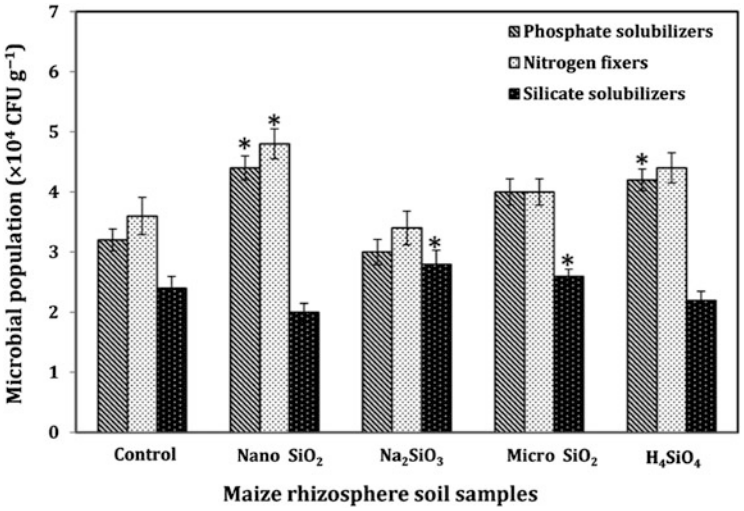

Fig. 6.3 Effect of silica sources on the microbial population in maize rhizosphere

6.4.4 Mechanism of SSBs

Silicon-solubilizing microbes secrete many organic acids during their metabolism activities that help in weathering of silicates. These organic acids provided H^+ ions to the medium and stimulated hydrolysis to produce acids such as oxalic acid, keto acids, citric acid, and hydroxy carbolic acid, which complex with cations and are rendered readily available to plants (Fig. 6.4). Barker et al. (1998) found that microbes are made readily available to silicate minerals by releasing of hydroxyl anions, protons, organic ligands, cellular polysaccharides, and enzymes. These bacteria alter silicates into soluble Si. SSBs increase the availability of soil nutrients, although Si is considering as a nutrient "anomaly" (Epstein and Bloom 2005). Actually, the biotic mechanism behind nano-silica uptake and its influence on soil microbes and silica availability requires thorough investigation. The maximum microbial population was found under the source of nano-silica (Fig. 6.5). Silicon concentrations in both plants and soils are pivotal in establishing the effect of Si, when applied as another silica source. Analysis of soil nutrients added with sodium silicates and calcium silicate has been done by Nanayakkara et al. (2008). All the same, findings on the influence of unique size-dependent qualities of nano-silica on soil microorganism populations and changes in soil silica content are meager. Although the effect of Si nano-particles on corn crop growth was shown in an earlier study, an in-depth assessment of the bio-components of the soils and possible utilizable mechanism of silica is lacking (Epstein and Bloom 2005). Growing some crops with poor management practices decreases Si concentration in soil, resulting in lower yields. In addition, soil microorganisms have great ability for converting various Si sources into a form readily taken up by the plants

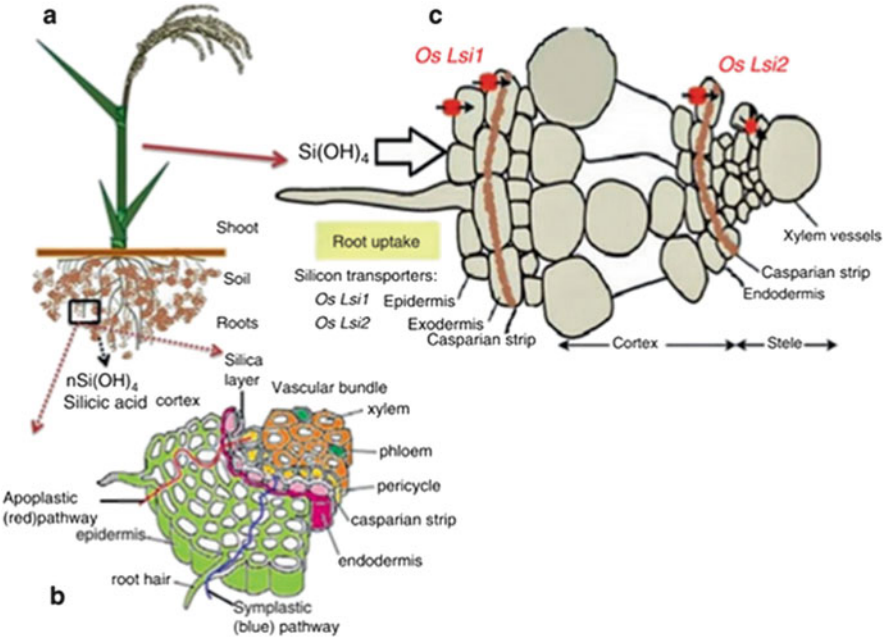

Fig. 6.4 Silicon transport mechanisms in plants

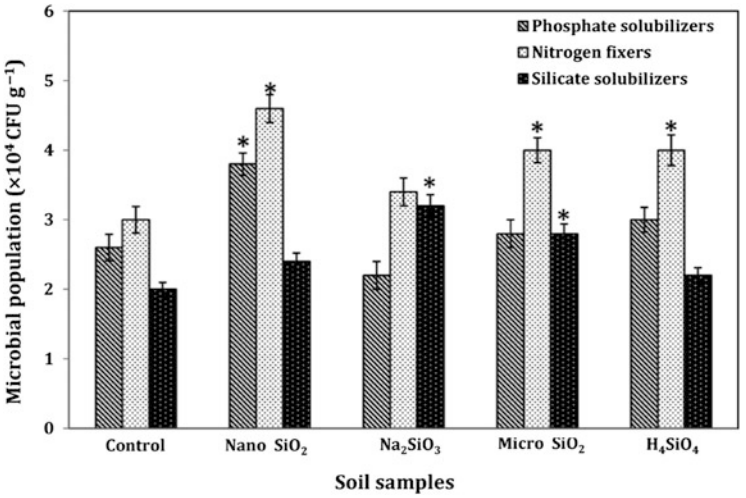

Fig. 6.5 Effect of silica sources on the microbial populations in soil

Table 6.1 Yields of sugarcane as influenced by different sources of silicon (Phonde and Banerjee 2015)

Source of silicon	Millable cane height (cm)	Cane yield (t/ha)	Sources content (%)	Commercial cane sugar yield (t/ha)	Benefit:cost ratio
Control	210.54	89.20	20.53	13.26	2.92
Bagasse ash	218.49	98.90	20.36	14.55	3.22
Fly ash	212.73	106.06	20.59	15.79	3.38
Pond ash	213.16	111.79	20.41	16.44	3.56
Calcium silicate	210.49	106.65	21.07	16.20	3.19
2.5% K_2SiO_3 spray	212.40	102.07	21.03	15.58	3.26
SEM±	4.78	3.1	0.23	0.54	0.10
CD ($P = 0.05$)	NS	9.7	NS	1.68	0.30

(Muralikannan and Anthoniraj 1998). Unfortunately, Si sources are not effectively taken up by the plants because they are not a direct source. Actually, synthetic silica-based fertilizer production cost is high and the uptake of silica is very slow (Table 6.1).

6.4.5 Si-Induced Mechanisms of Plant Resistance to Stress

Monosilicic acid or orthosilicic acid (H_4SiO_4) are the Si forms that are taken up by the roots of plants. Knight and Kinrade (2001) suggested that H_4SiO_4 concentration in soil solution ranges from 0.1 to 0.6 mM at the pH levels of most agricultural soils. Uptake of monosilicic acid or orthosilicic acid by lateral roots is via an active, passive, and rejective mechanism (Cornelis et al. 2011). It is considered that in higher Si accumulators the quantity of monosilicic acid adsorbed by active mechanisms is greater than content uptake by mass flow because of the higher density of Si transporters in roots and shoots, facilitating monosilicic movement across the root cell membranes. In rice crops, both radial transport and xylem loading of H_4SiO_4 are mediated by transporter Lsi1 and Lsi2 in roots and Lsi6 in shoots (Mitani and Ma 2005; Ma et al. 2007). Takahashi et al. (1990) classified plants as high accumulators, intermediate accumulators, or non-accumulators according to active, passive, and rejective absorption mechanisms, respectively. However, it was based solely on measuring Si in the leaves and does not measure this element daily in other parts of the plant. Some crops, including crimson clover (*Trifolium incarnatum*), coffee (*Coffea*), green onions (*Allium cepa*), radish (*Raphanus sativus*), Chinese cabbage (*Brassica rapa*), peppers, and tomatoes are now known to have more Si content in their roots than in the shoots (French-Monar et al. 2010; Huang et al. 2011). Thus, it is believed that rooting of all plants in the soil will have Si in their plant tissue and that the Si content may be greater than that of other essential minerals. Therefore, it is

not likely that there are plants that do not accumulate silica. Many workers continue to find that plants are categorized as high accumulators (10–100 g/kg, dry weight basis), and more are monocotyledons, such as rice, barley wheat, and sugarcane (Liang et al. 2007). Intermediate silica accumulation crops have 5–10 g/kg dry weight and are also monocotyledons, whereas dicotyledon plants, with less than 5 g/kg by dry weight, are low accumulators. The monosilicic acid taken up by root cells is accumulated in the leaf epidermal cells. If water is removed, the deposited H_4SiO_4 in the leaf becomes thickened into a hard polymerized silica gel (SiO_2. nH_2O), known as phytoliths. The accumulated Si in the leaf epidermal cells is immobile and cannot be translocated to new emerging leaves of the plants (Raven 1983).

Many reports are available regarding the benefits of silica in plants. Mainly, Si helps maintain productivity of plants under stressed situations (Epstein 1999; Li et al. 2007). The presence of the Si-induced mechanism enhanced plant resistance against natural and environmental stresses in the soils, root systems, and inside the plants. Some of the known mechanisms and actions that are involved externally and internally for induced plant resistance to more stresses are included in Table 6.2. The code position of Si and metals such as Al, Mn, and Cd in either soil and root solution and in the plants decreases concentrations of free metals at toxic levels in vegetation. Si-precipitated metals are not easily moved up, which reduces their toxic influence on plants (Richmond and Sussman 2003).

6.5 Zinc (Zn)

Zinc deficiency is the most common micronutrient deficiency and significantly affects crop production. It is an essential micronutrient needed by plants for higher growth, reproduction, and nutritional value. Zn is available in soils in the inorganic form, which commonly is not an available form for plant assimilation. It is found as a free element that drives and increases the rates of metabolic reactions in crop plants (Parisi and Vallee 1969). The levels of Zn in plant materials are very low, commonly in the order of 100 ppm or less in dry weight. The Zn requirement of plants is correspondingly small. Zn taken up by plants is less than 0.5 kg/ha/year. Zn concentrations ranging from 150 to 200 μg/g in dry weight is considered toxic to plants (Sauerbeck 1982). In practice, Zn deficiency is easily corrected by foliar spray or soil application through Zn-containing fertilizers. Application is usually in the range of about 5.0 kg/ha, which is effective for 3 years. $ZnSO_4$ is the most commonly used fertilizer because it has high solubility in the soil. Many microbes (bacteria) that are associated with roots of plants have great potential to improve plant growth and productivity through supplying mineral nutrients that are less mobile in the soils, such as Zn: these are the zinc-solubilizing bacteria (ZSBs) (Gandhi and Muralidharan 2016).

Table 6.2 Proposed silicon (Si) mechanisms associated with improved tolerance of plants to biotic and abiotic stresses

Number	Mechanisms	Specific actions
1.	External or involved in soil and root in preventing excessive uptake of metal	High [H_4SiO_4] increases soil pH, precipitates metal, e.g., Al, Cd, Fe, Mn (Lindsay 1979) H_4SiO_4 adsorbs Al hydroxides, diminishing the activity of Al in solution (Baylis et al. 1994) Mobile Al is strongly adsorbed on surfaces of silica (Schulthess and Tokunaga 1996) Si induces oxidizing capacity of roots facilitating the conversion of plant-available Fe^{2+} to a less plant-preferred Fe^{3+} (Ma and Takahashi 2002) Si induces release of OH^- by roots, which raises soil pH (Wallace 1993)
2.	Reinforces plant protective layer and mechanical structure	Silica in shoots enhances structural component of plant and creates a hard outer layer (Bélanger et al. 2003) Improves overall mechanical strength and protective layer of plant (Hayasaka et al. 2008)
3.	Mediated/primed mechanisms of defence	Increased production of glucanases, phytoalexins, and PR-1 proteins (Rodrigues et al. 2004, 2005) Enhanced deposition of phenolic-based compounds (Bélanger et al. 2003; Rodrigues et al. 2003) Up- and downregulation of a number of unique defensive and metabolic genes (Brunings et al. 2009; Ghareeb et al. 2011) Interferes with the synthesis and/or action of fungal ethylene (Van Bockhaven et al. 2014) Sequestration of cations and enhancing activity of some protein molecules (Fauteux et al. 2005)
4.	Internal or in planta	Enhances plant antioxidant systems (Inal et al. 2009) Silica deposits in cell wall react (co-precipitate) to heavy metals, impairing their translocation inside the plants (Richmond and Sussman 2003; Ma et al. 2007) Prevents accumulation of Na of salt-stressed plants through Si-induced reduction in transpiration (Yeo et al. 1999)

6.5.1 Significance of Zn in Plants

Zinc is a key element for plants with a significant role in structural constituents or regulation cofactors of a wide range of various enzymes activated by Zn that are involved in carbohydrate metabolism, maintenance of cellular membrane integrity, protein synthesis, regulation of auxin synthesis, and pollen formation (Alloway 2008). Zinc is also necessary for the integrity of cellular membranes to preserve the structural orientation of macromolecules and the ion transport system. Its interaction with phospholipids and sulfhydryl groups of membrane proteins contributes to the maintenance of membranes. Zinc is essential for the synthesis of tryptophan, a precursor of IAA, and is also active in the production of growth hormones such as auxins (Cakmak 2000). Zinc seems to affect the capacity for water uptake and transport in plants and also reduces the adverse effects of short periods of heat and salt stress.

6.5.2 Zn Status in Soils

Soils inherit their minor elements, including zinc, mainly from rocks through geochemical and pedo-chemical weathering processes. The average Zn content of the lithosphere zone is about 800 ppm (Goldschmidt 1954). Zn is generally found in the range from 10 to 300 mg/kg in many minerals. The level of Zn in soils is very much related to the parent materials. Soils derived from granite and gneiss can be low in total Zn (Helmke et al. 1977). Similarly, total Zn is low in highly leached, acid, or sandy soils such as those found in many coastal areas. Quartz in the soil dilutes Zn because the reported concentrations of Zn in quartz are very low, from 1.0 to <5–8 μg/g (Alloway 2008). Zn deficiency is becoming the most common nutrient problem; any practices that enhance Zn uptake and its transportation to sink have significant practical relevance. The presence of Zn in the soil depends on pH, type, intensity of weathering, climate, and other predominating factors during the process of soil formation (Saeed and Fox 1977). Zn deficiency can be found in every part of the world, and almost all crops respond positively to application of Zn. The deficiency occurs in a wide range of semi-arid areas: calcareous types of soils, tropical regions with highly weathered soils, and sandy-textured soils in several different climatic zones tend to be more seriously affected. More than 30% of the cultivable lands of the world contain a low level of Zn (FAO).

Zn is the essential micronutrient for all plants and microorganisms on Earth. Zn occurs in the Earth layers at 0.008%. It is significant in nutrition for prokaryotic and eukaryotic microorganisms as cofactor or metal activator in various enzymatic processes (Hughes and Poole 1991). Zn deficiencies are observed worldwide, mainly under the rice-based ecosystem of Asia Pacific regions (Tisdale et al. 2009) and in different orders of soils such as aridisols, mollisols, vertisols, and alfisols (Srivastava and Gupta 1996). The lowest Zn content in soils was found in spodosols (28 mg/kg), mollisols (30 mg/kg), luvisols (35 mg/kg), and vertisols (36 mg/kg); higher levels were found in ultisols (43 mg/kg), alfisols (44 kg/ha), entisols (47 mg/kg), histosols (58 mg/kg), fluvisols and inceptisols (60 mg/kg), aridisols (61 mg/kg), and oxisols (72 mg/kg) (Katyal and Sharma 1991; Kiekens 1995). More than 90% Zn in soils is available in the insoluble form and cannot be adsorbed by plants, whereas exchangeable Zn ranges from 0.1 to 2 mg/kg in soils (Singh 2011). In India, the total area under Zn deficiency is about 10 million hectares (ha). In the Indo-Gangetic Plains regions, about 85% of the area is under rice-wheat cropping systems, and their yield limiting factor is Zn, mainly because of calcareous and alkaline soils. In India, soybean-wheat systems removed around 7 tonnes grain/ha/year Zn from the soils and total uptake was about 416 g/ha/year. Indian soils showed deficiency around 50%, which is below the critical limit (0.5 mg/kg of available Zn) (Prasad 2010).

6.5.3 Roles of Zn in Plants

Among the micronutrients, Zn is an essential element present in enzymatic systems as cofactor and metal activator of various enzyme activities. Plant growth promotion requires Zn is an important essential micronutrient as it is a key part of many

metabolic enzymes, and its poor translocation in plants advised that a fixed supply of available zinc be obtained for proper growth and development of plants. Zinc is the first element known to be essential for human, animals, plants, and many microbes (Kabata-Pendias 2000). It is also required for regulation of carbonic anhydrase for fixation to carbohydrate in crop plants (Tisdale et al. 1984). Zn finger transcription factors are required for the development and function of floral tissues such as anthers, tapetum, pollen, and pistil secretary tissues in many plants (Marschner 1995).

Zn is a component of the active catalytic centre of the enzyme carbonic anhydrase, which increases the rate at which equilibrium is achieved between CO_2 and bicarbonate ions in solution. The reaction is very fast (a turnover time of 10^{-6}) and, therefore, the concentration of the enzyme and thus of zinc of this particular component of leaf tissue is very small (Rains 1976). It has more influences on plant life processes such as nitrogen metabolism, uptake of nitrogen, and quality of protein; chlorophyll synthesis and photosynthesis; and tolerance to biotic and abiotic stresses (Potarzycki and Grzebisz 2009). Zn shows superiority against plant insect pests and in disease resistance, protein metabolism, photosynthesis, pollen development, and cell membrane integrity (Kumawat et al. 2015; Gurmani et al. 2012) and improved levels of antioxidant enzyme and chlorophyll content in tissues of plants (Sbartai et al. 2011). An inadequate supply of Zn will reduce production, productivity, and quality attributes of produce. Thus, for proper growth and development of vegetation or plants, a fixed minimum level of Zn is essential.

6.5.4 Deficiency of Zn in Plants

Zinc is an essential nutrient for plants in a very small amount. In Zn uptake by plants from soils, adequate levels of dissolved Zn are needed for optimal growth of crops (Reed and Martens 1996). Necessary Zn for optimal growth and development of plants is 15–20 mg/kg dry weight (Marschner 1995). Deficient Zn levels are usually about 0–15 mg/kg dry weight (Boehle and Lindsay 1969). That Zn is essential was first discovered in maize, which is known as “white bud” (Maze 1915); in maize crops, chlorotic bands developed on either side of the leaf midrib. Zn deficiency was previously reported in rice crops by Nene (1966) at GBPUAT (Govind Ballabh Pant University of Agriculture and Technology), Pantnagar, India. Because Zn is associated with many enzymes, its deficiencies cause various disorders in the plants. In young plants, interveinal areas have dark brown necrotic lesions. These areas may be pale green, yellow, or white. The deficiency symptoms first appear on young leaves as zinc is immobile under conditions of deficiency. These leaves remained small, cupped upward, and developed interveinal chlorosis and necrotic spots on the top of the leaf surface which later merge to make a brown necrotic and brittle patch. The most common features of Zn deficiency in plants include stunted growth, smaller leaves, shortened internodes and petioles, chlorosis, pollen sterility, and spikelet sterility. Zn deficiency can have a negative impact on grain quality; plants

susceptible to injury by excessive light or temperature and to infection by fungus diseases may also increase (Cakmak 2000). The most identifiable symptoms in plants is loss of turgidity, where the plants fall over and float on the water surface. Zn deficiency may also affect the uptake and flow of water into vegetation and reduce the negative effects of short or long spells of temperature and salinity stresses (Tavallali et al. 2010; Peck and McDonald 2010). Zn deficiency also has an important role in the inhibition of RNA synthesis. Many more symptoms and responses by plants lead to Zn deficiency, as follow: mottled leaves from interveinal chlorosis, wilting caused by loss of turgidity in the leaves, and basal chlorosis of leaves, delayed development of the plant, and "bronzing" of leaf (Tripathi et al. 2015).

6.5.5 Zn-Solubilizing Bacteria (ZSBs)

Zn-solubilizing microorganisms have great potential as compared to chemical sources of plant nutrients such as fertilizers. Use of microorganisms in sustainable crop production and restoration of fertility is gaining more interest. Zn-solubilizing microbes have been discovered from the soils of many crops and tested as plant growth-promoting factors (Goteti et al. 2013; Sunithakumari et al. 2016). Within 7 days of application, applied Zn fertilizers partially cater the plant need as 96–99% of given zinc is converted into various insoluble forms; this mainly depends on the type of soil and physicochemical reactions (Saravanan et al. 2004). Hence, the insoluble form of Zn can be converted into soluble form by treated bacterial cultures with the ability for Zn solubilization. This shortage can be managed by zinc-solubilizing microbes, which have great ability to convert many unavailable forms of metals to a readily available form. These microbes can convert unsolubilized zinc such as zinc phosphates, zinc oxide, and zinc carbonates in good amounts, which is not a common feature among the microbes in the top surface soils (Cunninghan and Kuiack 1992). ZSBs are capable alternatives that can cater essential zinc to plants through solubilizing complexed zinc into soils. Several genera of microbes, such as *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Thiobacillus thiooxidans*, and *Thiobacillus ferrooxidans*, have been found as Zn solubilizers (Saravanan et al. 2007). The solubilized metals are formed by chelated ligands, protons, and the oxido-reductive system available on cell surfaces and in cell membranes. These microbes have many beneficial features to the plants such as producing phytohormones, siderophores, vitamins, antibiotics, and antifungal substances (Goteti et al. 2013). Rosas et al. (2009) found that when seed is treated with *Pseudomonas aurantiaca* in sandy loam soil in Argentina the grain yield of wheat increases by 36%. A positive correlation between Zn content and protein content in grain was observed by Cakmak et al. (2010). Seed inoculation with ZBS improved methionine content in the grains of wheat varieties compared to no inoculation; Zn inoculants may help to better Zn bio-availability and to produce better grains. Goteti et al. (2013) reported that seed inoculated with *Bacillus* and *Pseudomonas* increased in root volume (RV), shoot length (SL), total dry matter (TDM), leaf areas (LA), and also nutrient content in the

Table 6.3 Biometric growth parameters of maize treated with Zn-solubilizing bacteria (ZSBs) and inorganic sources of Zn

Treatment	Root volume (RV) (ml)	Shoot length (SL) (cm)	Total dry matter (TDM) (g)	Leaf area (LA) (cm^2)
Control	9.8^{j} (±0.45)	78.8^{h} (±3.63)	9.16^{h} (±0.422)	627.7^{i} (±28.93)
$ZnSO_4$	13.8^{h} (±0.64)	8.51^{fg} (±3.92)	15.25^{a} (±0.703)	1161.3^{a} (±53.52)
Priming	15.0^{fg} (±0.69)	96.0^{c} (±4.42)	12.87^{b} (±0.593)	861.0^{f} (±39.68)
B61	15.0^{fg} (±0.69)	97.8^{b} (±4.51)	11.36^{d} (±0.523)	908.3^{e} (±41.86)
B40	15.7^{de} (±0.72)	92.1^{d} (±4.24)	11.98^{c} (±0.552)	955.5^{d} (±44.04)
B116	16.7^{c} (±0.77)	110.1^{a} (±5.07)	12.78^{b} (±0.589)	1113.8^{b} (±51.33)
B114	16.2^{cd} (±0.75)	92.4^{d} (±4.26)	9.81^{fg} (±0.452)	901.7^{e} (±41.56)
B118	16.3^{c} (±0.76)	89.0^{e} (±4.10)	12.08^{c} (±0.557)	1041.8^{c} (±48.02)
P33	15.3^{e-g} (±0.71)	95.8^{c} (±4.42)	12.08^{c}(±0.557)	982.5^{d} (±45.28)
P29	18.3^{b} (±0.84)	84.7^{fg} (±3.90)	12.96^{b} (±0.597)	1147.5^{ab} (±58.02)
P74	14.8^{g} (±0.68)	75.5^{i} (±3.48)	10.13^{f} (±0.467)	851.7^{fg} (±39.25)
P17	9.8^{j} (±0.45)	73.5^{i} (±3.39)	7.38^{i} (±0.340)	611.8^{i} (±28.2)
P21	19.8^{a} (±0.91)	96.0^{c} (±4.43)	10.61^{e} (±0.489)	790.7^{h} (±36.44)
ZSB	12.8^{i} (±0.59)	86.3^{f} (±3.98)	9.67^{g} (±0.446)	859.7^{f} (±39.62)
FYM	15.5^{ef} (±0.71)	83.5^{g} (±3.85)	9.08^{h} (±0.418)	819.3^{gh} (±37.76)
LSD	0.57	2.0	0.42	35.5

Modified after Goteti et al. (2013)
[a-j]denotes the values are significant to other based on Multiple Duncan's test

leaf of corn plants (Table 6.3). Several studies have also been reported on solubilization of insoluble Zn forms by ZSBs (Di Simine et al. 1998; Fasim et al. 2002). The unavailable zinc can be converted into the available form by applying a microorganism that can solubilize the insoluble zinc (Saravanan et al. 2003). Among the microorganisms, an group of soil bacteria known as plant growth-promoting rhizobacteria (PGPR) have a role in nutrient cycling and, therefore, have attracted special attention for such bio-inoculants in sustainable agriculture (Weller and Thomashow 1994; Glick et al. 1999). In this context, application of beneficial rhizosphere microorganisms to convert insoluble zinc into the soluble form for plant assimilation and to achieve objectives of low-cost input is highly essential for sustainable agriculture (He et al. 2010).

6.5.6 Mechanism of Zn-Solubilizing Bacteria

PGPR are soil-borne microbes that colonize in the root zones, multiply, and compete with other rhizobacteria to improve the growth of plants (Kloepper and Okon 1994). These microbes improve the growth of plants through mobilization/solubilization and help in nutrient absorption or by releasing phytohormones or bio-control agents to save plants from many pathogens (Glick 2012). Many PGPR have been reported

to be effective Zn solubilizers. This type of rhizobacteria enhances growth and development of plants through colonization in the root zones and by solubilizing complex Zn compounds into simpler ones to make Zn available to vegetation. ZSBs solubilize Zn by many pathways, that is, acidifications. These bacteria generate organic compounds into soils that sequester Zn cations and lower the pH of nearby soils (Alexander 1997). Anions can also chelate Zn and improve its solubility in the soil (Jones and Darrah 1994). Other possible pathways include secretion of siderophores and protons, the oxido-reductive system on cell membranes, and chelated ligands for the solubilization of Zn (Agnihorti 1970; Saravanan et al. 2011). The most important mechanism is the excretion of organic acid by various bacteria as observed for solubilization of Zn in soil (Nguyen et al. 1992). The association of ZSBs and roots of higher plants are involved in the mobilization or solubilization, bio-fortification, and mineralization of Zn pools, as ZSBs can solubilize Zn from inorganic and organic pools of the total Zn present in the soils to increase Zn availability to plants (Fasim et al. 2002). These microbes are known as being more effective for Zn solubilization by their conjunction with roots of plants, producing root exudates that act as chemo-attractants (Shakeel et al. 2015). Di Simine et al. (1998) reported solubilization of Zn phosphate by strains of *Pseudomonas fluorescens*. It was observed that secretion of gluconic acids in the culture media helps in mobilization/solubilization of Zn. In this study, it was also found that lower pH can help solubilizing bacteria to generate organic acids and allow high production of available Zn in a culture medium. Inoculation with bacteria can improve bio-available Zn in rhizospheric soils and Zn concentration in the plants (Whiting et al. 2001; Biari et al. 2008). Saravanan et al. (2007) reported that 5-ketogluconic acid was exuded by *Gluconacetobacter diazotrophicus*, which helps in solubilizing Zn present in soils in insoluble form. Isolated bacterium strains when used as individuals and in combination with other strains significantly enhanced growth of plants and uptake of Zn by a rice crop as compared to control treatment and also Zn fertilizers alone (Vaid et al. 2014). Zn content in soil was increased by use of ZSBs as a inoculant; this approach has been practiced in cereals but was often neglected for fodder crops. ZSBs can solubilize the insoluble sources of Zn such as zinc oxide and zinc carbonate because most soils have high Zn concentration but a much less insoluble Zn form. Both *Bacillus* spp. and *Pseudomonas* spp. have the capability to solubilize these sources of Zn in the soils (Saravanan et al. 2003). Many soil microorganisms may be useful to various plant species by many pathways such as solubilization/mobilization of plant elements and also as bio-control agents (Khalid et al. 2009) (Fig. 6.6).

Vaid et al. (2014) found that inoculation with ZBSs in paddy field produced higher plant growth and 42.7% improved Zn content in grains of paddy. Many others strain has found for improve Zn concentration in the grains and straw of wheat and soybean and also increases reduced the zinc deficiencies in the soils. Zn-solubilizing microbes, mainly *Bacillus* spp., that enhance growth attributes, yields, and bio-fortification in maize, soybean, and wheat crops, have also been differentiated by many investigators (Kumar et al. 2016a; Khande et al. 2017).

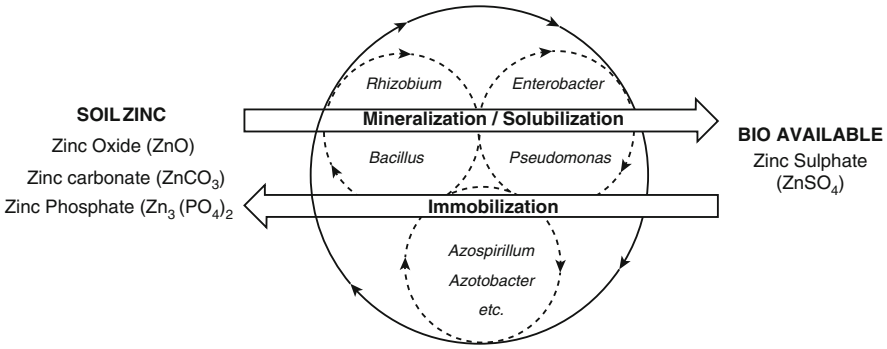

Fig. 6.6 Mechanism of Zn-solubilizing bacteria

6.6 Future Perspectives and Scope in Agriculture

Microbial diversification is among the most important components of overall world biological diversity. The latest technologies exploring microorganism diversity have found that a large proportion of microorganisms is still undiscovered, and their role in the ecological aspects is largely unknown. Several microbes are widely known for solving major agricultural needs such as crop productivity, plant protection, and maintenance of soil fertility. Many significant findings relative to PGPR and their roles in sustainable agriculture have appeared in the past few years, but studies of the impact of SSBs and ZSBs in field crops are meager. Until recently, silicon chemical dynamics in the soil have been poorly studied. The chemical dynamics between silica and other soil factors affects the quantity of available Si liberated into soil solution, a possible challenge assuming that based on the quantity of 2:1 layered silica minerals that has been found, most of the soils in the United States are able of providing a higher content of silica to the plants. Si- and Zn-solubilizing microorganisms have yet to fulfill their promise as commercially available bio-inoculants in many crops. Improvement of the effective strains that can work in different environmental behaviours and soil types may prove a boon in farming. Identification of efficient and potential Si- and Zn-solubilizing bacteria carrying other growth-promoting characteristics not only helps in enhancing the quality of crop production, animal feeds, and soil health, but also searches for its uses in bio-remediation in those areas affected by high metal contamination. In this regard, an important research work focus is required to better understood whether these are solubilizers or mobilizers of other minerals such as phosphorus, differing from Zn. Under solubilization of toxic compounds, their resistance toward toxic ions, mechanisms of solubilization, survival in rhizospheric soils, and improvement of solubilization minerals needs to be evaluated.

6.7 Conclusions

Application of inorganic fertilizers in the soils enhances the yields but kills beneficial microbes with huge harmful effects on the plant–soil ecosystem. To solve this problem, plant growth-promoting rhizobacteria (PGPR) is a better alternative. PGPR are multifunction microbes with an important role in the sustainable agriculture industry. They are significant in improving soil fertility, suppressing pathogens, and enhancing the growth of plants in sustainable agriculture. Increasing demands for food grain production with significantly reduced use of inorganic fertilizers and pesticides are currently a large challenge. The inoculation of nutrient solubilizers through seed or soils has been proved environmentally safe and also improves the yields by proving favourable environments and nutrients in the rhizosphere. The mechanism of the microbials including nutritional balance and hormonal regulation stimulate tolerance against harmful pathogens and provide nutrients to the plants by the solubilization process. Plants require macro/micronutrients for their optimum growth and development. These plant nutrients are provided by fertilizers, and organic inputs are absorbed by the plant roots with water. Some microbes have an important role in Si and Zn solubilization. Zn-solubilizing microbes solubilize zinc and improve the growth and yields of crops. Zn-solubilizing microbes are able to solubilize zinc oxide, zinc phosphate, and zinc carbonate by production of organic compounds. For the recommendation of Si fertilizer, clay content, pH, EC, organic matter, and Al and Fe oxide are essential factors to consider. Use of low-cost industrial Si fertilization by product sources with high liming potential may become an agronomic practice in many crop production systems, mainly for alleviating biotic and abiotic stresses that may limit yields and maintain soil pH.

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Chapter 7
Status and Prospects of Bacterial Inoculants for Sustainable Management of Agroecosystems

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Abstract Bacterial inoculants are bacterial species that are applied directly or indirectly to enhance the growth and yield of plants. The application of bacterial inoculants is largely due to their compatibility and complementarity with natural processes of nutrient cycling, plant protection and other related biological processes in agroecosystems. As a nature-based solution, bacterial inoculants are able to drive many beneficial biological processes in agroecosystems with little or no negative impacts. However, their applications have been limited by factors such as awareness, production quality and quantity, storage and compatibility. Although there are studies that are already investigating many of these challenges, the future prospects of the application of bacterial inoculants will be determined by the adoption of new technologies that include multi-omics approach for improving the quality as well as applicability of these beneficial microorganisms.

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7.1 Introduction

The core objective for sustainable agriculture is promoting a healthy environment while producing sufficient yield of crops to meet the requirements of an increasing world population. Generally, the vision of sustainable agriculture guarantees biosafety, nutrient-rich yield and efficient soil nutrient utilisation as well as increased crop productivity without compromising environmental integrity or public health (Lesueur et al. 2016; Lichtfouse et al. 2009). The application of chemical inputs such as inorganic fertilisers, pesticides and herbicides in agriculture has, without doubt, led to increased crop productivity over the years (Chianu et al. 2012; Hermary 2007). Nevertheless, their excessive application and inefficient management have contributed to soil degradation and environmental pollution, along with associated human, animal and crop health risks (Wallace and Knausenberger 1997).

Globally, there is an evolving consensus that encourages the adoption of suitable practices for management of both the agroecosystems and the environment in general. Of great importance is the use of beneficial plant and soil microorganisms, also known as biofertilisers or inoculants. They are regarded as active biological agents, free of agrochemicals, but contain microorganisms that are known to drive the biogeochemical cycles (Szilagyi-Zecchin et al. 2016; Trabelsi and Mhamdi 2013; Sayyed et al. 2012). These microorganisms hold huge potential in improving crop health through their ability to produce plant growth-promoting (PGP) substances such as siderophores, antifungal metabolites and 1-aminocyclopropane-1-carboxylate acid (ACC) (Khan et al. 2016a; Vejan et al. 2016; Glick 2014).

Microbial inoculants are classified based on different factors, which include type and functional capabilities of microbial components, method of application and market segmentation of the inoculant product (Huang et al. 2014; Malusá et al. 2012; Lucy et al. 2004). Although inoculants could be made of bacteria, fungi or blue-green algae (BGA) in combination or separately, this chapter only focusses on bacterial inoculants. Effects of bacterial inoculants are expressed through enhancement of growth and development by nitrogen fixation, macro- and micronutrient solubilisation and the production of PGP substances (Hassen et al. 2016; Singh et al. 2016; Gupta et al. 2007). In addition, these inoculants have secondary roles such as inducing systemic resistance on plants as well as biocontrol capabilities of pathogenic microorganisms. In this chapter, we write about different types of bacterial inoculants and their applications. In addition, future prospects of bacterial inoculant applications in the agroecosystem are also discussed.

7.2 Bacterial Inoculants as a Nature-Based Solution

A combination of factors that include climate change and the increasing world population and anthropogenic pollution of soils and water bodies pose a significant challenge to crop productivity (García-Fraile et al. 2015). Although cross-interactions between physicochemical and biological properties of the soil are important for plant productivity, microbes are key drivers of many processes in the soil-plant interphase (Huang et al. 2014). The interactions of plant and its beneficial microbes, especially in the soil, is important for maintenance of plant health and perhaps the continued existence of plants (Jain and Khichi 2014; Patel et al. 2014). Due to their biological origin and potential beneficial influence on the environment, fertilisers consisting of beneficial microbes have become an indispensable part of sustainable environmental practices (Vessey 2003). They are utilised not only for soil productivity but to also deal with many environmental and socioeconomic challenges such as climate change, water security, soil and water pollution, mineral purification, food security, plant and human health and disaster risk management (Raimi et al. 2017; Adeleke 2014; Patel et al. 2014).

Of the diverse types of soil beneficial microorganisms used for inoculant formulation, the bacterial group also known as plant growth-promoting rhizobacteria (PGPR) are, perhaps, the most promising with various agricultural applications (Glick 2014; Suyal et al. 2016; Vessey 2003). Activities of these microbes in the soil contribute to plant nutrient uptake, regulation and control of microclimate and hydrological processes, plant disease control and detoxification of noxious chemicals in the soil (Fig. 7.1) (Ambrosini et al. 2015). Examples of these beneficial rhizosphere bacteria include *Rhizobium*, *Azospirillum*, *Azotobacter*, *Azomonas, Bradyrhizobium*, *Pseudomonas* and *Bacillus.* In appreciation of their huge beneficial roles in promoting plant growth, these bacterial species have been widely utilised for the production of commercial inoculants (Malusà et al. 2016; Singh et al. 2016; Ahemad and Kibret 2014). Harnessing these essential beneficial microbes for increased crop productivity is a strategy towards achieving the objectives of sustainable agricultural production. Sustainable agriculture supports the development of a safe ecosystem for all plants and animals by promoting efficient use of diverse resources through the integration of biochemical, economic and physical sciences to develop new and eco-friendly techniques (Patel et al. 2014; Lichtfouse et al. 2009; Gupta et al. 2007). Hence, the adoption of an environmentally friendly nutrient management approach fits well into this scope.

7.3 Sources of Microbes Used for Inoculant Formulation

A large number of bacteria used for inoculant formulation are present in the rhizosphere and phyllosphere (Fig. 7.1). Some also exist as endophytic or free-living bacteria, for example, bacterial endophytes inhabit inter- and/or intracellular healthy tissues of host plants, for the entire or a part of their life cycle, without causing damage or disease (Singh et al. 2017; Shridhar 2012; Andrews and Harris 2000). The plant-endophyte

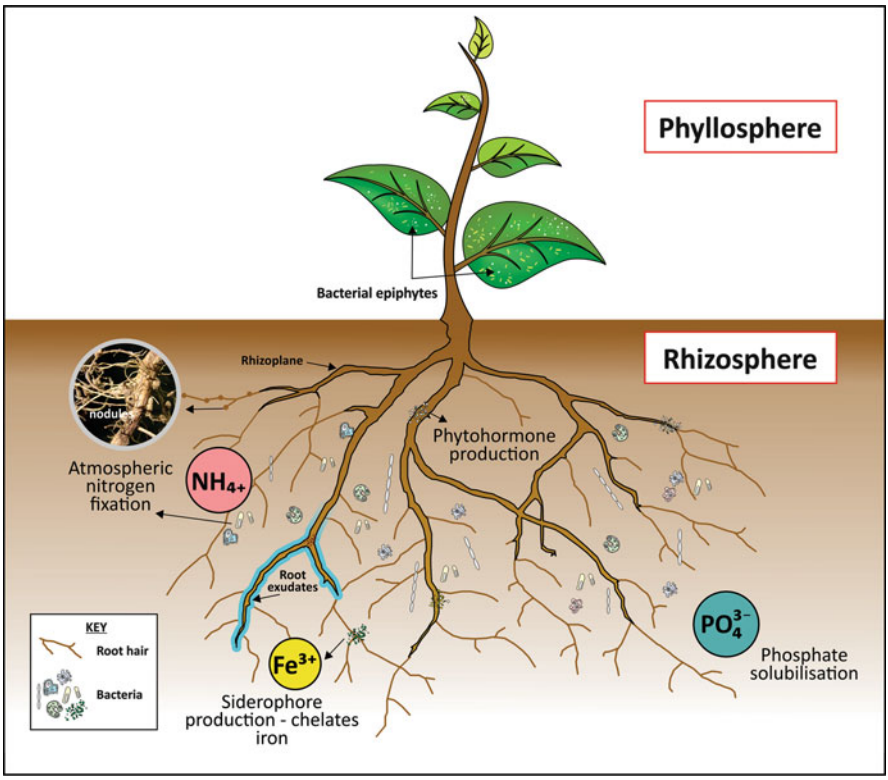

Fig. 7.1 Schematic overview of mechanisms of action and habitat of bacteria used for inoculant formulation. Different soil bacteria found in the phyllosphere and rhizosphere are involved in plant and soil nutrient management through atmospheric nitrogen fixation, nutrient solubilisation and the production of plant growth-promoting substances

association, mostly found in vascular plants, has been shown to enhance plant growth and development by protecting the host plant from pathogenic attack and improving their adaptability in adverse conditions. The endophytes accomplish this by secreting bioactive compounds such as alkaloids, steroids, flavonoids, phenols and azadirachtin (Singh et al. 2017). They exist in the host roots, leaves, stems, meristems, reproductive structures as well as seeds. Endophytes have been considered essential components of biodiversity that can be harnessed for sustainable production of bacterial inoculants for increased agricultural production (Gupta et al. 2012; James 2000).

Furthermore, epiphytic bacteria used for the production of inoculants are found on plant surfaces such as leaves, stems, buds, roots and flowers (Andrews and Harris 2000; Lindow and Brandl 2003). Various studies have reported bacteria as a major colonist of plant leaves with their population averaging up to 10^8 cells/g of leaf (Andrews and Harris 2000). This large population of bacteria on leave surfaces is an indication of the potential contributions of bacterial epiphytes to many essential global processes as well as plant behaviour and physiological condition (Lindow and Brandl 2003).

Other beneficial microbes freely inhabit the rhizosphere, the narrow region of the soil that is in close proximity to the plant roots. The rhizosphere is directly influenced by the microbial colonists, respiration and metabolism of the plant root (Zhang et al. 2010; Chung et al. 2005). The rhizosphere has a higher concentration and diversity of bacteria than any other part of the soil. Soil bacteria use root exudates as a source of nutrition while in return promote plant growth through soil nutrient management processes including nitrogen fixation, phosphorus solubilisation, sulphur oxidation as well as siderophore production and stimulation of the production of various phytohormones (Fig. 7.1) (Huang et al. 2014). The nutrient management in the rhizosphere is tailored towards high-efficiency crop production by enhancing the supply of nutrients in the plant root zone, regulating root architecture and physiological traits as well as influencing biological processes (Zhang et al. 2010). These processes are crucial and are reflected in the properties of bacteria that are considered in the formulation of specific and efficient inoculant products (Huang et al. 2014). Some of these processes occur at the rhizoplane, the surface of plant roots, comprising the epidermis and outer cortex, where microbes and plant exchange different types of nutrients and metabolic products (Huang et al. 2014; Johri et al. 2003). Microorganisms attach to the rhizoplane using structures such as flagella, fimbriae and polysaccharides. Generally, the rhizoplane and rhizosphere appear as a whole; this is because the thin boundary that separates the two habitats is difficult to differentiate (Johri et al. 2003).

7.4 Types of Bacterial Inoculants and Their Mechanisms of Action

7.4.1 Nitrogen-Fixing Bacterial Inoculants

Although the atmosphere consists of approximately 80% nitrogen, atmospheric nitrogen (N_2) is inaccessible to plants due to its stability. However, it may become accessible when converted to compounds such as ammonia and nitrate during biological nitrogen fixation (BNF) (Fig. 7.2) (Chianu et al. 2010; Guinness and Walpole 2012; Bloem et al. 2009). Biological nitrogen fixation is usually carried out by prokaryotic microorganisms that are collectively known as diazotrophs. Diazotrophs interact with host plant root in the soil under symbiotic or non-symbiotic associations to fix N (Bloem et al. 2009). Some of the well-known diazotrophs including symbiotic (rhizobia and *Frankia*) and non-symbiotic (free-living and associative) N-fixers of great importance in BNF are discussed in the section below.

Biological nitrogen fixation involves different biological and chemical transformations and/or processes that are performed by various rhizosphere beneficial microbes. Such processes are key components of the N cycle during which organic nitrogen and atmospheric nitrogen are transformed to ammonia through

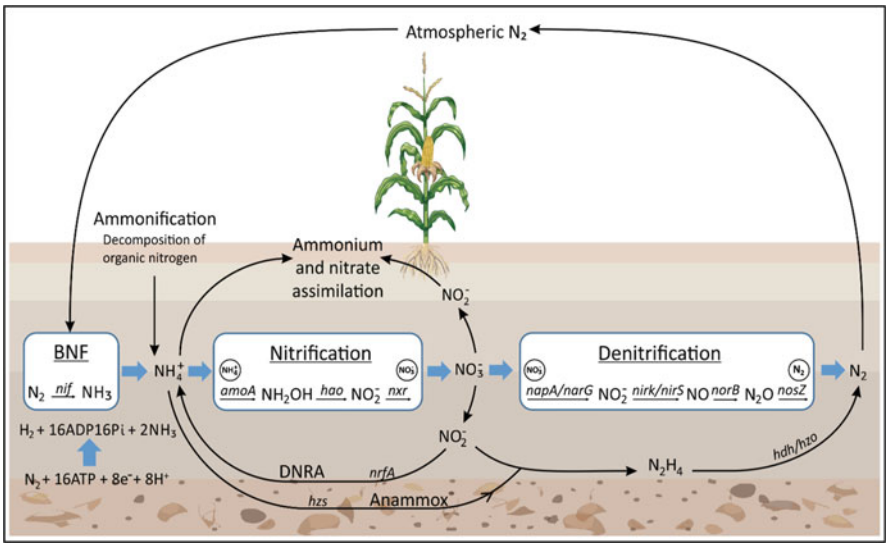

Fig. 7.2 Overview of the nitrogen cycle showing biological nitrogen fixation (BNF), nitrification and denitrification processes. The genes involved in the processes are in italics on the arrows that indicate the path of the reaction, where nitrogenase (*nif*), ammonium monooxygenase (*amoA*), hydroxylamine oxidoreductase (*hao*), nitrite oxidoreductase (*nxr*), periplasm nitrate reductase (*nap*), respiratory nitrate reductase (*nar*), nitrite reductase (*nir*), nitric oxide reductase (*nor*), nitrous oxide reductase (*nos*), multiheme nitrite reductase (*nrf*), and hydrazine synthase (*hzs*) are all enzymes involved in the reactions. The enzyme *nrf*A is involved in the dissimilatory nitrate reduction to ammonia (DNRA), while *hzs* is involved in the anaerobic ammonium oxidation (anammox). Adapted from Kox and Jetten (2015), Klotz and Stein (2008)

ammonification and BNF, respectively (Zehr and Kudela 2011; Klotz and Stein 2008). The cycle also involves the regulation of organic nitrogen in the soil through mineralisation and immobilisation. Mineralisation is the release of ammonia and nitrate during microbial decomposition of organic matter, whereas immobilisation occurs when soil microorganisms take up ammonia and nitrate for cell metabolism and growth. Mineralisation involves two major processes: ammonification and hydrolysis. The former transforms organic nitrogen into ammonia, while the latter converts ammonia to ammonium (Zehr and Kudela 2011).

Diazotrophs fix dinitrogen gas from abiotic to biotic environments employing a mechanism that involves the enzyme called nitrogenase (*nif*) (Zhang et al. 2017). Nitrogenase is an oxygen-sensitive enzyme complex that comprises dinitrogenase reductase and dinitrogenase, which both function in reducing the atmospheric nitrogen into a reactive form of ammonia and nitrate (Fig. 7.2) (Swain and Abhijita 2013; Shridhar 2012). The ammonium produced may be converted to nitrites (NO_2^-) and then nitrates (NO_3^-) through nitrification process (Fig. 7.2) (Zehr and Kudela 2011). In this process, ammonium is usually converted to nitrites by bacteria called *Nitrosomonas* spp., which possess key enzymes such as ammonium monooxygenase (*amoA*) and hydroxylamine oxidoreductase (*hao*) (Kox and Jetten

2015). The toxic nitrite produced is then converted to nitrate by *Nitrobacter* spp., using the nitrite oxidoreductase (*nxr*) (Fig. 7.2) (Klotz and Stein 2008). Nitrate is further transformed into nitrogen through denitrification process. In this process, nitric oxide (NO) and nitrous oxide (N_2O) released from the reduction of nitrate (NO_3^-) and nitrite (NO_2^-) are subsequently reduced to atmospheric nitrogen by nitrite reductase (*nir*), nitric oxide reductase (*nor*) and nitrous oxide reductase (*nos*) (Kox and Jetten 2015; Klotz and Stein 2008). Denitrification process completes the N cycle, and microbes such as *Pseudomonas* are involved in this process.

7.4.1.1 Symbiotic Nitrogen Fixers

Historically, rhizobia have been a major bacterial inoculant used for enhancement of plant and soil health. They are a group of well-known soil bacteria that are efficient in BNF (Somasegaran and Hoben 2012; Oldroyd et al. 2011). Most rhizobia belong to the family *Rhizobiaceae* and inhabit the intracellular spaces of the host in a symbiotic association. This synergy may be mutualistic, resulting in the formation of specialised structures called nodules (Fig. 7.1). Such mutualistic symbioses are most prominent in *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium* and *Sinorhizobium* in association with several hundreds of legume plants (Oldroyd et al. 2011; Peoples et al. 2009). The nodule-forming, rhizobia-legume association has enormous agronomic and ecological significance due to its substantial role in global BNF (Fig. 7.2). For instance, legumes cultivated with *Rhizobium* inoculants fix up to 300 kg N/ha and can also supply over 90% of the total nitrogen requirement of the host plants through BNF (Swain and Abhijita 2013; Hayat et al. 2010). By and large, rhizobial inoculants are most efficient in agricultural soils when the rhizobia in the local soil are lacking, less efficient or have low population (Lupwayi et al. 2000).

Another important nitrogen-fixing bacterium is *Frankia*. The first isolated species of *Frankia, F. alni* strain CpI1, which was isolated from the root nodules of *Comptonia peregrina*, is commonly referred to as CpI1 (*Comptonia peregrina* Isolate No.1) (Callaham et al. 1978). The soil actinomycete genus *Frankia* fixes nitrogen both in free-living and symbiotic association with the host, actinorhizal plants (Sellstedt and Richau 2013). It belongs to the family *Frankiaceae* and has been found to nodulate actinorhizal plants, which represent a diverse group of almost 220 species belonging to 8 plant families including Betulaceae, Casuarinaceae, Myricaceae, Rosaceae, Elaeagnaceae, Rhamnaceae, Datiscaceae and Coriariaceae (Santi et al. 2013). Its wide distribution, broad range of plant hosts and the ability to differentiate into sporangium and vesicles, which are specialised cells for nitrogen fixation, have increased its ecological importance (Santi et al. 2013; Boonkerd 1998). Similarly, the diazo-vesicles produced during the growth stage of *Frankia* can supply adequate amounts of nitrogen to the host plant under the symbiotic association. Thus, *Frankia* can support the growth of plants where nitrogen is a major limiting factor in the growth of the host (Sellstedt and Richau 2013). It has been reported that *Frankia* is responsible for about 15% of BNF in the world, mostly

in symbiotic relationship with plants and shrubs, especially dicot plants (Rascio and Rocca 2013). Under a symbiotic system, this important genus also secretes extracellular enzymes such as cellulases, pectinases and proteinases that are involved in bacteriolysis, hydrolysis and virulence (Santi et al. 2013).

7.4.1.2 Non-symbiotic Nitrogen Fixers

Free-Living Nitrogen Fixers

This group of N-fixers exist freely in the rhizosphere without necessarily having any association with the plant. Several non-symbiotic, free-living, N-fixing bacteria have been employed for the production of inoculants used on a large expanse of agricultural land. These include *Azotobacter*, *Beijerinckia*, *Bacillus*, *Pseudomonas* and *Clostridium* (Mirza and Rodrigues 2012; Ahmad et al. 2008). *Azotobacter* spp. are gram-negative bacteria belonging to the phylum *Proteobacteria* with extremely high rates of respiration, which makes it an efficient nitrogen fixer under nitrogen-deficient soil conditions (Hayat et al. 2010). *Azotobacter* species including *A. vinelandii*, *A. beijerinckii*, *A. nigricans*, *A. salinestri* and *A. chroococcum* are widely used in inoculant formulation. Apart from the nitrogen-fixing ability, *Azotobacter* also contributes to the production of PGP substances such as gibberellins, indole acetic acids and vitamins (Verma et al. 2001). Other free-living N-fixers that participate in BNF and also produce the aforementioned PGP substances are *Azoarcus* sp., *Klebsiella pneumoniae* and *Pantoea agglomerans* (Yanni et al. 2001; Reinhold-Hurek et al. 1993).

Associative Living Nitrogen Fixers

Other non-symbiotic nitrogen-fixing bacteria, including the genera of *Azospirillum* and *Enterobacter*, occur in an associative relationship with the host plant. The genus *Azospirillum* is a facultative endophyte, mostly inhabiting the intercellular space, vascular tissues or root surfaces of several kinds of cereal crops and grasses (Shridhar 2012; Wagner 2012). The species *Azospirillum brasilense* has been widely used on various crops to increase yield, while *Azospirillum diazotrophicus* has been reported to fix approximately 60–80% of nitrogen in sugarcane plantations (Ohyama et al. 2014; Lucy et al. 2004). Similarly, some of the species in the *Acetobacteraceae* family have the ability to fix N when in association with the host. These include *Swaminathania*, *Gluconacetobacter* and *Acetobacter*. For example, *Gluconacetobacter diazotrophicus* fixes nitrogen non-symbiotically or symbiotically, especially in association with sugarcane plants (James 2000). These bacteria have been isolated in countries such as Brazil, Argentina, the United States, Mexico and Egypt (Reis and Teixeira 2015). *Gluconacetobacter diazotrophicus* has been reported with the ability to colonise intracellular space of both leguminous and

nonleguminous plants without the formation of nodules. They produce enzymes such as cellulase, hemicellulase and pectinases that enhance host cell wall penetration (Dent and Cocking 2017). Under different field trials, the inoculant NFix® of *G. diazotrophicus* significantly increased crop yield such as maize, oilseed rape and wheat with or without the application of N fertilisers. It was suggested that the intracellular symbiotic N-fixation improved the level of photosynthesis and production of plant growth substances, which are essential for improvement of crop yield (Dent and Cocking 2017).

7.4.2 Solubilising Bacterial Inoculants

For increased crop productivity, agricultural soil must have adequate plant nutrients such as phosphorus, potassium, magnesium and zinc. These nutrients are frequently lacking and, when present, form stable complexes with iron, aluminium and calcium, which cannot be easily metabolised by plants (Shanware et al. 2014; Parmar and Sindhu 2013; Han and Lee 2005). This situation has resulted in limitations of plant growth due to nutrient deficiencies especially for phosphorus, which is the second most essential macronutrient after nitrogen for crop metabolism, growth and development (Cordell et al. 2009; Roy et al. 2006). Hence, solubilisation and mobilisation of insoluble nutrients in the soil using bacterial inoculant technology are essential strategies in nutrient management.

7.4.2.1 Phosphate-Solubilising and Phosphate-Mobilising Bacterial Inoculants

Phosphorus is essential for the formation and effective functioning of key plant enzymes. In spite of the large reservoir of phosphorus, it remains inaccessible by plants (Jenkins and Jenkins 2005). To improve crop productivity, phosphorus fertilisers are commonly used to augment phosphorus-deficient agricultural soils. However, most of the phosphorus fertilisers applied are immobilised, leaving a minimal amount available for plant use. Thus, phosphate-solubilising and phosphate-mobilising bacteria are essential for alleviating this situation (Mukhuba et al. 2018; Ma et al. 2011; Jenkins and Jenkins 2005). Phosphate-solubilising bacteria (PSB) have been in use since 1950 after it was first reported by Pikovskaya in 1948 (Krasilinikov 1957). Its application in crop cultivation, being a sustainable alternative to inorganic phosphorus fertiliser application, supports the world's campaign for the green revolution. Most PSB belong to the genera *Pseudomonas*, *Klebsiella*, *Serratia*, *Rhodococcus*, *Flavobacterium*, *Bacillus*, *Arthrobacter*, *Xanthomonas* and *Micrococcus* (Bello-Akinosho et al. 2016; Suyal et al. 2016; Mohammadi 2012). Some of the most efficient phosphorus solubilisers that have

been reported in different studies include *Enterobacter*, *Erwinia*, *Bacillus (B. polymyxa*, *B. megaterium*, *B. subtilis)* and *Pseudomonas (P. striata*, *P. rathonis)* (Adeleke et al. 2017; Pindi and Satyanarayana 2012; Bhattacharyya and Jha 2012; Mohammadi 2012).

There are different mechanisms through which beneficial rhizosphere bacteria solubilise insoluble phosphate. Such mechanisms are based on the form of available phosphorus, either inorganic or organic phosphorus (Mukhuba et al. 2018; Adeleke et al. 2017). Other factors such as soil pH, temperature and nutritional content as well as bacterial growth and physiological status greatly affect solubilisation efficiency (Goldstein and Krishnaraj 2007; Chung et al. 2005). For organic phosphorus, a major mechanism of solubilisation is by mineralisation through the secretion of phosphatase, an enzyme which hydrolyses organic phosphate to release phosphorus (Goldstein and Krishnaraj 2007). Conversely, the PSB solubilise inorganic phosphate by secreting low-molecular-weight organic acids (oxalic, citric, malic, fumaric, acetic and lactic acids), siderophores as well as hydroxyl and carboxyl groups (Fig. 7.3) (Adeleke et al. 2017; Sarkar et al. 2017). These chemical substances use a chelating mechanism to bind the cation to the insoluble phosphate compounds thereby releasing the soluble form of phosphate (Mohammadi 2012; Richardson and Simpson 2011). Many phosphorus-solubilising bacteria can effectively solubilise $Ca_3(PO_4)_2$ and phosphorite to monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{2-}) ions, which are easily taken up by plants (Oliveira et al. 2009).

The field efficiency of phosphate inoculants is dependent on several factors such as bacterial inoculant type, soil carbon and nitrogen, available phosphorus and level of hydrogen ions in the soil. Most *Enterobacter* and *Klebsiella* sp. are able to solubilise $Ca_3(PO_4)_2$ more efficiently than other phosphate compounds such as $FePO_4$ and $AlPO_4$ (Chung et al. 2005). Similarly, the metabolic activities of bacterial inoculants also directly contribute to the solubilisation of phosphorus through the efflux of protons and organic ions (Richardson and Simpson 2011).

Apart from the aforementioned, bacteria inoculants can also improve the ability of plants to acquire available phosphorus in the soil through hormonal stimulation of root growth, development and elongation (Adeleke et al. 2017; Goldstein and Krishnaraj 2007). In addition, variations in the soil sorption balance may increase the amount of orthophosphate ions in soil solutions. This may also enhance the mobility of organic phosphorus through microbial turnover (Richardson and Simpson 2011; Richardson et al. 2009).

7.4.2.2 Potassium-Solubilising Bacterial Inoculants

Major compounds of potassium including mica, muscovite, illite, orthoclase and biotite are unavailable for plant use (Raimi et al. 2017; Meena et al. 2014). This situation has adversely affected crop productivity in many agricultural fields. However, rhizosphere bacteria are capable of solubilising insoluble potassium compounds through the secretion of biochemical substances such as metabolites,

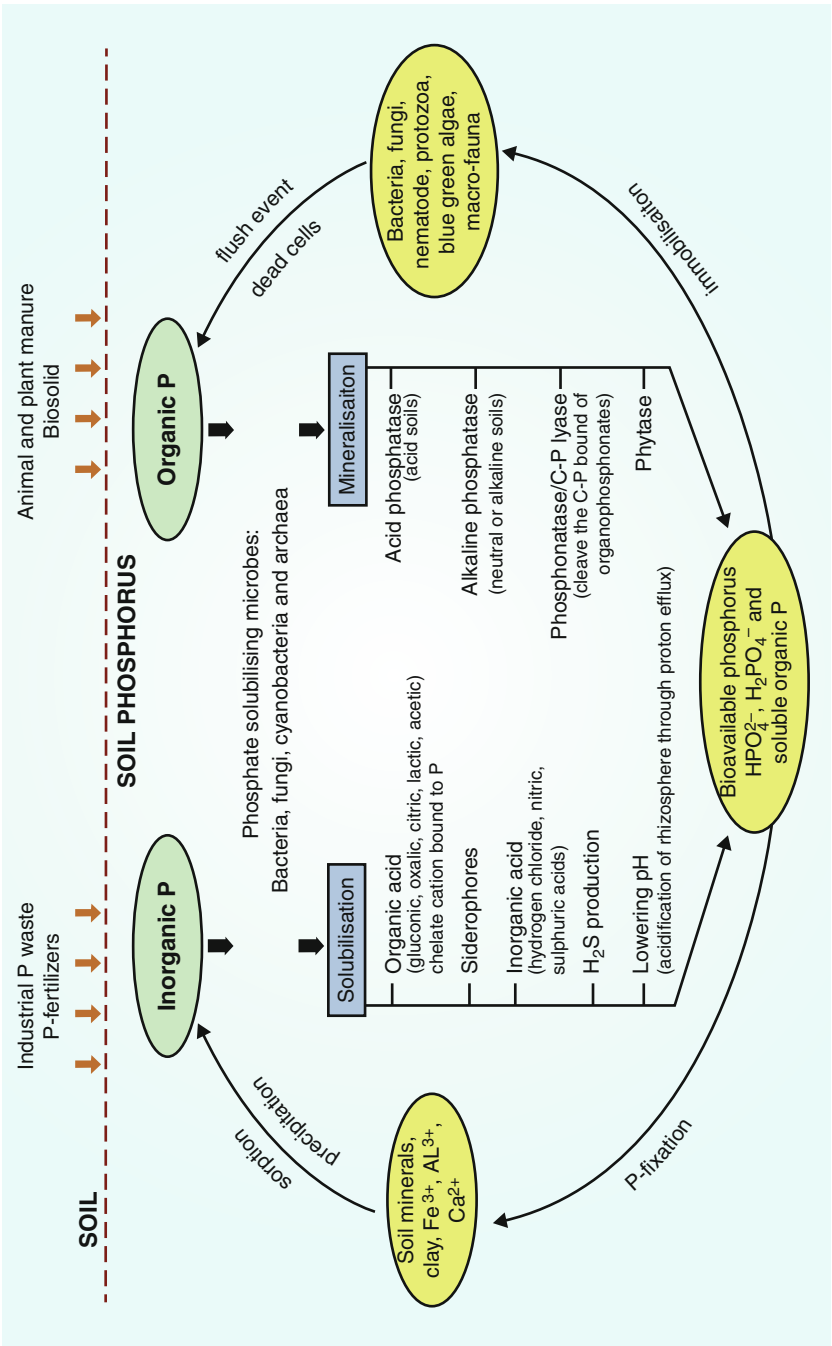

Fig. 7.3 Phosphorus cycle showing different mechanisms by which organic and inorganic soil phosphorus is solubilised. Source: Raimi et al. (2017)

organic ligands, hydroxyl anions and enzymes (Shanware et al. 2014; Han and Lee 2005). Bacteria with this ability are referred to as potassium-solubilising bacteria, and their solubilisation efficiency greatly depends on soil, microbial type and the form of potassium compounds (Meena et al. 2014; Shanware et al. 2014). Several bacterial genera such as *Acidothiobacillus*, *Bacillus*, *Pseudomonas*, *Burkholderia*, *Frateuria* and *Paenibacillus* are widely used for the production of K-solubilising inoculants. Important species of *Bacillus* with high K-solubilising and K-mobilising capabilities include *B. mucilaginous*, *B. edaphicus* and *B. circulans* (Parmar and Sindhu 2013; Sangeeth et al. 2012; Adeleke et al. 2010). These bacteria directly solubilise potassium by secreting viscous-like substances such as exopolysaccharides that invade silicate mineral and chelate silicon to release soluble potassium (Parmar and Sindhu 2013; Hutchens et al. 2003). It has been reported that organic ligands such as exudates, enzymes, secondary metabolites, siderophores and organic compounds (oxalic, gluconic, citric and lactic acids) aid in the solubilisation of potassium from its parent compounds such as feldspar and aluminosilicate (Sarkar et al. 2017; Hutchens et al. 2003). The application of potassium bacterial inoculants on agricultural soil is a sustainable measure to increase plant-available potassium in the soil, thereby reducing the cost of potassium fertiliser application for increasing crop production.

7.4.2.3 Micronutrient-Solubilising Bacterial Inoculants

Various micronutrients including zinc, iron and manganese are essential for the survival and multiplication of plants and microorganisms (Roy et al. 2006). Under different soil conditions, such as pH and oxygen levels, these compounds are transformed into various mineral complexes and become isolated, thereby preventing plants from accessing them (Adeleke et al. 2017). Under the oxic condition, iron occurs primarily as iron (III), an insoluble compound that forms hydroxides and oxyhydroxides (Hayat et al. 2010). These important elements drive the enzymatic and metabolic processes of plants and are needed in low quantity for metabolism. However, their absence or presence at high concentrations hinders plant growth and development (Berraho et al. 1997). To deal with this challenge, soils deficient in micronutrients are usually treated with fertilisers, but the majority of applied fertilisers are immobilised in the soil. For example, in zinc-fertilised soil, approximately 25% of applied zinc is available, with less than 4% of this being used by plants (Mahdi et al. 2010). However, bacterial inoculants such as *Bradyrhizobium*, *Rhizobium*, *Bacillus*, *Pseudomonas* and *Thiobacillus* are well known for the production of metabolic by-products and siderophores, which have a high affinity for soil micronutrients such as zinc and iron. These inoculants have been widely employed to overcome soil nutrient immobilisation in several agricultural soils (Ndakidemi et al. 2011; Esitken et al. 2010; Altomare et al. 1999).

7.4.3 Plant Growth Regulators Synthesised by Bacterial Inoculants

Bacteria and plant interactions in the rhizosphere have been used as indicators of soil and plant health (Huang et al. 2014). Several soil beneficial bacteria promote soil fertility and plant health through the production of different growth-promoting substances, also known as growth regulators. The production of these regulators may be facilitated through direct or indirect mechanisms (Chaiharn and Lumyong 2011; Hayat et al. 2010). Apart from participating in soil nutrient management, beneficial bacteria directly participate in plant growth promotion through biosynthesis of different plant hormones including auxins, gibberellins, cytokinins and 1-aminocyclopropane-1-carboxylate (ACC) acid, which is an ethylene precursor (Khan et al. 2016a; Karadeniz et al. 2006). These phytohormones have been found to increase leaf and root length as well as yield in plants, while also improving the interactions between plant and the rhizosphere microbes (Vacheron et al. 2013). Different types of auxins exist, and some of these include 1-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA), phenylacetic acid (PAA), indole-3-pyruvic acid (IPyA) and indole acetic acid (IAA) (Patten and Glick 1996). The most common and physiologically active auxin in plants is indole acetic acid (IAA), which promotes accelerated and long-term responses in plants. Indole acetic acid affects plant root architecture and cell division, elongation and differentiation, thereby stimulating increased root development (Patten and Glick 2002). Bacteria such as *Bacillus subtilis,* which are efficient in producing IAA, have been reported to promote tuber elongation and increased number of sprouts when used on *Dioscorea rotundata* (Swain et al. 2007). Similarly, inoculant of *Azospirillum* producing IAA-mediated ethylene stimulated an increase in the number of root hairs, root surface area and total biomass in tomato plants (Ribaudo et al. 2006). Rhizosphere beneficial bacteria including *Azospirillum* and *Paenibacillus* also produce indole-3-butyric acid, tryptophol and indole-3-ethanol, which indirectly contribute to plant growth promotion (Solaiman and Anawar 2015; Hayat et al. 2010). Approximately 80% of isolated rhizosphere bacteria have been reported to produce IAA (Patten and Glick 1996), while about 90% of isolated bacteria from the rhizosphere of different crops were found to be involved in cytokinin production, under in vitro cultivation (Barea et al. 1976). According to Vacheron et al. (2013), the biosynthesis of cytokinins has also been documented in bacteria such as *Bradyrhizobium japonicum, Pseudomonas fluorescens* and *Bacillus licheniformis*. Similarly, gibberellic acid produced by *Bacillus megaterium*, *B. aureus* and *Klebsiella pneumoniae* stimulates increased flowering, stem and internode elongation as well as fruit setting and growth in different plants (Kumar et al. 2014; Zalewska and Antkowiak 2013; Karadeniz et al. 2006). Maize, tomato and rice planted with gibberellic acid had a substantial increase in growth and yield when compared to the control (Kumar et al. 2014; Zalewska and Antkowiak 2013; Fulchieri et al. 1993). 1-Aminocyclopropane-1-carboxylate acid (ACC) plays an essential role in the biosynthesis of ethylene hormone found in higher plants (Khan et al. 2016a, Onofre-Lemus et al. 2009).

Ethylene hormone acts as a modulator of growth and development in plants. Although ethylene is a key factor in plant defence response to a wide range of stress, high levels of ethylene could be detrimental to plant growth. Fortunately, ACC can be degraded by bacterial ACC deaminase, an enzyme that indirectly facilitates plant growth (Glick 2014). Rhizosphere bacteria with ACC deaminase function as a sink for ACC by producing alpha-ketobutyrate and ammonia from ACC hydrolysis, instead of ethylene (Onofre-Lemus et al. 2009). This process lowers the amount of ACC and ethylene levels in plants, thereby promoting steady plant growth and development, through the reduction of damages such as plant death and growth inhibition usually caused by high concentration of plant ethylene (Glick 2014; Hayat et al. 2010; Onofre-Lemus et al. 2009; Saleem et al. 2007).

Furthermore, rhizosphere bacteria also produce siderophores, particularly under iron-deficient soil. Siderophores are low-molecular-weight (~200–2000 Da) substances with an extraordinary chelating ability for iron (Ahmed and Holmström 2014). A wide range of siderophores have been reported in different bacteria, and most of these are catecholates (enterobactin), carboxylates (rhizobactin) and hydroxamates (ferrioxamine B). Most of the soil iron is not readily available for rhizosphere beneficial microbes and plant use (Shanmugaiah et al. 2015; Ahmed and Holmström 2014). The bacteria producing siderophores are able to overcome this condition through iron-chelation mechanism (Sarkar et al. 2017; Radzki et al. 2013). The mechanism of siderophore-bound iron transport systems varies between gram-positive and gram-negative bacteria (Ahmed and Holmström 2014). In gram-negative bacteria, the Fe(III)-siderophore complexes bind to TonB-dependent outer membrane receptor and cross the membrane through an energy-dependent system involving outer membrane receptor proteins, periplasmic binding proteins and inner membrane transport proteins (Fukushima et al. 2013; Braun and Hantke 2011). Subsequently, the complex is transported into the cytoplasm through the cytoplasmic membrane by an ATP-binding cassette (ABC) transport system after the Fe(III)-siderophore complex, bounded by periplasmic binding protein have been released into the periplasmic space (Ahmed and Holmström 2014; Noinaj et al. 2010). Finally, the Fe(III)-siderophore complex is reduced to Fe(II). For gram-positive bacteria, the membrane receptors are absent due to lack of the outer membrane. Hence, the Fe(III)-siderophore complexes are bound by periplasmic binding proteins that are attached to the cell membrane due to lack of periplasmic space. Similar to gram-negative bacteria, the Fe(III)-siderophore complexes are then transported into the cytoplasm using ATP-binding (ABC) transport system (Fukushima et al. 2013; Braun and Hantke 2011). Some of the bacterial species used for inoculum formulation and their plant growth-promoting functions are presented in Table 7.1.

Table 7.1 Bacterial species used for inoculum formulation and their plant growth-promoting functions

Bacterial genera	Species	Function	Reference
Sinorhizobium	*Sinorhizobium meliloti*	Fix-N	Villegas et al. (2006)
Bradyrhizobium	*B. japonicum*, *B. elkanii*, *B. betae*, *B. canariense*, *B. liaoningense*	Fix-N, P-solubilisation, siderophore and IAA production	Antoun et al. (1998), Wu et al. (2011)
Azospirillum	*A. brasilense*, *A. lipoferum*, *A. amazonense*	Fix-N, P-solubilisation, IAA and siderophore production	Rodrigues et al. (2008), Thakuria et al. (2004)
Azotobacter	*Azotobacter chroococcum*	Fix-N, P-solubilisation, gibberellin, IAA, kinetin and siderophore production	Ahmad et al. (2005), Verma et al. (2001)
Azoarcus	*A. communis*, *A. indigens*	N-fixer	Reinhold-Hurek et al. (1993)
Bacillus	*B. mucilaginous*, *B. megaterium*, *B. licheniformis*, *B. edaphicus*, *B. subtilis*, *B. cereus*, *B. pumilus*, *B. circulans*	K- and P-solubilisation, gibberellin, auxin, and cytokinin production	Parmar and Sindhu (2013), Mohammadi and Sohrabi (2012), Karadeniz et al. (2006)
Burkholderia	*B. unamae*, *B. tropica*	1-Aminocyclopropane-1-carboxylate (ACC), N-fixer, IAA, P-solubilisation and siderophore	Onofre-Lemus et al. (2009)
Enterobacter	*E. asburiae*	IAA, P-solubilisation, siderophore ammonia	Ahemad and Khan (2010)
Klebsiella	*Klebsiella* sp.	IAA, P-solubilisation, siderophore ammonia	Ahemad and Khan (2011)
Pseudomonas	*Pseudomonas putida*, *P. jessenii*, *P. aeruginosa*, *P. chlororaphis*	P-solubilisation, siderophore and IAA	Parani and Saha (2012), Shaharoona et al. (2008)
Alcaligenes	*Alcaligenes faecalis*	P-solubilisation, IAA and siderophore production	Sayyed et al. (2010)
Acinetobacter	*Acinetobacter* spp.	IAA, P-solubilisation and siderophore	Rokhbakhsh-Zamin et al. (2011)
Rhizobium	*Rhizobium cicero*, *R. phaseoli*, *R. undicola*	Siderophore, Fix-N, IAA	Berraho et al. (1997), Ghosh et al. (2015)
Serratia	*Serratia nematodiphila*	IAA, siderophore, HCN and P-solubilisation	Dastager et al. (2011)
Flavobacterium	*Flavobacterium* sp.	IAA, P-solubilisation	Soltani et al. (2010)

Adapted from Raimi et al. (2017), Ahemad and Kibret (2014)

7.5 Applications of Bacterial Inoculants in Agroecosystems

7.5.1 Bacterial Inoculants for Increased Crop Productivity and Soil Restoration/Maintenance

The application of bacterial inoculants in agriculture has robust benefits in enhancing soil fertility and crop productivity (Raimi et al. 2017; Hassen et al. 2016; Singh et al. 2016). The efficiency of agronomic input is enhanced where inoculants are used in combination with other integrated nutrient management methods (Duarah et al. 2011; Kumar et al. 2010; Shaharoona et al. 2008). In general, these benefits lead to the reduction of inorganic fertiliser application, while also improving the economic status and profitability of farmers (Singh et al. 2016; Suyal et al. 2016; Geetha and Joshi 2013; Catroux et al. 2001; Bashan 1998). Cost-effectiveness of bacterial inoculants is usually estimated based on the fraction of the value of possible benefits correlated to the total real costs of applied inoculants over a specific period of time (Mulongoy et al. 1992). For legume inoculants, the benefits are based on the N-fixing capability of the product. For example, white clover plant had cost/benefit ratio of 416 with a N-fixing capability of 200 kg/ha, while soybean had a cost/benefit ratio of 17 and fixes about 100 kg of N/ha from inoculation which cost as low as half a dollar (US$ currency) per kg of bacterial inoculant (Mulongoy et al. 1992). In addition, the cost of bacterial inoculants that will provide the same quantity of nutrient supplied by mineral fertiliser is low. For example, NoduMax® inoculant costs only $5 per ha in application as opposed to $100 per ha cost of urea fertiliser needed to supply the same quantity of nutrients (N2Africa 2015).

The soil is the farmer's most precious asset and must be made productive through a systematic application of nutrients. It has been estimated that about 28.8 million tons of plant nutrients are needed for the production of 321 million tons of grain crops by the year 2020. Due to high market price and unavailability, only 21.6 million tons will be supplied through chemical fertiliser application, leaving a shortfall of 7.2 million tons (Pathak et al. 2017). This deficit is a major challenge for increasing food supply, especially in developing nations. However, the application of bacterial inoculants, which is more economically viable, is an efficient nutrient management technique for augmenting the gap (Chianu et al. 2010; Graham and Vance 2003).

7.5.2 Availability of Soil Nutrients and Increased Crop Yield

Crop yield, especially for legumes, is improved when cultivated with nitrogen-fixing bacterial inoculants such as *Sinorhizobium*, *Bradyrhizobium*, *Rhizobium* and *Azorhizobium*, which can fix appreciable amounts of soil nitrogen through BNF (Wagner 2012; Oldroyd et al. 2011). The symbiotic relationship of the *Rhizobium*-leguminous plant has been reported to fix between 24 and 584 kg N/ha annually under different crop and soil types (Martínez-Romero 2009; O'hara et al. 2002). For

example, soybean yield and soil organic matter were improved under *Rhizobium*-inoculated soil which was attributed to the biological fixation of approximately 80% of nitrogen (Smaling et al. 2008). In addition, *Frankia* and *Casuarina equisetifolia* symbiotic relationship resulted in the fixation of up to 362 kg N/ha, whereas *Azotobacter*, a free-living bacteria, contributes about 15 kg N/ha/year (Elkan 1992). Depending on crop types, co-inoculation of *Azotobacter* and *Azospirillum* increases the yield of crops in the range of 5%–10% (Pathak et al. 2017). Likewise, the increased growth of *Phaseolus vulgaris* (common bean) was attributed to *Rhizobial* inoculant application (Ndakidemi et al. 2011). In addition, pomegranate (*Punica granatum* L.) treated with inoculants containing N-fixing bacteria (*Azotobacter chroococcum*) and arbuscular mycorrhiza fungi (*Glomus mosseae)* had increased growth and yield (Aseri et al. 2008). The combined treatment of the inoculants enhanced microbial activities, nutrient uptake as well as the activities of dehydrogenase, alkaline phosphatase and nitrogenase in the plant rhizosphere compared to the control (Aseri et al. 2008).

Similarly, solubilising bacteria also have positive influence on crop growth and development. For instance, *Bacillus magisterium* var. *phosphaticum* applied on sugarcane plants stimulated plant growth and yield with high sugar content (Sundara et al. 2002). In the same vein, the cultivation of rice (*Oryza sativa*) and yardlong bean (*Vigna unguiculata*) with P inoculants such as *Pseudomonas*, *Bacillus* and *Erwinia* was also found to promote seed germination (germination index > 2.5) as well as increased shoot, root length and biomass (Duarah et al. 2011). Peanut (*Arachis hypogaea*) and sunflower (*Helianthus annuus*) had high yield when inoculated with *Bacillus* inoculants (Wang et al. 2014; Ahmed and El-Araby 2012). In addition, *Pseudomonas aeruginosa* strain $PSBI_3$-1 and *Aerococcus* sp. strain $PSBCRG_1$-1 solubilise tricalcium phosphate at different sodium chloride concentrations for plants grown under saline soil, while *Burkholderia cepacia* increased maize plant yield under sodium chloride concentration of up to 5% (Alori et al. 2017; Srinivasan et al. 2012).

Furthermore, under low P and K soil, eggplant (*Solanum melongena*), pepper (*Capsicum annuum* L.) and cucumber (*Cucumis sativus* L.) plants were reported to have improved mineral uptake with an increase in nutrient (NPK) content and yield of crops when cultivated with a combination of potassium and phosphate inoculants (Han and Lee 2005, 2006). The potassium and phosphate inoculants contained *Bacillus megaterium* var. *phosphaticum* and *Bacillus mucilaginosus,* respectively (Han and Lee 2005). Similarly, under soil inoculation with K-solubilising *Bacillus edaphicus*, an increased yield of rape (*Brassica napus* L.) and cotton (*Gossypium hirsutum* L.) was achieved (Sheng 2005). Inoculants of *Pseudomonas*, *Mycobacterium* and *Bacillus* have also been reported with high ability to increase the growth and yield of maize (*Zea mays*) plants (Egamberdiyeva 2007).

In iron-deficient soil, inoculants producing siderophores caused an increase in the yield of groundnut (*Arachis hypogaea*) and tomato (*Solanum lycopersicum*) plants compared to the control (Radzki et al. 2013; Sayyed et al. 2010). Likewise, mung bean (*Vigna radiata* L.) had increased chlorophyll content and yield under iron-deficient soil when inoculated with *Pseudomonas* strain (GRP3) (Sharma et al. 2003). In addition, available soil iron is of great importance for effective functioning

of N-fixing bacterial inoculants. This is because iron is necessary for the formation of iron-molybdenum and iron proteins that play crucial roles in the effective functioning of the nitrogenase, an important enzyme in BNF (Sickerman et al. 2017). Thus, for increased N-fixation, especially under iron-deficient soil, siderophore-producing bacterial inoculants are essential (Hassen et al. 2016; Duval and Hungate 2008). These observations highlight the positive influence of inoculant application in increasing crop nutrient uptake and productivity.

7.5.3 Biocontrol Ability of Bacterial Inoculants

The iron-chelation mechanism of siderophores creates an indirect competition for soil iron amongst rhizosphere microbes. This process reduces the available soil iron, thereby indirectly suppressing pathogens and their ability to cause diseases (Shanmugaiah et al. 2015; Sayyed et al. 2010). For example, the fusarium wilt of potato and maize has been controlled by siderophore-producing *Pseudomona*s and *Bacillus* inoculants, through their ability to make iron unavailable to the pathogen (Beneduzi et al. 2012). In the same vein, inoculants of *Pseudomonas aeruginosa* have been widely used for controlling bacterial blight disease caused by *Xanthomonas oryzae* pv. *oryza* and *Rhizoctonia solani* (Yasmin et al. 2017). *Fusarium* spp. and *Pythium* spp., mainly attacking both maize and wheat crops, have also been controlled with inoculants of *Bacillus* spp. and *Burkholderia cepacia* (Whipps 2001). The application of inoculants for biocontrol of crop pest and diseases is a sustainable alternative to pesticide application.

On the other hand, the direct inhibition of pathogens by bacterial inoculants is usually through their metabolic activities and production of antibiotics (Solanki et al. 2012; Akgül and Mirik 2008). For example, *Fusarium udum* Butler and *Erwinia carotovora* cause *Fusarium* wilt of pigeon pea (*Cajanus cajan* L.) and soft rot of potato (*Solanum tuberosum*), respectively, thereby reducing the productivity of these crops (Sharma et al. 2016; Pérombelon 2002). However, these pathogens can be controlled by inoculants of *Pseudomonas fluorescens* and *Sinorhizobium* that synthesise chitinase and β-1,3-glucanase (Gupta et al. 2013; Kumar et al. 2010). These enzymes are able to break down the cell wall components of fungal pathogen. Chitinases hydrolyse chitin, the major components of fungal cell walls, while glucanases catalyse hydrolytic cleavage of the glucosidic linkages in the (1, 3) β-glucan and break down the glucans present in the fungal cell wall (Gupta et al. 2013). Furthermore, plant-microbe interactions in the rhizosphere can strengthen the defence mechanisms of plants against pest attack through cyanogenesis, a process through which hydrogen cyanide is produced (Rudrappa et al. 2008). The cyanogenic defence substances produced in the legume-*Rhizobium* symbiotic relationship promote resistance in plants against herbivore attack (Thamer et al. 2011; Kempel et al. 2009). Similarly, about 26% reduction in the population of predatory insects was achieved when maize (*Zea mays*) plants were cultivated with bacterial inoculants (Megali et al. 2015).

7.5.4 Volatile Organic Compounds

One of the major groups of secondary metabolites produced by rhizosphere bacteria is known as volatile organic compounds (VOCs). Volatile organic compounds are essential components of plant growth regulators that have been found to stimulate increased crop productivity through induced resistance of plants to pathogens and as a direct source of plant nutrients (Santoro et al. 2011). These metabolites play an essential role in plant-microbe signal communication (Insam and Seewald 2010). Some of the well-known VOCs include acetone, 3-butanediol, terpenes, jasmonates and isoprene. These compounds have a high vapour pressure, low boiling point and low molecular mass (<300 Da). Several factors have been reported to affect the production of microbial VOCs in the soil. These factors include the pH, moisture content, temperature, oxygen level and nutrient content of the soil (Insam and Seewald 2010). The microbial growth stage also influences VOCs production. Several studies have shown that microbial VOCs can indirectly affect root development, secretion of hormones and plant growth (Piechulla et al. 2017; Schulz-Bohm et al. 2017; Ryu et al. 2004). For example, in a study by Santoro et al. (2011), the biosynthesis of essential oils and increased growth parameters observed in *Mentha piperita* (peppermint) were attributed to the VOCs produced by *Pseudomonas fluorescens*, *Bacillus subtilis* and *Azospirillum brasilense*. Similarly, biocontrol potential of different species of *Pseudomonas* and *Bacillus* has been attributed to the antibacterial activities of their various VOCs (Schulz-Bohm et al. 2017). Volatile organic compounds such as benzothiazole and 1-methylnaphthalene produced by *Pseudomonas fluorescens* WR-1 have bacteriostatic effects against *Ralstonia solanacearum,* a tomato pathogen (Raza et al. 2016). Likewise, benzaldehyde and 1,3-butadiene produced by *Bacillus* spp. suppress the growth of *R. solanacearum* and induces systemic resistance in tobacco plant against bacterial wilt diseases (Tahir et al. 2017).

7.6 Bacterial Inoculants for Environmental Sustainability

7.6.1 Bioremediation of Polluted Agricultural Soil

Of recent, rhizosphere beneficial bacteria have found application in soil bioremediation, especially in toxic metal-polluted soils (Adeleke et al. 2012; Adeleke 2014; El-Kabbany 1999). Bioremediation process is an eco-friendly and cost-effective technique that employs microorganisms to effectively remove or reduce pollutants of water, soil and sediments. This process is based on the ability of microbes such as bacteria to degrade organic and inorganic substances in polluted environment (Adeleke 2014; Chorom et al. 2010). In addition, the diverse rhizosphere beneficial processes such as nutrient cycling, biochemical synthesis, detoxification as well as

soil structure conservation have been harnessed in bioremediation (Jiao et al. 2015; Panda and Mishra 2007).

The main advantage of using bacterial inoculants for bioremediation of polluted soil in agroecosystems is the potential additional capabilities of microorganisms to drive the processes involved in nutrient cycling. For instance, *Rhizobacteria* in association with arbuscular mycorrhizal fungi (AMF) have been used to clean up toxic heavy metal-contaminated agricultural soil (Khan 2014). Such approach will allow the ecosystem, especially the agroecosystem, to benefit comprehensively from the bioremediation process. Similarly, Bello-Akinosho et al. (2016) in an in vitro study also reported the potential of *Pseudomonas* sp. strain 10–1B in the degradation of polycyclic aromatic hydrocarbons (PAH) as well as in soil fertility management. Several beneficial bacteria including *Burkholderia, Pseudomonas, Bacillus, Rhizobium* and *Enterobacter* have also found application in bioremediation (Bello-Akinosho et al. 2015, 2016, 2017; Jain and Khichi 2014; Mathew et al. 2014). *Burkholderia* spp. have been used to remediate Cd- and Pb-polluted agricultural soil (Jiang et al. 2008), while species of *Bacillus*, *Streptococcus*, *Pseudomonas* and *Micrococcus* have also been reported with bioremediation potential for Cd-, Pb- and Cu-contaminated soil (Mani and Kumar 2014; Fulekar et al. 2012). Importantly, the twofold functions, viz. soil nutrient management and bioremediation, have made rhizosphere beneficial bacteria a significant soil fertility management technology for increasing agricultural land productivity in polluted soils (Raimi et al. 2017).

7.6.2 Drought or Water Stress Resistance

Plant-microbe interactions have vital influences on the diversity, abundance and survival of both plants and their associated microbes (Huang et al. 2014; Whipps 2001). Due to this close interconnection, stress and sudden changes in the abiotic environment of plants also affect their associated microbial communities (Naylor and Coleman-Derr 2018). One of such environmental stress conditions is drought, which adversely affects crop productivity. Under repeated water stress conditions, interactions between plants and microbes have evolved adaptive strategies (Cruz-Martínez et al. 2009). This involves improved association of plants with microbes. These microbes can directly or indirectly improve the metabolism and development of the host plant, thereby making such plants drought-resistant (Naylor and Coleman-Derr 2018). Many of the root-associated bacterial communities of plants cultivated under drought conditions have the capability to enhance water stress tolerance through their growth-promoting mechanisms (Kaushal and Wani 2016). The production of antioxidant defence substances, VOCs, dehydrins, PGP substances and exopolysaccharides (EPS) and modification of phytohormone levels are some of the common mechanisms used by bacteria to enhance water stress resistance of plants (Cruz-Martínez et al. 2009; Glick 2014; Kaushal and Wani 2016). Unfortunately, no single bacterial isolate possesses all these attributes. Hence, utilisation of a microbial consortium rather than single isolates could be important in the formulation of bacterial inoculants with

drought-resistant capabilities (Naylor and Coleman-Derr 2018). For example, in a study conducted by Khan et al. (2016b), a consortium of ten endophytic strains improved water stress resistance of hybrid poplar (*Populus* sp.) through multiple distinct drought response pathways.

Another example is the ability of such consortium to produce a combination of PGP substances such as auxins, cytokinins, gibberellins, siderophores and ACC, which promote high water stress tolerance in plants (Kaushal and Wani 2016; Molina-Romero et al. 2017). Hence, inoculants known for the production of these PGP substances have immense application in drought-prone environments (Figueiredo et al. 2010; Wang et al. 2012). For instance, cucumber (*Cucumis sativus*) plants inoculated with a consortium of PGPR strains (*Bacillus cereus* AR156, *Bacillus subtilis* SM21 and *Serratia* sp. XY21) under drought stress conditions had increased leaf proline and chlorophyll content, darker green leaves and improved root recovery intension when compared to the control (Wang et al. 2012). Similarly, a bacterial consortium formulated with *Pseudomonas putida* KT2440, *Sphingomonas* sp. OF178, *Azospirillum brasilense* Sp7 and *Acinetobacter* sp. EMM2 improved the yield of maize (*Zea mays*) compared to the control. This was attributed to the abilities of the strains to solubilise phosphorus and produce high levels of siderophore and IAA (Molina-Romero et al. 2017). According to Gururani et al. (2013), *Bacillus* inoculant, which produces ACC and siderophores, enhanced water stress tolerance of potato (*Solanum tuberosum*). Also, pepper (*Capsicum annum*) and tomato (*Solanum lycopersicum*) plants inoculated with *Achromobacter piechaudii* ARV8 had increased water stress resistance when cultivated under water-stressed soil conditions (Mayak et al. 2004).

7.7 Current Status and Hurdles in the Formulation of Efficient Inoculants

Efficient bacterial inoculants must not only have the ability to enhance plant growth, but they should also be highly potent with sufficient capabilities to dominate in the rhizosphere environment (Lupwayi et al. 2000). It is also important to ensure that inoculants have high association compatibility with the plant host and other beneficial rhizosphere microbes, as well as a broad range of beneficial functions with diverse crops (Herridge et al. 2002). In addition, bacteria used for inoculant production must be easily multiplied (both in the laboratory and field), environmentally friendly and have the capability to perform under various ecological conditions (Reddy and Giller 2008). Quite a number of rhizosphere bacteria have been reported to possess a combination of the aforementioned abilities. As earlier highlighted, no single inoculant can effectively perform all these functions under the different ecological conditions. This has encouraged the formulation of inoculants with microbial consortium, which perform diverse field functions (Herrmann and Lesueur 2013). In addition, it is also necessary to screen and select beneficial plant growth promoters under different ecological conditions for the formulation and production

of efficient bacterial inoculants for increased crop productivity (Arora et al. 2010). For instance, several species of *Pseudomonas*, *Bacillus*, *Azospirillum* and *Azotobacter* have found extensive applications in soil nutrient enhancement, not only for their high nutrient solubilisation capability but also for their abilities to produce different PGP substances and fix appreciable amounts of nitrogen, especially under extreme environmental conditions (Bello-Akinosho et al. 2016; Ghosh et al. 2015; Parani and Saha 2012; Sharma et al. 2003).

In spite of the need for increased production and application of inoculant in sustainable agriculture, there exist some challenges that limit full commercialisation of inoculants. One of the limiting factors is the field efficacy, which affects the overall acceptability and success of the products (Parnell et al. 2016). Generally, the field efficacy of inoculants cannot always be guaranteed. Several successful laboratory and greenhouse experiments are rarely translated to field success. In addition, several quality assessments have shown that poor-quality inoculant products unable to improve crop productivity are sold in the agro market (Herrmann et al. 2015; Olsen et al. 1996; Raimi and Adeleke 2018). More so, efforts to formulate inoculants that can perform under all ecological conditions have been unsuccessful (Stephens and Rask 2000). Specific plants recruit a range of beneficial bacteria based on the plant's metabolites or exudates in the form of carbon, VOCs and organic acids (Parnell et al. 2016). Moreover, efficiency of inoculants on different crops may differ due to differences in their associated microbial community, developmental stages, environment and nutrient availability or preferences (Herrmann and Lesueur 2013).

Furthermore, the success of inoculants greatly depends on the target crop, product availability and cost as well as ease of application and environmental challenges. Developing an efficient product suitable under different field conditions, which combines all the aforementioned characteristics, has become a major challenge in the inoculant industry (Stephens and Rask 2000). Another important factor is the carrier formulation for inoculant production. This is a challenge that affects product application, quality and field efficiency. It is essential that carrier materials support the growth of specific inoculant strains and maintain the desired population of these strains over an acceptable shelf life. Unfortunately, carriers for consortium products are usually less selective; a desired quality that is required to support the diverse microbial strains used for consortium product formulation. However, the disadvantage of the less selective carrier is the potential to support growth of other microbial contaminants. This is a major challenge affecting the formulation of good-quality inoculants, especially the consortium products (Herrmann et al. 2015; Olsen et al. 1996).

An additional challenge in the production of efficient inoculants is the lack of stringent quality control measures. Better quality control system should be put in place to assess the quality of the numerous emerging products in the market as well as the activities of the growing industry (Lupwayi et al. 2000). It is essential that the products meet all quality criteria through regular quality assurance performed by the manufacturers during production processes. In addition, quality control assessment by independent bodies or government should be performed regularly to confirm quality standards of inoculants (Herrmann and Lesueur 2013).

7.8 Commercial Bacterial Inoculant Products

Bacterial inoculants have been established for over a century, with the first reported inoculant, Nitragin®, produced by a Dutch scientist, Hiltner L. in 1896 (Bhattacharjee and Dey 2014). The growing need for sustainable agricultural production has increased awareness and use of bacterial inoculants. This has caused an increase in the commercialisation and market share of inoculants with different types of products being supplied to the agromarket (Raimi et al. 2017). Recently, the majority of inoculants produced and used are mostly rhizobia products, which constitutes approximately 79% of the global inoculant demand. This may be attributed to the major role nitrogen plays in crop productivity. Apart from rhizobia, the phosphate-solubilising inoculants account for approximately 15%, while other inoculants including mycorrhizal products make up the remaining percentage (Transparency Market Research 2014; Suyal et al. 2016). According to Transparency Market Research (2014), the bioinoculant global market demand is growing and has been estimated to increase at a robust cumulative average growth rate (CAGR) of approximately 13% from 2017 to 2025. It is projected to be valued at US$4.09 billion in 2025 from the value of US$1.25 billion as at 2016. *Azospirillum* sp. and *Bacillus subtilis* are commonly used for the formulation of commercial free-living PGPR products, *Bacillus subtilis* has been used under different trade names such as Serenade® and Kodiak® for crops including beans, pea, rice, maize and soybean (Transparency Market Research 2014). Another important bacterial species in inoculant production is *Agrobacterium radiobacter*, which have been produced by different manufacturers under the trade names Diegall® and Nogall®. These products are used for the cultivation of fruit, trees and ornamentals. Similarly, *Pseudomonas fluorescens* has been produced under trade names such as Conquer® and Victus®, used on various types of crops (Suyal et al. 2016). Some of these inoculant products are listed in Table 7.2.

7.9 Conclusions

Bacterial inoculants play several essential roles in agroecosystems. Their direct and indirect impacts on plant growth and development are expressed through various mechanisms including nutrient solubilisation and mobilisation as well as the production of PGP substances. Therefore, traditional nutrient management strategies, which are greatly dependent on the application of agrochemical inputs such as inorganic fertilisers and pesticides must realign with contemporary integrated nutrient management systems such as bacterial inoculant technology. In spite of the many success stories attributed to the use of bacterial inoculants for improving agricultural production, many questions regarding their sole utilisation to improve soil quality and enhance plant health remain unanswered.

Table 7.2 Global representation of inoculants, bacterial components and manufacturers

Continent	Product	Active component	Manufacturer
Africa	Firstbase, Biostat, Landbac, Waterbac, lifeForce	*Bacillus* sp.	Microbial solution (Pty) Ltd, South Africa
	Likuiq Semia	*Bradyrhizobium elkanii*	Microbial solution (Pty) Ltd, South Africa
	Nitrasec Alfalfa (Lucerne)	*Sinorhizobium meliloti*	Microbial solution (Pty) Ltd, South Africa
	Organico	*Bacillus* spp. *Enterobacter* spp., *Pseudomonas*, *Stenotrophomonas*, *Rhizobium*	Amka Products (Pty) Ltd, South Africa
	Soil Vital Q	*Bacillus subtilis*, *Bacillus thuringiensis*, *Azotobacter chroococcum*, *Pseudomonas fluorescens*, *Lactobacillus* sp.	BioControl Products SA (Pty) Ltd
	Bac up	*Bacillus subtilis*	BioControl Products SA (Pty) Ltd
	Azo-N	*Azospirillum brasilense*, *Azospirillum lipoferum*	BioControl Products SA (Pty) Ltd
	Azo-N Plus	*Azospirillum brasilense*, *Azospirillum lipoferum*, *Azotobacter chroococcum*	BioControl Products SA (Pty) Ltd
	B-RUS, Extrasol	*Bacillus subtilis*	Ag-Chem Africa (Ltd) Ltd, South Africa
	NAT-P	*Pseudomonas fluorescence*	BioControl Products SA (Ltd) Ltd
	N-Soy	*Bradyrhizobium japonicum*	BioControl Products SA (Ltd) Ltd
	SoilFix	*Brevibacillus laterosporus*, *Paenibacillus chitinolyticus*, *Lysinibacillus sphaericus*, *Sporolactobacillus laevolacticus*	BioControl Products SA (Ltd) Ltd
	Composter	*Bacillus* spp.	BioControl Products SA (Ltd) Ltd
	N-Bean	*Rhizobium phaseolus*	BioControl Products SA (Ltd) Ltd
	Histick	*Bradyrhizobium japonicum*	BASF SA (Pty) Ltd, South Africa
	Nodumax	*Bradyrhizobia*	IITA Business incubation platform, Nigeria
	BIOFIX	Rhizobia	MEA Fertilizer Ltd, Kenya
	Soyflo	*Bradyrhizobium japonicum*	Soygro (Ltd) Ltd, South Africa

(continued)

Table 7.2 (continued)

Continent	Product	Active component	Manufacturer
	Rhizostim	*Azospirillum* sp.	Soygro (Ltd) Ltd, South Africa
	Mazospirflo	*Azospirillum brasilense*	Soygro (Ltd) Ltd, South Africa
Europe	Legume fix (common bean)	*Rhizobium* spp.	Legume Technology (UK)
	Legume fix (soybean)	*Bradyrhizobium japonicum*	
	Twin N	*Azorhizobium* sp., *Azoarcus* sp., *Azospirillum* sp.	Mapleton Ltd, UK
	Nitrasec	*Rhizobium tropici*	Lage y Cía. S.A, Uruguay
Australia	Bio-N	*Azotobacter* spp.	Nutri-Tech Solution, Australia
	B.Sub	*Bacillus subtilis*	Nutri-Tech Solution, Australia
	Accelerate	*Bacillus polymyxa*, *Streptomyces* spp.	Nutri-Tech Solution, Australia
	Bioplex	*Azotobacter* spp.	Nutri-Tech Solution, Australia
	Myco tea	*Azotobacter chrococcum*, *Bacillus polymyxa*	Nutri-Tech Solution, Australia
	Twin-N	*Azorhizobium*, *Azoarcus*, *Azospirillum*	Mapleton Int. Australia
	NIB PGPR peat inoculant	*Pseudomonas* sp.	Murdoch University, Australia
North and South America	Vault NP	*Bradyrhizobium japonicum*	Becker Underwood, USA
	Chick Pea Nodulator	*Mesorhizobium ciceri*	Becker Underwood, USA
	Cowpea peat inoculant	Rhizobia	Becker Underwood, USA
	Excalibur Gold	*Natural bacteria for field seed*	America's Best Inoculant, USA
	Graph-Ex	*Bradyrhizobium japonicum*	America's Best Inoculant, USA
	Green gram peat and Groundnut peat	Rhizobia	Becker Underwood, USA
	Myco Apply Soluble Maxx	*Bacillus licheniformis*, *B. megaterium*, *B. pumilus*, *B. amyloliquefaciens*	Mycorrhizal Application, Inc. USA
	Vault HP	*Bradyrhizobium* spp.	BASF, Canada

(continued)

Table 7.2 (continued)

Continent	Product	Active component	Manufacturer
	PHC Biopak	*Bacillus azotofixans*, *B. licheniformis*, *B. megaterium*, *B. polymyxa*, *B. subtilis*, *B. thuringiensis*	Plant Health Care Inc. USA
	PHC Biopak colonise AG	*Paenibacillus azotofixans*, *Bacillus licheniformis*, *B. megaterium*, *B. polymyxa*, *B. subtilis*, *B. thuringiensis*	Plant Health Care Inc. USA
	Rizo-Liq (green gram, common bean, soybean, groundnut, chickpea)	*Bradyrhizobium* sp. (green gram, ground nut and soybean), *Mesorhizobium ciceri* (chickpea), *Rhizobium* spp. (common bean)	Rizobacter, Argentina
	Rizo-Liq Top	*Bradyrhizobium japonicum*	Rizobacter, Argentina
Asia	Bioplant	*Clostridium*, *Achromobacter*, *Streptomyces*, *Aerobacter*, *Nitrobacter*, *Nitrosomonas*, *Bacillus*	Artemis & Angelio Co. Ltd, Thailand

Adapted from Raimi et al. (2017), Herrmann et al. (2015)

Furthermore, several research works have focussed on rhizobia, possibly because of its huge biological N-fixation capability, especially in symbiosis with legumes (Reis and Teixeira 2015; Zahran 1999). However, beyond rhizobia-legume interactions, there is more to be discovered and developed for improving N-fixation, particularly in nonleguminous crops. Similarly, bacterial inoculants that have multiple field applications (e.g. nitrogen fixation, nutrient solubilisation and syntheses of PGP substances) should be further investigated for efficient inoculation and sustainable crop production.

Globally, to improve quality, acceptance and adoption of bacterial inoculants, ideas should be borrowed from new technologies that include multi-omics approach. This approach could lead to the development of 'super-inoculants' that can be used not only to improve plant health but also to eliminate unwanted microbes that directly or indirectly inhibit plant development. This could involve development of a biomarker strategy for manipulating plant microbiome ecosystems, thus improving the production of efficient bacterial inoculants for sustainable management of agroecosystems.

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Chapter 8
Plant Nutrient Management Through Inoculation of Zinc-Solubilizing Bacteria for Sustainable Agriculture

Satyavir S. Sindhu, Ruchi Sharma, Swati Sindhu, and Manisha Phour

Abstract The agricultural practices adopted to enhance agricultural productivity have adversely affected our environment and the natural resources. Moreover, food security for the ever-increasing human population also demands improvement in the quality of agri-produce. Due to the very low concentration of micronutrients in cereals, human beings are suffering the deficiency of these micronutrients. Approximately one-third of the total population in developing countries is at high risk of Zn deficiency because they depend on cereals for their daily caloric intake. Indiscriminate use of agro-chemicals and chemical fertilizers to increase crop yield has caused considerably negative impact on environmental sustainability and has resulted in deficiency of micronutrients in soil and plants. The micronutrient deficiency has further resulted in loss of plant enzyme functions, cell damage, oxidative stress and metabolic disturbances and subsequently affected crop productivity. Increased interest in low-input agriculture in recent years has emphasized the use of biological inoculants (bacteria and/or fungi) to increase the mobilization of key nutrients (nitrogen, phosphorus, potassium and zinc) to crop plants. Zinc (Zn) is a crucial micronutrient for plants, microorganisms and humans. Therefore, effective strategies are required to overcome Zn deficiency in edible crops, to enhance the grain Zn content and to minimize the adverse effects of Zn deficiency on humans. Recently, inoculation of zinc-solubilizing bacteria has been recommended to overcome the zinc deficiency in plants and human beings. Zinc-solubilizing bacteria alone or with organic manures has been found to increase the bioavailability of native and applied zinc to the plants. Several bacteria including *Acinetobacter*, *Bacillus* and *Pseudomonas* have been reported to solubilize zinc. Thus, the production and management of biological fertilizers containing zinc-solubilizing bacteria can be an effective alternative to chemical fertilizers. The current knowledge about the characterization of zinc-solubilizing microorganisms (ZnSMs), complexity of the Zn-solubilization mechanisms and the interactions of biofertilizers under the field conditions leading to improved crop productivity is discussed in this chapter.

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8.1 Introduction

Plants require a variety of nutrients for optimum growth and metabolism. The inorganic forms of nutrients are absorbed along with water by the plant roots. Some of the micronutrients play a vital role in balanced crop nutrition and physiological functions and are therefore essential for plant growth and crop production. The common micronutrients important for plant metabolic activities are iron, copper, zinc, boron, nickel, manganese, molybdenum and chloride (Uchida 2000). Deficiency of any one of these micronutrients in the soil could retard plant growth, even if all other macro- or micronutrients are present in sufficient quantity (Yu and Rengel 1999). Most of the soils in world are deficient in micronutrients due to harvesting of micronutrients from the soil by growing of high-yield crops, increased use of NPK fertilizers containing lesser amounts of micronutrients and less use of organic manures and compost.

Among the different micronutrients, zinc is important for healthy growth, reproduction and metabolism of crop plants (Hughes and Poole 1989; Perumal et al. 2017). Zinc serves as an important component in a variety of enzymatic reactions, redox reactions and metabolic processes (Gandhi et al. 2014). Zinc has been reported to perform many critical functions in biological systems, including protection of structural and functional integrity of biological membranes, photosynthesis, biomass production, chlorophyll formation, nodulation, lipid and protein metabolism, carbohydrate synthesis, enhanced stress tolerance and reproductive processes (Thenua et al. 2014; Yu et al. 2017). Zinc is also required for the synthesis of phytohormones like auxins and cytokinins, which help in growth regulation and stem elongation in plants (Hussain et al. 2015). It is used for protection from free radicals and conversion of starches to sugars. It also plays a vital role in regulation of the gene expression needed for the tolerance of environmental stresses in plants (Cakmak 2000).

In areas where zinc deficiency is widespread in crops, there is a high risk for the health of livestock and humans. Zn plays a critical role in humans maintaining the activity of enzymes and is found responsible for controlling over 300 enzymatic reactions (Tapiero and Tew 2003). Solanki et al. (2016) reported that fertility problems have increased in the past few years in humans and animals in areas where zinc deficiency is more pronounced. The deficiency of important micronutrients such as iron and zinc may often lead to impairment in brain development and wound healing, and the person becomes immune-compromised to common infectious diseases such as pneumonia, diarrhoea and malaria (Prasad 2013). Mostly, the zinc and iron deficiencies are caused by a diet deficient in micronutrients or their non-bioavailability (Welch and Graham 2004).

Zinc deficiencies are commonly found in 30% of the global soils (Sharifi and Paymozd 2016) and have resulted in large losses in yield and quality of several crops and legumes worldwide. The low solubility of zinc in spite of its high abundance in soils is mainly responsible for widespread occurrence of zinc deficiency problem in crop plants (Cakmak 2008). In India, up to 50% of the agricultural land, particularly

the whole of the Indo-Gangetic belt, is reeling under zinc deficiency and expected to further increase up to 63% by 2025 (Sunitha Kumari et al. 2016). The deficiency of zinc results in remarkable reduction in plant height and occurrence of whitish brown patches, which turn necrotic subsequently. This led to serious consequences when crop plants were grown on zinc-deficient soils, which resulted in grain yield reduction of up to 80%. Zn deficiency is very common in rice cultivation, and it stands next to nitrogen and phosphorus deficiency. Severe deficiency causes a decrease in the number of tillers and delay in crop maturity (Wissuwa et al. 2006). Mostly, chemical fertilizers are applied to overcome these nutritional constraints, and the impact of zinc application on increasing crop yields has been recorded on most crops, both under irrigated and rainfed conditions. Usually, the addition of 25 kg/ha $ZnSO_4$ heptahydrate, equivalent to 5 kg/ha zinc, is generally recommended for every year or alternate years for soil application. But, they are not cost-effective, and added fertilizers readily get converted into non-accessible insoluble form to plants.

Availability of zinc from insoluble sources is regulated by many factors, among which biochemical reactions of rhizospheric microorganisms play an important role in converting unavailable forms of zinc into available forms (Singh et al. 2005; Bapiri et al. 2012; Zamana et al. 2018). From the exogenous application of soluble zinc sources, only 20% of applied zinc is available for plant uptake (Bapiri et al. 2012). The unavailable or immobilized zinc, i.e. zinc phosphate, zinc oxide and zinc carbonate, is reverted to available forms by the inoculation of bacterial strains which can solubilize it by release of organic acids and decrease in pH (Wang et al. 2013; Sharma et al. 2014).

8.2 Importance of Zinc (Zn) in Metabolism of Plants, Humans and Microorganisms

The essentiality of zinc as a micronutrient in plants and animals is phenomenal (Das and Green 2013), and Zn is observed as the 23rd most copious element on Earth with five stable isotopes (Broadley et al. 2007). Zn^{2+} has distinct characteristics of Lewis acid and is considered to be redox-stable (Barak and Helmke 1993; Sinclair and Kramer 2012; Hafeez et al. 2013). Interestingly, Zn plays a prominent role in many biochemical reactions because it is a structural constituent or a regulatory cofactor for different enzymes and proteins. At the organism level, the significant role of 'zinc finger' as a structural motif is well established in regulation of transcription (Klug 1999; Englbrecht et al. 2004; Broadley et al. 2007).

8.2.1 Responses of Zinc in Plant Metabolism and Growth

Zinc performs several important functions in different plants. It is involved in the regulation of carbonic anhydrase for fixation to carbohydrates in plants and also promotes metabolism of carbohydrate, protein and auxin and pollen formation (Marschner 1995). Zinc has been found to govern the functioning of biological membranes and to perform defence mechanism against harmful pathogens. The presence of Zn in superoxide dismutase and catalase as a cofactor has been shown to protect plants from oxidative stress. Moreover, Zn is the component of all the six enzyme classes, i.e. oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases, which perform catalytic role in various biochemical reactions in plants.

Zinc is a component of the Rubisco structure, and therefore, it activates several biochemical reactions in the photosynthetic metabolism (Brown et al. 1993; Alloway 2004a, b). Zn has been found to inhibit the production of high toxic hydroxyl radicals in Haber–Weiss reactions in the thylakoid lamellae, due to its high affinity with cysteine and histidine (Brennan 2005; Disante et al. 2010; Tsonko and Lidon 2012). The uptake and availability of water to plants have also been found to be affected by the availability of Zn (Barcelo and Poschenrieder 1990; Tsonko and Lidon 2012). In addition, Zn is also involved in the formation of complexes with DNA and RNA (Pahlsson 1989; Coleman 1992). Due to its involvement in the tryptophan synthesis (precursor for indole acetic acid production), Zn has been reported to play an active role in signal transduction (Brown et al. 1993; Alloway 2004a, b; Hansch and Mendel 2009). By combining with phospholipids and sulphydryl groups of membrane proteins, Zn is also involved in the regulation of membranes. Based on its prominent role in different functions, the Zn concentration required for proper growth of the plant is estimated to be 15–20 mg Zn kg^{-1} dry weight (Marschner 1995). The Zn deficiency in plants may cause different symptoms and responses including necrosis at root apex and inward curling of leaf lamina, mottled leaf due to inter-veinal chlorosis, bronzing and internode shortening and size reductions in leaf. Significant losses in crop quality and quantity have been reported worldwide due to Zn deficiency in crops and legumes.

8.2.2 Effect of Zinc in Humans

Zinc is a structural component of several body enzymes in the human body. Deficiency of Zn may result from unsatisfactory consumption and inappropriate absorption of Zn in the body. More than 30% of world's population is found to suffer from severe Zn deficiency (Welch 2002), and Zn deficiency is the fifth most important risk factor responsible for illness and death of humans in the developing world (Cakmak 2009). Zinc has been reported to improve the immune system of humans (Walker and Black 2007; Gibson et al. 2008). Due to the deficiency of Zn, human body suffers from hair and memory loss, skin complications and weakness in

body muscles. Insufficient Zn intake during pregnancy may cause stunted brain development of the foetus (Graham 2008; Benton 2008). Moreover, infertility has also been perceived in Zn-deficient men. Zinc deficiency may also cause congenital diseases like acrodermatitis enteropathica (Zimmermann and Hilty 2011; Kumar et al. 2016; Sharma et al. 2016). Zn deficiency in human beings is widespread in India, Pakistan, China, Iran and Turkey, and interestingly, these are the regions with Zn-deficient soils (Hotz and Brown 2004; Joy et al. 2015).

The detection and diagnosis of zinc deficiency in the human body is usually carried out by measuring zinc concentration in serum and other tissues (Hambidge and Krebs 2007). A common recommendation for an average male is for intake of 11 mg Zn per day, whereas an average female needs 9 mg of Zn daily. A female needs 13–14 mg of Zn on a daily basis during pregnancy and lactation because the requirement for zinc intake increases during this period (Hotz and Brown 2004). Zn has been found abundant in the rice husk and grains. Zn-rich foods include beef, pork, chicken and breakfast cereals; nuts like roasted peanuts, almonds, walnuts and oats; and dairy products such as yogurt, cheese and milk (Cakmak 2002; Masood and Bano 2016; Velazquez et al. 2016).

8.2.3 Role of Zinc in Microorganisms

The role of zinc in the nutrition and physiology of both eukaryotic and prokaryotic microorganisms is widely studied (Hughes and Poole 1989). Zinc deficiency in fungi and bacteria is accompanied by impairment of the formation of pigments such as melanin, chrisogenin, prodigiosin, subtilisin and others (Chernavina 1970). A few fungal genera possess immense potential of solubilizing zinc and tolerating a high zinc level. *Aspergillus niger* was found to grow under 1000 mg Zn, and this fungus is used to quantify zinc in soils containing low zinc (2 mg kg^{-1} available zinc) (Bullen and Kemila 1997). Lichens and conifers are conspicuous for their high zinc content, and the highest concentration of zinc has been found in poisonous mushrooms (Vinogradov 1965). Some bacteria, viz. *Thiobacillus thiooxidans*, *T. ferrooxidans* and facultative thermophilic iron oxidizers, have been reported to solubilize zinc from sulphide ore (sphalerite) (Hutchins et al. 1986).

8.2.4 Zinc Tolerance and Toxicity in Plants and Microbes

Zn is toxic to cellular organisms at high concentrations, but it is an indispensable component of thousands of proteins in plants, humans and microorganisms. Hence, adequate supply of Zn is critical for growth and development of organisms. Therefore, further efforts are required to understand the concept of application, acquisition and assimilation of zinc in plants. The exposure of leaf with elevated level of Zn, i.e. above 0.2 mg g^{-1} dry matter, has been found to cause multiple abnormal

functioning in plant. This toxicity level resulted in deterioration of leaf tissue, and the productivity of plant is lowered by making their growth stagnant. Soybean and rice plants were found to show sensitivity toward toxic Zn concentration (Chaney 1993). Similarly, leafy vegetable crops, viz. spinach and beet, tend to accumulate a high concentration of Zn, and therefore, effect of Zn toxicity was observed in these crops (Boawn and Rasmussen 1971).

Zinc is also toxic to prokaryotic and eukaryotic microorganisms at higher concentrations, and therefore, zinc solubilization might limit the bacterial growth. Variable effects on the growth and activities of different microorganisms were observed by supplementation of zinc in the medium. For example, 10 mM concentration of Zn^{2+} decreased the survival of *Escherichia coli* but enhanced the survival of *Bacillus cereus*, whereas it did not significantly affect the survival of *Pseudomonas aeruginosa* and *Norcardia coralline* (Babich and Stotzky 1985). Saravanan et al. (2003) studied zinc tolerance limit of bacterial isolates ZSB-O-1 and ZSB-S-2, and population reduction was reported even at 25 mg L^{-1} of $ZnSO_4$ within 24 h. Nweke et al. (2006) assessed toxicity of Zn^{2+} on four planktonic bacteria by measuring dehydrogenase activity after exposing bacterial strains to various zinc concentrations (0.2–2.0 mM). Dehydrogenase activity was progressively inhibited at concentrations greater than 0.2 mM, indicating that these bacterial strains are sensitive to Zn^{2+} stress. Rajkumar et al. (2008) isolated a metal-resistant bacterial strain SM3 from a serpentine soil, and the strain was characterized as *Bacillus weihenstephanensis*. This strain exhibited resistance to nickel and zinc even at a concentration of 700 mg L^{-1} and also exhibited the capability of solubilizing phosphate both in the absence and presence of nickel, copper and zinc metals.

8.3 Prevalence of Zinc in Soil and Factors Affecting Zinc Availability

Zinc is found in the Earth's crust at a concentration of 0.008%, and more than 50% of Indian soils exhibit deficiency of zinc (Katyal and Rattan 1993; Ramesh et al. 2014). The worldwide prevalence of Zn deficiency in crops is due to low solubility of Zn rather low Zn availability in soil (Iqbal et al. 2010). The soluble zinc sulphate ($ZnSO_4$) is added as fertilizer to improve plant growth and crop productivity, but constraints are faced in absorbing zinc from the soil, because only 1–10% of total available zinc is utilized by the crop and 90% of applied zinc is transformed into different mineral fractions (Zn-fixation), which are not available for plant absorption (crystalline iron oxide bound and residual zinc). Zinc fixation is closely related to cation exchange in acidic soils, whereas under alkaline conditions, Zn fixation occurs by means of chemisorptions of zinc on calcium carbonate, which formed a solid solution of $ZnCaCO_3$ and by complexation by organic ligands (Alloway 2008).

The content of zinc and capacity to supply Zn for optimal crop growth varies widely in agriculture soils (White and Zasoski 1999). Soils deficient in their ability

to supply Zn to crops are widespread all over the world including Australia (Sillanpaa 1990), China (Lui 1991) and India (Takkar 1996; Singh 2008; Behera et al. 2009b). The zinc applied to agriculture fields as zinc sulphate (soluble) gets converted to different insoluble forms like $Zn(OH)_2$ at high soil pH, $ZnCO_3$ in calcium-rich alkali soils and zinc phosphate in near-neutral to alkaline soils (with large application of P fertilizers) and ZnS under reducing conditions particularly during flooding (Sarathambal et al. 2010). Several factors have been found to affect Zn availability depending on the soil conditions. For example, solubility of Zn has been reported to decrease with the increase in pH (Anderson and Christensen 1988), high organic matter and bicarbonate content, high magnesium-to-calcium ratio and high availability of P and Fe (Wissuwa et al. 2006). Usually, extractable Zn was found to decrease with an increase in soil pH due to increased adsorptive capacity, formation of hydrolysed forms of zinc, possible chemisorption on calcium carbonate and co-precipitation in iron oxides (Cox and Kamprath 1972; Alloway 2008).

Zn deficiency is usually more prevalent in calcareous soils with high pH (Liu et al. 1983; Katyal and Vlek 1985). The problem of Zn deficiency is also more acute in sandy acidic soils having low organic matter content and low level of available plant nutrients (Rautaray et al. 2003). The acidic soils in India cover about 49 million ha of area, whereas more than 800 million ha of acidic soils are found worldwide (Sharma and Singh 2002). Therefore, soil acidity is causing a huge problem by affecting food production across Asia, Africa and Latin America, and it is imposing heavy costs on farmers in Europe and North America. Excessive accumulation of phosphorus in the soil has also been found to interfere on zinc uptake by plants, and thus, it has been found to cause zinc-imposed deficiency in plants (Salimpour et al. 2010).

After 7 years of continuous cropping of wheat (*Triticum aestivum*)—rice (*Oryza sativa*), wheat and maize (*Zea mays*) and chickpea (*Cicer arietinum*)—bajra (*Pennisetum typhoides*) decrease of soil pH was reported in a sandy loam soil (Chandi and Takkar 1982). These crop rotations showed diverse effects on labile Zn fractions in soil due to their effect on soil pH. Moreover, differential uptake of Zn by the crops was observed from different soil Zn fractions. Behera et al. (2009a) reported decline in organic matter and carbonate-bound Zn in an inceptisol as a result of intensive cropping with maize and wheat for more than three decades. Soil organic matter content was also reported to affect the availability of Zn (Lindsay 1972; Moody et al. 1997). High levels of organic matter increased exchangeable and organic fractions of Zn and decreased the oxide fractions of Zn in soil because of reducing conditions to enhance Zn availability for uptake by the plants.

Thus, Zn management in acidic soils is an emerging area of concern for obtaining higher crop yield. Soil surveys illustrating the geographic distribution of soil zinc availability will provide a better understanding of the nature and extent of zinc deficiencies and toxicities observed in plants, livestock and humans (White and Zasoski 1999). To evaluate the bioavailability of Zn in soils, several extractants are being used which include mineral acids, chelating agents, buffered salts and neutral salts. Diethylene triamine pentaacetic acid (DTPA) is the most widely used soil extractant for extraction of plant-available Zn in different soil types, but other

extractants like ethylenediaminetetraacetic acid (EDTA), hydrochloric acid, ammonium bicarbonate-DTPA (ABDTPA), Mehlich 1 and Mehlich 3 are also widely used (Alloway 2008). The unavailability of zinc fertilizers at the time of need, poor quality of zinc fertilizers available in the market and lack of awareness of the farmers about effects of micronutrient on plant and human health are the major challenges faced by the farmers (Das and Green 2013).

8.4 Occurrence of Beneficial Microorganisms in the Rhizosphere

The plant–soil interface around living roots, termed as rhizosphere, is a narrow zone of soil that provides niche to various microorganisms including fungi, bacteria, actinomycetes, algae and nematodes (Prashar et al. 2014). Nearly 5–21% of all photosynthetically fixed carbon by plants is being transferred to the rhizosphere through root exudates (Marschner 1995; Flores et al. 1999). These root exudates support the growth of specific microbial populations and thereby markedly affect interactions between plants and the soil environment (Doornbos et al. 2012; Mendes et al. 2013). Phenolic metabolites released in root exudates attract particular rhizospheric and soil microbes and successfully manipulate the resident soil microbial population (Brimecombe et al. 2001).

Some plants shape their rhizosphere microbiome with the recruitment of beneficial bacteria or fungi (Berendsen et al. 2012), and host genotype also influences the overall composition of these microbial communities (Badri et al. 2013; Bulgarelli et al. 2015). In addition, edaphic and environmental factors also affect the composition of root microbiome (Hacquard et al. 2015). Legume plants release a specific kind of flavonoids in the root exudates, which interact with nodulation gene *nod*D of the host-specific rhizobia to establish symbiosis with legume plants (Bertin et al. 2003; Hassan and Mathesius 2011), which provide fixed nitrogen supply to the plant (Marschner et al. 2011; Oldroyd 2013). Some plant roots release strigolactones to attract mycorrhiza for improving phosphate supply (Akiyama et al. 2005). Recently, the changing climatic conditions were found to alter the rhizosphere biology by modifying rates of root exudation and biogeochemical cycling (Hawley et al. 2017). These rhizosphere bacteria improve plant growth by (1) supplying nutrients to crops; (2) producing plant hormones; (3) inhibiting the activity of plant pathogens; (4) improving soil structure; (5) reducing abiotic and biotic stress and (6) causing bioaccumulation or microbial leaching of inorganics and heavy metals (Ehrlich 1996; Sindhu et al. 2014).

Some beneficial rhizosphere microorganisms improve the plant growth and yield through nutrient cycling by providing mineralized nutrients (Bulgarelli et al. 2013; Sindhu et al. 2016, 2019). Beneficial plant growth-promoting rhizobacteria (termed as PGPR) include a wide range of genera, i.e. *Acinetobacter*, *Alcaligenes*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Serratia*, etc.

(Sturz et al. 2000; Shoebitz et al. 2009). These rhizobacteria produce plant growth regulators/hormones, solubilize phosphorus and potassium, fix atmospheric inert nitrogen and act as elicitors for tolerance of abiotic and biotic stresses (Yang et al. 2008; Bhattacharyya and Jha 2012; Pérez-Montaño et al. 2014). Some bacteria produce phytohormones such as indole acetic acid (IAA), gibberellins (GA_3) and cytokinins, which alter root architecture and stimulate plant growth (Spaepen et al. 2007; Duca et al. 2014). Some species of *Pseudomonas* (e.g. *P. fluorescens*), *Streptomyces* and *Bacillus* have been found to inhibit the proliferation of the pathogens (Bhattacharyya and Jha 2012; Sharma et al. 2018b). Other PGPR strains have been reported to induce tolerance in plants to abiotic stresses. For instance, *Paenibacillus polymyxa*, *Achromobacter piechaudii* and *Rhizobium tropici* were found to ameliorate the drought stress in *Arabidopsis*, tomato (*Solanum lycopersicum*) and common bean (*Phaseolus vulgaris*), respectively, by accumulation of abscisic acid and due to degradation of reactive oxygen species and ACC (1-aminocyclopropane-1-carboxylate) (Mayak et al. 2004b; Yang et al. 2008). Salinity tolerance in plants was improved by inoculation of *Achromobacter piechaudii* and *B. subtilis* (Mayak et al. 2004a; Zhang et al. 2008; Choudhary and Sindhu 2016). Endophytic bacteria isolated from wild rice (*Oryza alta*) plants were found to supply fixed nitrogen to their host plants (Baldani et al. 2000; Chaudhary et al. 2012).

Infestation of plants with a pathogen has been reported to alter the soil microbiome composition through shifts in root exudation profile (Chaparro et al. 2013). For example, the presence of the pathogenic fungus *Fusarium graminearum* in the rhizosphere of barley triggered the exudation of many phenolic compounds that prevented fungal spore germination (Lanoue et al. 2009). The rhizobacterium Pst DC3000 was chemoattracted by secretion of L-malic acid by roots in response to infection of foliage. The interaction of the *B. subtilis* strain FB17 with the *Arabidopsis* plants altered the expression of host plant genes, which are involved in regulation of auxin production, metabolism, defence and stress responses and also caused modifications in cell wall (Lakshmanan et al. 2012). The hormones involved in plant immunity, i.e. salicylic acid and jasmonic acid, were also found to affect the root microbiome (Lebeis et al. 2015). Therefore, further understanding of the rhizosphere biology is required for promoting beneficial plant–microbe interactions as a low-input biotechnology for sustainable agriculture (Ryan et al. 2009; Dubey et al. 2016).

8.5 Characterization of Zinc-Solubilizing Bacteria from Rhizosphere

The soluble form of zinc fertilizers are applied to the field soils to surmount the Zn deficiency. These chemical fertilizers are very costly and cause pollution in soil, air and water. Therefore, an eco-friendly and cost-effective approach is required to supplement the Zn deficiency by inoculation of Zn-solubilizing microorganisms.

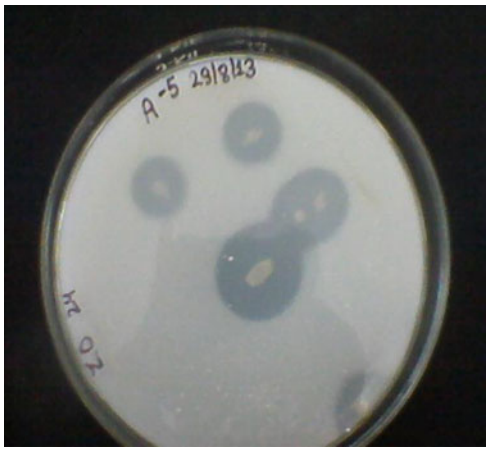

Fig. 8.1 Solubilization zone formed by zinc-solubilizing bacteria

Recently, the use of beneficial microorganisms is advocated for sustainable agriculture and restoration of soil fertility (Sindhu et al. 2019). For improving Zn availability in field soils, solubilization of insoluble Zn compounds [ZnO, $ZnCO_3$, $Zn_3(PO_4)_2$] by plant growth-promoting rhizobacteria has been reported (Saravanan et al. 2007a, b; Sharma et al. 2012; Krithika and Balachandar 2016; Gontia-Mishra et al. 2016) (Fig. 8.1). The inoculation of Zn-solubilizing bacteria (ZSB) has been found to increase the availability of soluble zinc for plant assimilation and eventually resulting in plant growth promotion.

Bacteria including *Thiobacillus thiooxidans*, *T. ferrooxidans* and facultative thermophilic iron oxidizers were reported to solubilize zinc from sulphide ore (Hutchins et al. 1986). Simine et al. (1998) isolated a zinc-solubilizing *Pseudomonas fluorescens* strain from forest soil. Zinc-solubilizing ability of *Bacillus* sp. (isolated from zinc ore) and *Pseudomonas* sp. (isolated from paddy soil) was assessed using zinc oxide, zinc sulphide and zinc carbonate in both plate and broth assays (Saravanan et al. 2003). A strain of *Gluconacetobacter diazotrophicus* was isolated that caused zinc solubilization and also showed anti-nematode activity against *Meloidogyne incognita* (Saravanan et al. 2007a, b). Sindhu (2014) obtained 38 bacterial isolates from rhizosphere soil of different crops and screened these isolates for solubilization of various insoluble zinc sources, i.e. zinc oxide, zinc sulphide and zinc carbonate. All the rhizobacterial isolates solubilized zinc oxide with solubilization index ranging from 1.56 to 36.00. Only three isolates solubilized zinc sulphide with the index varying from 1.96 to 4.00, and 33 isolates solubilized zinc carbonate with index 3.36 to 25.00. Fourteen rhizobacterial isolates showing zinc solubilization index more than 15.00 on zinc oxide-containing plates were also screened for phosphorus (P) solubilization and IAA production. All the 14 bacterial isolates solubilized P with an index ranging from 1.56 to 14.87, and only 11 isolates showed IAA production that varied in the range of 4.06–8.77 μg mL^{-1}.

Sharma et al. (2014) isolated 48 endophytic bacteria from soybean (43) and summer mungbean (5) rhizosphere. The zinc-solubilizing ability of these isolates

was studied in Tris minimal medium separately amended with inorganic zinc compounds, viz. zinc oxide (ZnO) and zinc phosphate $Zn_3(PO_4)_2$ by plate assay method. Only two bacterial isolates solubilized ZnO, while other two isolates solubilized $Zn(PO_4)_2$ on Tris minimal medium. Due to their efficiency of phosphate solubilization, zinc solubilization and IAA production, endophytes 1J (*Klebsiella* spp.) and 19D (*Pseudomonas* spp.) were found to be the most promising bacterial isolates for stimulation of plant growth. Similarly, Gandhi et al. (2014) isolated 240 zinc-solubilizing bacterial strains from rhizosphere of rice, and of them, 15 isolates were found efficient zinc solubilizers. From eight different agricultural fields of Coimbatore district of Tamil Nadu, 35 zinc-solubilizing bacteria were isolated (Sunitha Kumari et al. 2016). Five bacterial isolates were selected as the best strains based on their solubilization efficacy and were identified using the 16S rRNA sequencing method. Of the five bacterial isolates, *Pseudomonas aeruginosa* showed maximum solubilization of zinc in the broth and also decreased the pH from 7 to 3.3.

Perumal et al. (2017) isolated six zinc-solubilizing bacterial strains from the rhizosphere of maize. Bacterial isolate ZSB SM-1 was found to be most effective in solubilization of insoluble zinc substances, viz. zinc oxide, zinc carbonate and Zn-EDTA. The insoluble Zn compounds were effectively solubilized at 0.1% concentration as compared to 0.2% concentration. Dhaked et al. (2017) isolated four potassium-solubilizing bacteria (KSB), eight zinc-solubilizing bacteria (ZnSB) and two zinc-solubilizing fungi (ZnSF) from rice, maize, cotton and sorghum rhizosphere soil. Screening of the KSB isolates for solubilization of insoluble zinc oxide showed that the solubilization zone for zinc oxide ranged from 6 to 16 mm. The isolate ZnSB-3 showed maximum solubilization zone of 16 mm, and the solubilization efficiency ranged from 150% to 333.33%. The isolate ZnSF-1 showed maximum solubilization zone of 85 mm followed by ZnSF-2 with 34 mm for ZnO. The solubilization zone ranged from 6 mm to 25 mm for ZnP. The isolate ZnSB-8 showed maximum solubilization zone of 25 mm for zinc phosphate, and solubilization efficiency ranged from 157.14% to 500%.

8.6 Mechanisms Involved in Solubilization of Zinc by Zinc-Solubilizing Bacteria

Zinc-solubilizing bacteria increase the availability of zinc in the rhizosphere through different mechanisms, which ultimately improve the uptake of soluble zinc by the plant (Fig. 8.2). Different mechanisms employed by zinc-solubilizing bacteria to improve zinc bioavailability are discussed below.

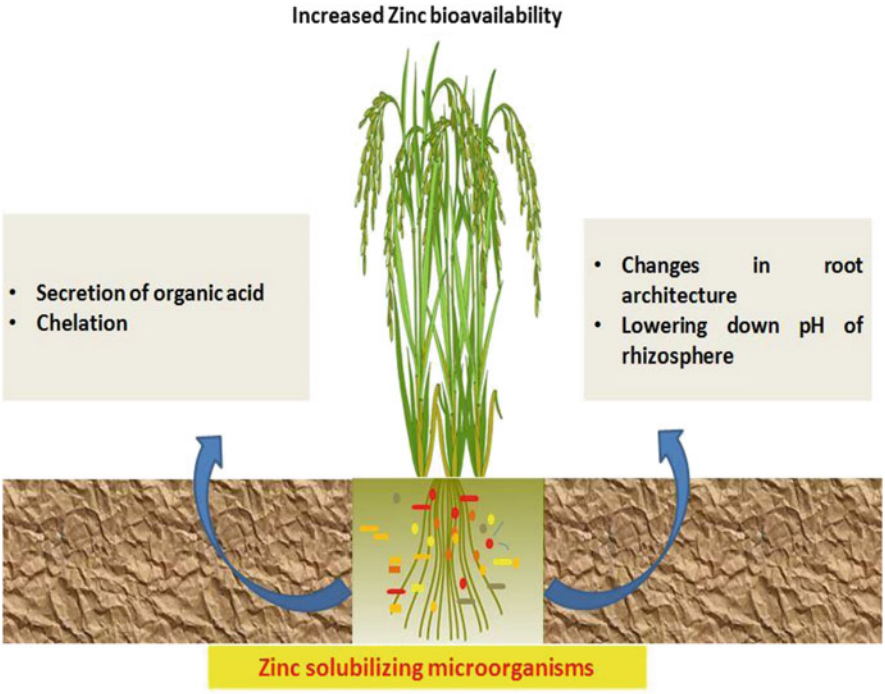

Fig. 8.2 Mechanisms involved in solubilization of zinc by microorganisms in the rhizosphere of crop plant

8.6.1 Lowering the pH of Rhizosphere

Plant growth-promoting bacteria have been reported to release organic acids and extrude protons, which lowers the pH of the rhizosphere (Fasim et al. 2002; Wu et al. 2006; Parmar and Sindhu 2018). For example, the secretion of 2-ketogluconic acid and gluconic acid by *Pseudomonas fluorescens* resulted in solubilization of zinc phosphate in the culture. Furthermore, coinoculation of *Pseudomonas* and *Bacillus* spp. in broth culture lowered down the pH, which solubilized zinc sulphide, zinc oxide and zinc carbonate (Saravanan et al. 2004). The availability of micronutrients in soil is also influenced by the pH of the soil, and it has been reported that decrease in one unit of pH resulted in 100 times increase in the availability of Zn in the soil (Havlin et al. 2005). The role of low pH has also been correlated with potassium solubilization in efficient potassium-solubilizing strains, i.e. *Bacillus subtilis* ANctcri 3 and *Bacillus megaterium* ANctcri 7 isolated from rocks in Kerala (Anjanadevi et al. 2016). Similarly, inoculation of arbuscular mycorrhizae (AM) was found to lower the soil pH in the rhizosphere, and it contributed to release of zinc from mineral fraction (Subramanian et al. 2009). However, the reduction in rhizosphere pH varied among different microorganisms (Giri et al. 2005). Wu et al. (2006) observed a decrease in pH up to 0.47 units with bacterial inoculation due to

the release of organic acids and H^+, which ultimately improved the Zn solubilization and uptake by plants.

8.6.2 Zinc Chelation

Chelation of zinc by soil/rhizosphere microorganisms is another dominant mechanism to improve Zn bioavailability and uptake by plant roots. Usually, the plant-available Zn fraction in the soil is less due to low persistency and high reactivity of Zn in soil solution. Zn-chelating compounds have been found to increase the bioavailability of zinc in the rhizosphere (Obrador et al. 2003). These chelating compounds are released by the plant roots and microorganisms present in the rhizosphere, which chelate the Zn and increase its availability in root zone of the plants. Various metabolites secreted by the rhizosphere microorganisms form complexes with Zn^{2+} (Tarkalson et al. 1998) and thereby reduce their reaction with the soil. Some bacteria, e.g. *Pseudomonas monteilii*, *Microbacterium saperdae* and *Enterobacter cancerogenesis*, have been found to synthesize Zn-chelating metallophores for enhancing water-soluble Zn, which is bioavailable in soil for plant uptake (Whiting et al. 2001). Tariq et al. (2007) reported release of fixed insoluble zinc by the biofertilizer strains containing *Pseudomonas* sp. (96-51), *Azospirillum lipoferum* (JCM-1270, ER-20) and *Agrobacterium* sp. (Ca-18) due to production of chelating agent ethylenediaminetetraacetic acid and made the zinc available for longer period to rice. Inoculation of *Penicillium bilaji* was found to enhance the bioavailability of zinc to plants through chelating mechanism (Kucey 1987).

8.6.3 Organic Acid Production

The production of organic acids like citric, oxalic and tartaric acids and the production of capsular polysaccharides by microorganisms were found to cause dissolution of the minerals illite and feldspar to release potassium (Vyas and Gulati 2009; Qureshi et al. 2017; Parmar and Sindhu 2018). The pH of the medium decreased from 7.0 to 2.05 after growth of bacterial and fungal cultures during bioextraction of potassium using feldspar. Species of *Bacillus* and *Pseudomonas* were found to produce organic acids, which decreased the pH in the root zone, and Zn was made available to plants (Saravanan et al. 2004). Some PGPR strains were reported to produce gluconic acids (Saravanan et al. 2011) or its derivatives such as 2-ketogluconic acid (Fasim et al. 2002), 5-ketogluconic acid (Saravanan et al. 2007a, b) and various other organic acids (Tariq et al. 2007) for solubilization of zinc. Zinc phosphate solubilization was studied by a strain of *Pseudomonas fluorescens* and gluconic acids produced in culture medium was found to help in solubilization of zinc salts (Simine et al. 1998). Similarly, *Bacillus* sp. AZ6 was found to solubilize insoluble zinc compounds by releasing organic acids like cinnamic acid, ferulic acid, caffeic acid, chlorogenic acid, syringic acid and gallic

acid in a liquid medium (Hussain et al. 2011). Martino et al. (2003) found that mycorrhizal fungi secreted organic acids to solubilize zinc from insoluble $Zn_3(PO4)_2$ and ZnO.

Enhanced production of organic acids was found to improve the available zinc in the culture broth. Desai et al. (2012) reported that higher availability of Zn is directly proportional to acidic pH of the culture broth. Solubilization of zinc phosphate occurred by both an increase in the H^+ concentration of the medium and the production of gluconic acid. Perumal et al. (2017) studied solubilization of insoluble zinc substances, viz. zinc oxide, zinc carbonate and Zn-EDTA using six bacterial strains isolated from the rhizosphere of maize. They concluded that solubilization of zinc from insoluble zinc substances might be due to production of acids by the culture, since the pH of the broth decreased from 7.0–7.3 to 3.0–4.8 after 10 days of inoculation.

8.7 Inoculation Effect of Zinc-Solubilizing Bacteria on Crop Growth and Yield

Micronutrient deficiencies in the soil have been found to reduce the quality and yield of the agriculture produce. It has been reported that more than 3 billion people worldwide experience micronutrient deficiency (Hennessy et al. 2014). Zn deficiency is reported as a global nutritional problem, and this deficiency is more severe in developing countries (Zamana et al. 2018). The Zn deficiency has been attributed to consumption of cereal grains having very low grain Zn concentrations, which are usually grown in Zn-deficient soils. Zinc deficiency can be minimized by nutritional diversification, food enrichment and biofortification. Zinc biofortification is a viable choice to augment the bioavailable concentrations of vital micronutrients in edible portions of crop plants through agronomic practices or genetic methods (Zamana et al. 2018). The quality of crop produce biofortification has been found to depend on the chemical properties of the soil, crop genotypes, agricultural management practices and climatic factors (Schulin et al. 2009). Attempts are being made worldwide to improve the genetic potential of crop plants for enhancing the micronutrient bioavailability in common staple food crops such as wheat, rice, maize, beans and oilseeds (Cakmak et al. 2010). Plant breeding approaches are being used to enhance the amount of a number of minerals concurrently available in edible tissues of food, whereas transgenic approaches are used to improve nutrient mobilization from the soil, transport to the shoot and leaf and build-up of mineral elements in bioavailable forms in edible tissues (Borrill et al. 2014). The plant breeding approach to increase micronutrient uptake by plant roots is tedious, and results take a long time, whereas the transgenic approach is costly.

Another eco-friendly alternative approach is the application of potential plant growth promoting microorganisms (PGPMs) to increase micronutrient uptake by roots. These PGPMs could facilitate the growth of crop plants by modulating of root architecture resulting in growth of deep root systems in nutrient-deficient soils and

Table 8.1 Effect of various zinc-solubilizing bacterial isolates on plant growth parameters

Zinc-solubilizing bacterial isolates	Effects on plant growth	Crop plant	Reference
Pseudomonas sp. strain ZSB-S-I	Improved the zinc content in plant tissues	Soybean	Saravanan et al. (2004)
Pseudomonas strain BA-8 and *Bacillus* strain M-3	Increased fruit yield per plant, i.e. 91.73% and 81.58% when treated with BA-8+M-3 and M-3, respectively	Strawberry	Esitken et al. (2009)
P. aeruginosa strain CMG860	Increase in root (144%) and shoot length (120%)	Rye	Shahab et al. (2009)
Bacillus isolates	Increase zinc accumulation in seeds	Soybean	Sharma et al. (2012)
Pseudomonas strains B_1 and B_2	Increased grain Zn concentration (31%)	Rice	Deepak et al. (2013)
Burkholderia strain BC and *Acinetobacter* strains AB and AX	Increased mean number of productive tillers (21.1%), number of grains per year (5.7%), thousand grain weight (10.1%), grain yield (18.1%) and straw yield (3.1%) and reduced phytic acid concentration (17.6%)	Wheat	Vaid et al. (2013)
Bacillus aryabhattai strains MDSR7, MDSR11 and MDSR14	Strains MDSR7 and MDSR14 substantially influenced mobilization of zinc and its concentration in edible portion, yield of soybean and wheat	Soybean and wheat	Ramesh et al. (2014)
Bradyrhizobium japonicum	Phosphorus supplementation caused increase in micronutrients uptake; but decrease in Zn content was observed in few organs	Cowpea	Nyoki and Ndakidemi (2014)
Bacillus strain AZ6	Increased shoot length (59%) and photosynthetic rate (90%)	Maize	Hussain et al. (2015)
Bacillus sp. and *Bacillus cereus*	Suppressed *Pyricularia oryzae* and *Fusarium moniliforme*, and enhanced Zn translocation toward grains and increased yield of basmati-385 (22–49%) and super basmati rice varieties (18–47%)	Rice	Shakeel et al. (2015)

the excretion of ligands/siderophores or acids/alkalis to mobilize micronutrients. Microbial transformation of unavailable forms of soil zinc to plant-available zinc by zinc-solubilizing bacteria could influence the mobilization and uptake of zinc in edible portion and may improve the yield of different cereals, legumes and horticulture plants (Table 8.1).

8.7.1 Zinc Uptake by PGPR and ZnSB

Saravanan et al. (2004) isolated zinc-solubilizing bacterial cultures from soil and ore (sphalerite) sources both by direct plating and by enrichment technique in the modified Bunt and Rovira medium incorporated with 0.1% zinc. Among these, ZSB-O-1 and ZSB-S-4 were characterized as *Bacillus* sp. and ZSB-S-2 as *Pseudomonas* sp. The results revealed that *Pseudomonas* sp. (ZSB-S-1) was able to correct the zinc deficiency in soybean plants when used along with 1% (w/w) zinc oxide. Tariq et al. (2007) inoculated plant growth-promoting rhizobacteria for mobilizing indigenous soil zinc in rice (*Oryza sativa* L.) and compared it with the available form of chemical Zn source as Zn-EDTA. Application of PGPR decreased the zinc deficiency symptoms and increased the total biomass (23%), grain yield (65%) and zinc concentration in the grains invariably. Positive effects on root length (54%), root weight (74%), root volume (62%), root area (75%), shoot weight (23%), panicle emergence index (96%) and higher Zn mobilization efficiency were observed in inoculated plants in comparison to the uninoculated control. Li et al. (2007) investigated the effects of *Burkholderia cepacia* on metal uptake by the hyperaccumulating plant *Sedum alfredii* with different concentrations of cadmium and zinc. Inoculation with bacteria significantly enhanced plant growth (up to 110% with zinc treatment), phosphorus uptake (up to 56.1% with cadmium treatment), and metal uptake (up to 243% and 96.3% with cadmium and zinc treatment, respectively) in shoots, the tolerance index (up to 134% with zinc treatment) and translocation of metals (up to 296% and 135% with cadmium and zinc treatment, respectively) from root to shoot.

Kuffner et al. (2008) obtained ten rhizospheric isolates (*Pseudomonas*, *Janthinobacterium*, *Serratia*, *Flavobacterium*, *Streptomyces* and *Agromyces*) from heavy-metal-accumulating willows. These isolates were analysed for plant growth promotion and zinc and cadmium uptake in *Salix caprea* plantlets grown in sterilized, zinc–cadmium–lead-contaminated soil. *Agromyces* strain AR33 was found to increase plant growth and also enhanced the total amount of zinc and cadmium extracted from soil. Iqbal et al. (2010) studied the inoculation effects of five bacterial isolates (U, 8M, 36, 102 and 111) on the growth of *Vigna radiata.* Bacterial isolates were applied alone or together with zinc phosphate [$Zn_3(PO_4)_2{\cdot}4H_2O$]. The maximum increase in root and shoot length was observed as a result of inoculation with the isolate 102. The fresh and dry weight of seedlings was also enhanced in comparison to control. Bacterial isolate 36 with amendment of 1 mM zinc phosphate resulted in a maximum increase of almost 1.7 times in the seedling length (35.1 cm) in comparison to control (19.3 cm), indicating that bacteria can be used as a biofertilizer for improving the growth of mungbean plants in presence of water-insoluble zinc phosphate.

Sharma et al. (2012) isolated 134 *Bacillus* isolates from soybean rhizosphere soils to select effective zinc solubilizers for increased assimilation of Zn in soybean seeds. Inoculation of *Bacillus* isolates significantly increased the Zn concentration in soybean as compared with uninoculated control (47.14 μg/g). Goteti et al. (2013)

screened ten zinc solubilizing strains on maize crop in a short-term pot culture experiment. Seed bacterization with zinc-solubilizing *Pseudomonas* sp. strain P29 significantly enhanced the concentrations of macronutrients and micronutrients such as manganese (60 ppm) and zinc (278.8 ppm) in comparison to uninoculated control. In similar studies, Vaid et al. (2014) assessed the capacity of three bacterial strains, i.e. *Burkholderia* strain BC and *Acinetobacter* strains AB and AX, isolated from a zinc-deficient rice–wheat field to improve Zn nutrition in Zn-responsive (NDR359) and Zn non-responsive (PD16) varieties of rice. Bacterial inoculation significantly enhanced the total zinc uptake per pot (52.5%) as well as grain methionine concentration (38.8%). Inoculation with bacteria either singly or in combination significantly increased the mean dry matter yield/pot (12.9%), productive tillers/plant (15.1%), grain yield (17.0%) and straw yield (12.4%) over the control and Zn fertilizer treatment. The phytate-to-zinc ratio in grains was also reduced by 38.4% in treatments with bacterial inoculations.

8.7.2 Inoculation Effect of AM Fungi on Zinc Uptake

Root colonization by arbuscular mycorrhizal (AM) fungi was found to increase the uptake of metal micronutrients, such as copper in white clover (Li et al. 1991), copper, zinc, manganese and iron in *Zea mays* (Liu et al. 2000) and zinc in field pea crops (Ryan and Angus 2003). Similarly, higher uptake of iron, manganese, zinc and copper was reported in wheat by inoculation of *Azospirillum* and mycorrhizae in comparison with uninoculated control plants (Ardakani et al. 2011). Inoculation of rice roots with arbuscular mycorrhizal fungi was found to increase zinc uptake and mobilization and showed enhanced growth of rice (Purakayastha and Chhonkar 2001). Higher Zn uptake and increase in wheat and maize growth was observed by inoculation of AM fungi in zinc-deficient soils after addition of zinc as a fertilizer (Kothari et al. 1990).

8.7.3 Application of ZnSB Along with Manure and Fertilizers

Strains of *Bacillus cereus* (N_2 fixing), *Brevibacillus reuszeri* (phosphorus solubilizing) and *Rhizobium rubi* (both N_2 fixing and phosphorus solubilizing) were inoculated on broccoli to evaluate their effect on plant growth, nutrient uptake and yield in comparison with manure (control) and mineral fertilizer application under field conditions (Yildirim et al. 2011). Bacterial inoculations with manure significantly increased yield, plant weight, head diameter, chlorophyll content and nitrogen, potassium, calcium, sulphur, phosphorus, magnesium, iron, manganese, zinc and copper content of broccoli in comparison to control treatment. Senthilkumar et al. (2014) reported that the combination of fertigation and a consortium of biofertilizers in banana significantly enhanced accumulation of secondary nutrients and micronutrients (Fe, Zn, V and Mn) in the leaves, pseudostem and fruits at harvest. Senthil et al. (2004) conducted a field

study to assess the effect of Zn-enriched organic manures and Zn-solubilizing bacteria on the yield, curcumin content of turmeric and nutrient status of the soil. When treated with farm yard manure (FYM) along with zinc-solubilizing bacteria, higher turmeric rhizome yield (21.6%) was observed in comparison with those treated with FYM alone (9.1%) and without manure (control). The dry rhizome yield showed the promising effect of Zn- and Fe-enriched coir pith or FYM. The highest values for available N, P and K contents in the soil were observed by use of FYM along with Zn-solubilizing bacteria. Significant effect on the availability of N, P and K was observed in treatment with inoculation of Zn-solubilizing *Bacillus* sp. The application of $ZnSO_4$, $FeSO_4$ and fortified FYM along with Zn and Fe and their foliar spray showed synergistic effect and enhanced the bioavailability of micronutrients as well as potassium.

The effect of micronutrients and inoculation of zinc-solubilizing bacteria was studied on the yield and quality of grape variety Thompson seedless (Subramoniam et al. 2006). Recommended doses of N, P and K fertilizers were applied along with foliar sprays of $ZnSO_4$ (0.2%) + boric acid (0.2%) + $FeSO_4$ (0.2%) + $MnSO_4$ (0.2%) + $MgSO_4$ (0.5%) + $CaCl_2$ (0.5%) + KNO_3 (0.5%) + urea (1%) at blooming and 15 days after blooming stages. Both the inoculation of zinc-solubilizing bacteria along with application of fertilizers and foliar sprays were recommended as cost-effective technology for increasing the grape yield. The fruits' quality such as juice content, TSS, titratable acidity, specific gravity, total sugar and TSS/acidity ratio were also higher in the treatment having inoculation of zinc-solubilizing bacteria along with fertilizers in comparison to control uninoculated treatment.

8.7.4 Coinoculation of Phosphorus- and Zinc-Solubilizing Bacteria

Phosphorus is the second major plant nutrient required for the proper growth and metabolic activities of a plant (Sindhu et al. 2014). Hu et al. (2006) isolated two phosphate- and potassium-solubilizing *Paenibacillus mucilaginosus* strains KNP413 and KNP414 from the soil of Tianmu Mountain. Both the strains effectively dissolved mineral phosphate and potassium, while strain KNP414 showed higher dissolution capacity. In a similar way, it is desired that coinoculation of phosphorus or potassium-solubilizing bacteria having zinc solubilizing activity may show synergistic effects leading to significant stimulation of the plant growth. Woo et al. (2010) isolated phosphate-solubilizing bacterial isolates from the rhizosphere of Chinese cabbage and found that 10 strains having higher phosphorus-solubilization potential also solubilized insoluble ZnO. Recently, Zeng et al. (2017) reported that production of organic acids by *Pseudomonas frederiksbergensis* strain JW-SD2 is correlated with phosphorus-solubilizing activity, and its effects on plant growth promotion of poplar seedlings were greater in the non-sterilized than sterilized soil.

To assess the impacts of *B. japonicum* inoculation and phosphorus supplementation on the uptake of micronutrients in cowpea, a field and pot house experiment was conducted (Nyoki and Ndakidemi 2014). Significant improvement in micronutrients uptake was observed in the *B. japonicum*-inoculated treatments over the control. Phosphorus supplementation (40 kg P/ha) also resulted in significant increase in the uptake of some micronutrients, while it caused decrease in Zn uptake in few plant organs. Significant interaction between *B. japonicum* inoculation and addition of phosphorus was observed with the root uptake of Zn for the field experiment. Sindhu (2014) tested three bacterial isolates MR1, CR2 and OR1 for zinc solubilization, and their inoculation effect was studied on growth and yield of mungbean crop under pot house conditions. The inoculation of isolate MR1 caused 72.6% increase in shoot dry weight in comparison to uninoculated control. Inoculation of mungbean with bacterial isolates MR1 and CR2 showed 104.8% and 72.0% increase in seed yield, respectively, as compared to uninoculated control. Treatment with $ZnSO_4$ at 25 kg ha^{-1} along with inoculation of isolate OR1 was found significantly superior to all other treatments and caused 184% and 92.6% increase in seed yield and shoot dry weight in comparison to uninoculated control. The selected two strains, CR2 (highest zinc solubilizer) and OR1 (highest plant growth promoter), were identified as *Bacillus stratosphericus* and *Bacillus altitudinis* by 16S rRNA gene sequence analysis. It was concluded that the *Bacillus altitudinis* isolate OR1 showing maximum plant growth promotion effect under pot house conditions could be exploited as a Zn-solubilizing biofertilizer for plant growth promotion of mungbean under field conditions.

8.7.5 ZnSB Role in Disease Control

Global crop yields are reduced by 20–40% annually due to pests and diseases (Strange and Scott 2005). Sustainable agricultural practices are revitalizing the interest of scientists in characterization of plant beneficial microorganisms having both nutrient mobilization and control of plant diseases by biological control agents. Recently, some of the microbial strains were isolated for solubilization/mobilization of phosphorous, potassium or zinc, and these strains also inhibited the growth of pathogenic fungi resulting in suppression of plant diseases (Sharma et al. 2018a, b; Parmar and Sindhu 2018). Zinc-solubilizing bacteria *Gluconacetobacter diazotrophicus* was found to possess antagonistic activities, and therefore, it was also used as a biocontrol agent against root nematodes and various fungal phytopathogens (Saravanan et al. 2007a, b). Shakeel et al. (2015) isolated *Bacillus* sp. and *Bacillus cereus*, which suppressed the growth of *Pyricularia oryzae* and *Fusarium moniliforme* (22%–29%), and their inoculation increased the yield of basmati rice variety 385 by 22–49% and super basmati rice varieties by 18–47%. Inoculation of zinc-solubilizing bacteria and their consortium in wheat along with $ZnSO_4.7H_2O$ at 5 mM significantly enhanced the plant height, chlorophyll content and grain number of wheat plants (Deepak et al. 2013).

8.7.6 Auxin Production by Zinc-Solubilizing Bacteria

Phytohormones have been found to affect the physiological processes of plants. Production of indole acetic acid (IAA) is more frequent among rhizosphere bacteria than other hormones such as gibberllic acid and cytokinins (Spaepen and Vanderleyden 2011). About 80% of rhizosphere bacteria have been reported to possess IAA production ability (Patten and Glick 1996; Jangu and Sindhu 2011). Skoog (1940) reported relationship between zinc solubilization and auxin production, which resulted in improvement of growth in higher plants. Shahab et al. (2009) tested efficient zinc phosphate-solubilizing bacteria for auxin production. These bacteria exhibited positive effects on the growth of root and shoot elongation of mung bean (*Vigna radiata*). Sindhu (2014) isolated 38 zinc-solubilizing bacteria from rhizosphere soil of different crops. Fourteen rhizobacterial isolates showing zinc solubilization index more than 15.00 on zinc oxide-containing plates were also screened for phosphorus solubilization and IAA production. All the 14 bacterial isolates solubilized P with an index ranging from 1.56 to 14.87, and only 11 isolates showed IAA production in the range of 4.06–8.77 μg mL^{-1}.

8.8 Conclusion

The widespread incidences of zinc deficiency in crop plants are correlated with low solubility of zinc compounds (Cakmak 2009). The chemical fertilizers are applied in the soil to improve crop productivity, which results in high costs to farmers, and excessive use of fertilizers is also responsible for environmental pollution. The development of sustainable agriculture system requires new eco-friendly technologies to minimize the use of chemical fertilizers while maintaining proper crop yields. Generally, a major part of added fertilizers gets converted to insoluble fractions and becomes unavailable to plants. Therefore, the application of PGPR having nutrient solubilization potential in agriculture will not only reduce the cost expenditure by minimizing the use of expensive agro-chemicals but also provide safe and healthy environment (Herrera et al. 1993; Glick 1995; Requena et al. 1997; Vessey 2003). Keeping in view the importance of zinc in various crops and role of Zn-solubilizing bacteria in making it available to the plants, identification of zinc-solubilizing bacteria is necessary to solubilize zinc in the soil. Recently, zinc-solubilizing bacteria have been isolated from the rhizospheric soil of different crops (Sunitha Kumari et al. 2016; Dhaked et al. 2017; Zamana et al. 2018). Inoculation of ZnSB ensures proper functioning and plant growth and presents a viable, self-sustainable, low input and eco-friendly alternative to chemical fertilizers for use in agro-ecosystems. These microbial strains capable of solubilizing zinc minerals can conserve our existing resources and avoid environmental pollution hazards caused by excessive use of chemical fertilizers. Thus, inoculation of microbial consortium possessing the capability of N, P, K and Zn mineralization is a cost-effective and

eco-friendly approach for enhancing crop yields in sustainable agriculture (Badr et al. 2006; Zhang et al. 2013: Dhaked et al. 2017; Sindhu et al. 2019). On the applied side, the coinoculation of zinc-solubilizing bacteria with growth-promoting rhizosphere bacteria or the inoculation of microbial consortia is preferable because these microorganisms might express beneficial functions more continually in a soil or rhizosphere system, even under ecologically different and/or variable conditions.

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Chapter 9
Endophytic Bacteria as a Modern Tool for Sustainable Crop Management Under Stress

Yachana Jha

Abstract Plant growth and development under both biotic and abiotic stress is enabled by the bacteria residing in plants, especially in the roots. Of many isolated endophytic bacteria, two isolates, *Pseudomonas aeruginosa* and *Pseudomonas pseudoalcaligenes*, on the basis of the presence of the *nifH* gene and growth elevation potential were selected as a tool to develop tolerance in crops under stress. Plants inoculated with such bacteria have better nutrient status under stress. Abiotic stress, especially salinity, causes consequences in altered protein profiling, production of low molecular weight chaperones, as well as production of nontoxic osmoprotectants in plants to overcome stress. Isolated endophytes also induce differential gene expression of β-1,3-glucanase and RAB18, which has been observed during RNA profiling. Such plants acquire better ability to survive under stressful environments. These findings suggest that the ecologically safe endophytic bacteria can be a modest and economic tool for regulating several plant metabolites and opposing stress to enhance crop production by assisting stress management in crop plants. Use of such beneficial bacteria in diverse agronomic systems to develop plants broadly resistant under both stressed and normal states is a current need.

Keywords Endophytic bacteria · Programmmed cell death · Biotic stress · Abiotic stress · Gene induction

9.1 Introduction

A major threat for agriculture sustainability is the continuous increase in the human population and reduction in the availability of land for farming (Shahbaz and Ashraf 2013). Various environmental factors that confine crop yield or rescind plant growth are known as stresses. Agricultural crop production has been hampered by several environmental stresses, in which soil salinity is the most disruptive. Salinity is

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responsible for the loss of cultivable land, stunted plant growth, and reduced crop production and quality. Indeed, in arid and semi-arid provinces, soil and water salinity has become one of the major stresses, which can brutally limit crop production (Tester and Bacic 2005). Plant growth is severely affected by salinity as it alters the osmotic potential of the soil extracts, enhances particular ion effects, and also changes the nutritional status of soil as well as acquisition by the plant roots. Separately or in combination, all such factors affect biochemical, physiological, and molecular aspects of plant growth and development (Shao et al. 2008). However, the plant develops various mechanisms such as modification in the biochemical, physiological, and morphological patterns to develop tolerance/resistance against such stresses.

The food requirements of the increasing population have been fulfilled initially by conventional agriculture, but to meet the growing food requirements there has been a massive increase in the use of chemical fertilizers and pesticides, which make soil infertile as well as hostile for farming (Santos et al. 2012). The excess use of chemical fertilizers pollutes agricultural soils, and the chemicals are also incorporated into our food chain. Endophytic bacteria are microorganisms that reside in plants and provide positive effects on plant growth. Endophytic bacterial establishment depends on the capability of bacteria to invade plant host cells for their niche as required for multiplication. Endophytic bacteria may act as efficient tools for the growth of plants under different adverse conditions. Endophytic bacteria not only help in plant growth, but also have the ability to act as a biocontrol agent for protecting plants against numerous plant pathogens, increase nutrient assimilation, and develop tolerance for its better growth. With increased awareness about our health, more efforts have been put into the production of 'nutrient-rich high-quality food' in justifiable conduct toward confirming bio-safety. So, it is the need of the hour to develop a technique for the application of biologically based products as an alternative to agro-chemicals (Raja 2013).

9.2 Isolation, Identification, and Inoculation of Endophytic Bacteria

The name endophyte consists of two Greek words—"endon," which means within, and "phyton," which means plant. Endophytes are a group of microbes including bacteria and fungi that reside in the plant cell for their entire life and assist the plant for better survival. In this association, microbes reside in the plant without causing any detrimental effect on the plant host. Such microbes include *Azoarcus*, *Pantoea*, *Gluconobacter, Burkholderia*, *Pseudomonas, Klebsiella,* and *Herbaspirillum* (Kandel et al. 2015). Such microbes have some common features comprising the ability to solubilize phosphate, synthesize plant hormones, and secrete siderophores and antibiotics to enhance plant ability to survive against adverse environmental conditions (Gaiero et al. 2013). Some unique endophytes also have the ability to fix

atmospheric nitrogen as they have the gene for biological nitrogen fixation, which converts dinitrogen gas (N_2) into ammonium and nitrate for their host, which can be easily used by the plant (Santi et al. 2013).

For this study, endophytic bacteria were isolated from the paddy and *Suaeda nudiflora* wild mosque plant roots as per our published method (Jha et al. 2011). Initially, semi-solid NFb medium is used for the isolation of endophytic bacterial strain; the appearance of a white veil-like pellicle after 1 week of inoculation below the surface of the semi-solid NFb medium confirmed the presence of endophytic bacteria. The NFb medium has bromothymol blue, a pH indicator dye that changes in plate color from green to blue to indicate a shift in pH toward alkalinity from nitrogen fixation by such bacterial growth. The endophytic bacteria are then purified and transferred to NFb agar plates, then on nutrient agar plates to be maintained for further study. The isolates able to grow on NFb medium indicate the ability of the microbes for nitrogen fixation. This ability has further been confirmed by amplification of the nifH gene in this bacterial isolate, which indicates the existence of nitrogenase reductase (nifH) for nitrogen fixation. For this, total genomic DNA has been isolated from the bacteria and subjected to polymerase chain reaction (PCR) amplification with gene-specific primers, resulting in a 420 bp PCR product on agarose gel (Fig. 9.1). The bands are then eluted from the gel and sequenced, and subjected to nucleotide BLAST for the DNA sequence data match, which matched with the predicted nifH sequence.

Isolates were identified on the basis of their 16*S* rDNA gene sequences by using total genomic DNA of the isolates subjected to PCR amplification with 16*S* rDNA-specific primers 16SF:5′-AGAGTTTGATCCTGGCTCAG-3′ and 16SR:5′-AGGTTACCTTGTTACGACTT-3′, followed by sequencing as in our published method (Jha and Subramanian 2013a). Bands of 1.5 kb are obtained as discrete bands in agarose gel (Fig. 9.2) of both bacterial isolates, followed by sequencing. The sequences obtained are used for construction of the phylogenetic trees after nucleotide BLAST. The 16*S* rDNA sequence of isolates and sequence of

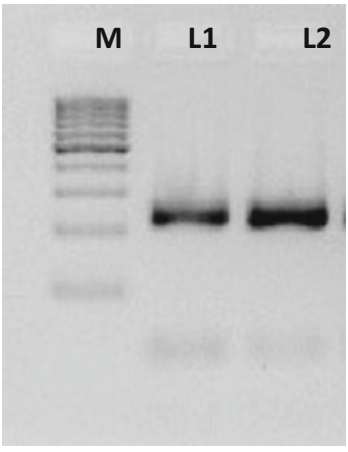

Fig. 9.1 Agarose gel showing amplified *nifH* gene of isolates. M—100 bp marker; L-1 and L-2—lanes of *nifH* gene of *Pseudomonas pseudoalcaligenes* and *Pseudomonas aeruginosa*, molecular weight about 420 bp each, respectively

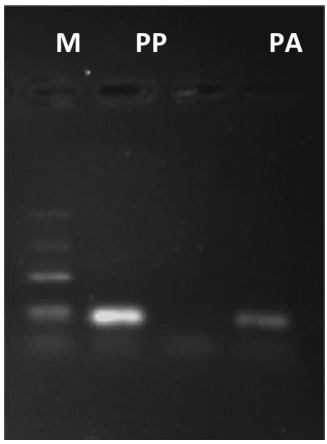

Fig. 9.2 Agarose gel showing amplified 16*S* rDNA of two bacterial isolates. M—1 kb marker; PP—*P. pseudoalcaligenes*, and PA—*Pseudomonas aeruginosa*, 16*S* rDNA amplicons

related genera are compared from the database using the neighbor-joining (NJ) algorithm and maximum likelihood (ML) method. By molecular analysis, two isolates are identified as *Pseudomonas pseudoalcaligenes* and *Pseudomonas aeruginosa*, with Gene Bank accession numbers EU921258 and JQ790515, respectively. Both the molecular analyses showed that isolates from the N-free semi-solid enrichments medium are nitrogen-fixing endophytic bacteria.

These isolates are used for maize seed inoculation with some modification as per our published methods (Jha and Subramanian 2013b). First, seed are properly washed with sterile distilled water and, to check for possible contamination, the seeds are aseptically transferred on tryptone glucose yeast extract agar medium and incubated at 30 °C in the dark. For inoculation with the isolates, germinated seedlings devoid of any contamination are used. The maize plant is transferred on 400 ml Hoagland's nutrient medium, having 400 ml micronutrients and 1% agar in 40 ml distilled water in culture tubes, to analyze the consequences of the isolated bacteria on the various biochemical parameters. The isolated bacteria are added to the medium at a concentration of 6×10^8 cfu ml^{-1}, and an equal volume of both cultures is mixed in a concentration of 6×10^8 cfu ml^{-1} for the mixture of both bacterial cultures. Then, the culture tubes are transferred to the growth chamber at 27 °C in a 12-h light–dark cycle. 2,3,5-Triphenyl tetrazolium chloride (TTC) staining has been used for bacterial association with the plant root.

For staining, the maize root is surface sterilized with sodium hypochlorite and incubated in TTC stain for 12 h. The stained roots are cross sectioned and observed under an image analyzer microscope (Carl Zeiss) (Jha and Subramanian 2011). The association of bacteria within the root cortex region can be clearly visualized as red-colored cells under the microscope (Fig. 9.3). The study showed that both individual bacterial isolates and their mixture have positive response on various growth parameters (as shown in Table 9.1).

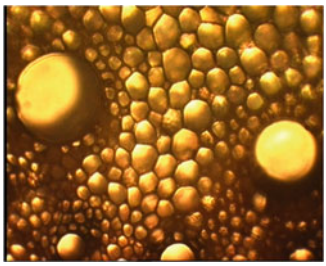

Fig. 9.3 Micrograph of section of maize plant root shows association of bacteria in root cortex as dark spot by triphenyltetrazolium chloride (TTC) staining

9.3 Nutrient Availability for Plant Uptake

Plant nutrient dynamics has been affected by a sequence of extremities such as cold, salinity, and drought because of global climate change. Nutrient availability in the soil, and nutrient assimilation, acquisition, and distribution in farm crops are largely disturbed by various environmental stress factors (Etienne et al. 2018). The nutrient dynamics of the plants including transportation via xylem and phloem to reproductive structures or mobilization from senescing leaves are strongly affected by stress. Such stress also affects nutrient assimilatory activities, redistribution of inorganic nutrients, and water fluxes.

Endophytic bacteria have the capability to surge accessibility of nutrients by assimilating nutrients in the plant root, thus preventing their leaching. Such bacteria, capable of solubilizing phosphate, produce siderophores and phytohormones responsible for a greater amount of phosphate and iron ion assimilation for the plants and plant growth development. The collaboration of endophytic bacteria and its consequences on plant growth response under stress is a composite. The synthesis of nucleotides, amino acids, and proteins requires nitrogen, which is one of the most limiting nutrients for plants. Atmospheric nitrogen is exclusively fixed by such bacteria into organic forms that can be integrated by the plants. So, in this study, endophytic bacteria having nitrogen fixation ability are isolated using an NFb agar plate, which has been further analyzed for the presence of the nifH gene (Fig. 9.1).

In our study, the foliar contents of P, K, Na, Ca, and N in endophytic bacteria-inoculated plants are assessed by using 1 g digested plant material in a tri-acid mixture in the ratio of 9:3:1 by using a specific filter on digital flame photometry. In our study, plants inoculated with endophytic bacteria always have higher nitrogen and carbon concentrations under normal and stress conditions. The plants inoculated with endophytic bacteria have a higher concentration of foliar phosphorus (P), whereas the concentration of sodium (Na) is higher in noninoculated control plants under stress. The concentration of foliar potassium (K) is always higher in plants inoculated with endophytic bacteria alone and in combination (Table 9.2). Water intake by the plant cell is also contributed by the osmotically active solute potassium under saline stress and helps the stressed plant in maintaining central metabolic activity for its survival under stress (Jha 2018a). The most imperative outcome of this study is that cation uptake is abridged in endophytic bacteria-inoculated plants,

Table 9.1 Effect of plant growth-promoting rhizobacteria (PGPR) strains on plant growth-promoting activity in maize plant

Treatment	Shoot length (cm)	Root length (cm)	Total plant height (cm)	Total biomass (g)	
				Fresh weight (g)	Dry weight (g)
Normal					
Control	14.50 ± 0.11	15.0 ± 0.14	29.50 ± 0.1	1.26 ± 0.13	0.65 ± 0.11
Control + *Pseudomonas pseudoalcaligenes*	26.66 ± 0.2	18.0 ± 0.12	44.66 ± 0.1	2.90 ± 0.22	0.42 ± 0.1
Control + *P. aeruginosa*	24.00 ± 0.21	22.5 ± 0.13	46.50 ± 0.21	3.20 ± 0.17	0.51 ± 0.31
Control + *P. pseudoalcaligenes* + *P. aeruginosa*	32.16 ± 0.13	26.0 ± 0.21	58.16 ± 0.2	3.70 ± 0.11	0.68 ± 0.17
Stressed					
Control	11.3 ± 0.13	12.2 ± 0.17	23.21 ± 0.1	0.78 ± 0.11	0.26 ± 0.2
Control + *P. pseudoalcaligenes*	24.1 ± 0.11	32.6 ± 0.13	56.76 ± 0.21	1.14 ± 0.2	0.92 ± 0.11
Control + *P. aeruginosa*	25.7 ± 0.1	33.1 ± 0.23	58.12 ± 0.15	1.72 ± 0.13	0.54 ± 0.3
Control + *P. pseudoalcaligenes* + *P. aeruginosa*	27.2 ± 0.3	33.8 ± 0.21	59.97 ± 0.14	2.31 ± 0.1	1.32 ± 0.1

Values represent mean ± SD; $n = 3$

Table 9.2 Effect of endophytic bacteria on mineral concentrations in maize under salinity

Treatment	N (mg kg^{-1})	P (mg kg^{-1})	K (mg kg^{-1})	Na (mg kg^{-1})	Ca (mg kg^{-1})
Normal					
Control	19.1^{d}	1885.1^{d}	$58{,}710^{d}$	720.2^{ab}	$11{,}674^{cd}$
Control + *P. pseudoalcaligenes*	24.4^{ab}	2139.1^{b}	$65{,}131^{b}$	714.3^{abc}	$12{,}541^{c}$
Control + *P. aeruginosa*	23.8^{c}	2091.3^{bc}	$64{,}223^{bc}$	704.7^{cd}	$12{,}787^{ab}$
Control + *P. pseudoalcaligenes* + *P. aeruginosa*	27.2^{a}	2293.2^{a}	$71{,}142^{a}$	791.3^{a}	$13{,}263^{a}$
Stressed					
Control	14.4^{d}	2314.2^{cd}	$35{,}162^{c\ d}$	613.1^{d}	$18{,}310^{ab}$
Control + *P. pseudoalcaligenes*	18.3^{b}	2615.2^{ab}	$38{,}343^{ab}$	723.2^{b}	$16{,}324^{c}$
Control + *P. aeruginosa*	17.1^{bc}	2536.0^{bc}	$37{,}320^{bc}$	713.3^{bc}	$15{,}336^{cd}$
Control + *P. pseudoalcaligenes* + *P. aeruginosa*	19.9^{a}	2758.4^{a}	$39{,}835^{a}$	745.5^{a}	$18{,}834^{a}$

Values are the means of replicates. For each Parameter, values in columns followed by the same letter are not significantly different at ($P \leq 0.05$). Values with different alphabets are significantly different at $P < 0.05$ (Duncan's test)

alleviating stress in plants. As interactions of such cations as Na^{+} and Ca^{2+} have significant effects on plant cell membrane character and transportation of ions, a change resulted in cytoplasmic Ca^{2+} activity necessary for many important physiological activities, such as ion transport, nutrition uptake, and water assimilation as well as plant growth under stress. The availability of important nutrients increased by the inoculation of endophytic bacteria alone or in groups under abiotic stress confers better tolerance to the plant against adverse environmental conditions.

9.4 Osmotic Stress Management

Many stresses that are induced by abiotic and abiotic factors directly affect agricultural crops. Such stressful conditions are responsible for the loss of crop yield, and losses from such stress are in the range of 50–82%, depending on stress type and crop sensitivity toward the stress. Such stress always limits crop production, and it is more prominent in the semi-arid and arid areas of the world because of the continued increase in soil salinity. As such, drought and salinity are the most common and prominent abiotic stresses, which result in numerous physiological and metabolic changes in plant response to such stress (Saharan and Nehra 2011). A cascade of response can be induced in plants ranging from a prime response such as alteration of osmotic and ionic concentration, stomatal closure, and reduced transpiration, to subordinate responses such as production of secondary metabolites and plant

hormones. Plants have an inherent ability to react against such stress via signal transduction pathways to modulate their metabolism. The major cause of salinity is mainly Na^+, Cl^-, and SO_4^{2-}, which imposes osmotic, ionic, and other secondary stresses such as oxidative stress and nutritional imbalances (Hussain et al. 2008). The turgor pressure and biomass production of the crop has also been affected by salinity. Accumulation in plants of important amino acids such as arginine, alanine, glycine, leucine, and serine, together with proline, takes place under such stress.

Proline widely accumulates in larger amounts in plants than other amino acids under salt stress. Glycine betaine and proline are well-known compatible solutes, with important roles in osmotic adjustment in stressed cells or organism salinity stress (Zhonghua et al. 2007). However, many economically important crop plants do not have the ability for the accumulation of such osmo-protectants, lacking the enzymes required for its biosynthesis. Most plant species can accumulate proline and betaine as compatible solutes, which have been considered as osmo-protectants. Our previous analysis confirmed that endophytic bacterially inoculated important crops such as maize and rice develop the ability to accumulate proline and glycine-betaine (Table 9.3) as a osmo-protectant, to acquire tolerance against saline stress (Jha 2017). So, the current need is to survey different mechanisms to enhance the salt tolerance ability of plants, to increase the accumulation of osmo-protectants in crop plants under saline stress. Plants have established many systems for physiological adaptation to overcome water deficiency, such as modification of root architecture to acquire water, production of osmo-protectants, regulation of water movement by aquaporins, and stomata to improve water use efficiency (Alavilli et al. 2016). Endophytic bacteria have an essential role in recovering plant growth and metabolism in stressed conditions. Endophytic bacteria allow the plant to overcome stress by regulating secondary metabolite production and accumulation of osmo-protectants to protect the plant from osmotic stress. However, the association of such bacteria with the plant may be affected by many more environmental components as well as plant-related components such as age of plant or plant species. Recently, many endophytic bacterial strains have been identified as having potential for improved crop growth under osmotic stress.

9.5 Modulation of Root Architecture

The root system was developed by terrestrial plants to explore the soil for better acquisition of nutrients for their sustained growth. Roots participate in the formation of definite microbial biological residence in plant-based systems, predominantly in the case of soil in contact with plant roots, that is, the endosphere. Also, the plant root system is responsible for interaction with diverse soil microbes and anchors the plant within the soil, helping the plant in procuring water and ions, vegetative growth, and nutrient storage (Berg and Smalla 2009). The plant root has many discrete regions with specialized functions, such as development of root hairs from differentiated epidermal cells to increase the surface area to enhance water and nutrient uptake

Table 9.3 Effect of endophytic bacteria on proline, glycine-betaine, gibberellic acid, 1-aminocyclopropane-1-carboxylate (ACC)-deaminase, and abscisic acid in maize under salinity

Treatment	Proline (mol min^{-1} $g^{-1)}$	Glycine-betaine (mol min^{-1} g^{-1})	Auxin (μg ml^{-1})	Gibberellic acid (μg ml^{-1})	ACC-deaminase (μg ml^{-1})	Abscisic acid (μg ml^{-1})
Normal						
Control	1.9^{d}	1.8^{d}	1.32^{cd}	0.8^{d}	1.25^{d}	2.45^{d}
Control + *P. pseudoalcaligenes*	2.4^{ab}	2.1^{b}	1.49^{c}	1.02^{c}	1.29^{bc}	2.83^{b}
Control + *P. aeruginosa*	2.8^{c}	2.3^{bc}	1.53^{b}	1.23^{b}	1.32^{b}	2.75^{bc}
Control + *P. pseudoalcaligenes* + *P. aeruginosa*	2.2^{a}	2.2^{a}	1.59^{a}	1.32^{a}	1.38^{a}	3.11^{a}
Stressed						
Control	1.3^{d}	1.2^{d}	0.95^{cd}	1.31^{d}	1.110^{ab}	3.12^{a}
Control + *P. pseudoalcaligenes*	2.2^{ab}	1.3^{b}	1.08^{c}	2.37^{b}	1.24^{c}	2.59^{bc}
Control + *P. aeruginosa*	2.6^{a}	1.4^{a}	1.13^{b}	2.84^{bc}	1.36^{cd}	2.76^{b}
Control + *P. pseudoalcaligenes* + *P. aeruginosa*	2.3^{c}	1.5^{bc}	1.24^{a}	3.13^{a}	1.84^{a}	2.28^{d}

Values are the means of replicates. For each Parameter, values in columns followed by the same letter are not significantly different at ($P \leq 0.05$). Values with different alphabets are significantly different at $P < 0.05$ (Duncan's test)

capacity. The functional efficiency of the root has a direct relationship with the level of plant–microbe interactions. The root is a complex organ having its own architecture, which includes the dispersion of main and lateral roots, root system topology, root length, and its strength. Numerous biotic and abiotic factors can affect the root system architecture, including endophytic bacterial associations (Jha and Subramanian 2014a).

Endophytic bacteria modify the plant hormone profile of the plant to alter the root tissue structure and root system architecture. The presence of denser root hairs in our study resulting from inoculation of the plant with endophytic bacteria has increased the surface area of the root to enhance the water as well as mineral uptake ability of the plant under stress, and enhanced root growth is proposed as a probable adjunct by which endophytic bacteria enhance plant growth. In our study, the consequence of endophytic bacteria on plant roots has been studied on plants inoculated with such bacteria. Cross sections of the roots are prepared after careful collection of plantlets with the roots after 35 days past sowing and are examined under an image analyzer microscope (Carl Zeiss). Inoculated as well as noninoculated plants showed no anatomical change in the xylem tissues, whereas development in root hairs and increase in root length have been recorded in inoculated plants. Plant hormones such as indole acetic acid and gibberellic acid produced by such bacteria may be responsible for the increased surface area of roots and root length, and number of root tips, thereby improving mineral and water uptake and plant growth response under saline stress (Egamberdieva et al. 2010).

9.6 Modification of Phytohormonal Activity

The production of phytohormones such as abscisic acid, gibberellins, cytokinins, ethylene, and indole acetic acid by plants is necessary for growth and development of the plant (Egamberdieva 2009). Among these the most imperative hormone physiologically required for plant growth and development is auxin. The study shows that plants inoculated with auxin-producing endophytic bacteria have improved formation of lateral roots, root growth, and root hairs, responsible for improved water and mineral uptake potential of the plant to handle water scarcity. Dimkpa et al. (2008) reported that *Azospirillum* by producing auxin increases the tolerance of the plant to drought stress. Endophytic bacteria have a key role in enhancing tolerance in the host plant by producing plant hormones and stimulating endogenous hormones under stress. Similarly, *Azospirillum brasilense* inoculated into the common bean under drought showed enhanced specific root length, root projection area, and specific root area compared to noninoculated controls.

The production of ethylene in the plant is regulated by environmental stress (Hardoim et al. 2008). The biosynthesis of ethylene is carried out by the enzyme 1-aminocyclopropane-1-carboxylate synthase, which converts *S*-adenosylmethionine into 1-aminocyclopropane-1-carboxylate (ACC), the direct precursor for ethylene production. Ethylene is responsible for endogenous regulation of plant homoeostasis

under stress conditions. Endophytic bacteria producing ACC-deaminase degrade plant ACC to acquire nitrogen and energy for its growth and subsequently decrease the toxic effect of ethylene, improving plant growth and stress tolerance ability (Jha and Subramanian 2014b). Reduced ethylene production in tomato and pepper seedlings was also reported after inoculation with the ACC-deaminase-producing bacterium *Achromobacter piechaudii* ARV8, which remarkably improved both fresh and dry weights under drought stress (Mayak et al. 2004).

Abscisic acid is the most important phytohormone that confers tolerance in crop plants under abiotic stress. Extreme environmental conditions such as high temperature and salinity considerably increase abscisic acid content in plants to activate the stress tolerance ability of the plant, to develop the adaptability of the plant to survive in such stressful conditions (Ng et al. 2014). ABA has multiple functions in plants. Under normal environmental conditions, it is required for the growth and development of the plant. Light usually stimulates stomatal opening for gas exchange, but ABA encourages complete or partial closure of the stomata. Stomatal closure resulted in reduced gas exchange and ultimately results in reduced transpiration to check water loss from the plant and reduce photosynthate production (Mittler and Blumwald 2015). ABA also modulates turgor pressure by increasing water influx of roots and decreasing transpiration to check water loss. In our study, both the isolates were analyzed for their ability to produce phytohormones such as auxin, gibberellic acid, ABA, and ACC-deaminase. The study shows that both isolates exhibited a significant amount of auxin production after 24 h incubation with tryptophan (Table 9.3). Auxin production increased with time by both isolates, with the maximum by *Pseudomonas aeruginosa* on the fifth day. Gibberellic acid production began after 72 h inoculation by both the isolates, and its production was also significantly higher in *Pseudomonas pseudoalcaligenes*. Gibberellic acid production was three times higher by *P. pseudoalcaligenes* compared to *P. aeruginosa*. Our study also showed enhanced production of ABA and ACC-deaminase activity by both isolates with time after inoculation. Nowadays, bacterial strains having the ability to produce ABA and having ACC-deaminase activity are screened in a wide range of genera such as *Enterobacter, Achromobacter, Serratia, Burkholderia, Pseudomonas, Agrobacterium, Rhizobium,* and *Bacillus* (Kang et al. 2010).

9.7 Biotic Stress Management

A group of physical and chemical barriers has been developed by plants to elude nearly all hostile interactions with a biotic stressor. The chemical barrier basically includes hasty accumulation of secondary metabolites to induce defense response such as induction of enzymes to block the enzymatic functions of pathogens or production of defense protein to prevent the growth of pathogens or secretion of toxic metabolites to kill the pathogens. The physical barrier of the plant includes cell walls, cell wall lignification, and cuticle to protect the plant from injurious biotic interactions. Plants develop multiple levels and forms of obstacles depending on the

atmosphere in which plants grow and on the plant species. Endophytes that reside in the plant have been modified to adapt to the plant in which they colonize by producing different types of potential metabolite. Endophytic bacteria have to live in a nutrient-rich environment, which is enormously competitive, so such bacteria have to develop the ability to survive in a predator-rich and competitor-rich environment. Therefore, endophytic bacteria have the ability to produce a variety of anti-pathogenic compounds or antibiotics for its establishment in the plant host. For their interaction with the host plant, endophytic bacteria also produce many more supporting metabolites as needed for the specific interaction. Such metabolites not only help in its interaction with host plant, but also have defense potentials and may also participate in interspecies or intraspecies signaling processes, as well as antibiosis function (Raaijmakers and Mazzola 2015). Nonpathogenic endophytic bacteria stimulate a defense mechanism in the plant called induced systemic resistance (ISR) to suppress disease. An improved defensive ability established by plants is known as induced when properly activated. The pathogen-induced systemic acquired resistance and endophytic bacteria-mediated induced systemic resistance have similarity, as in both induced resistance protects uninfected plant parts to develop more resistance against plant pathogens (Pozo and Azcon-Aguilar 2007) and are activated through diverse signaling paths. Therefore, plants have established a coordination that allows targeted and quick responses against biotic stress.

The molecular mechanism for this type of stress management is by induction and redirecting the genetic information toward it depending on the assembly of factors. Low molecular weight organic compounds produced by microbes have the ability to prevent the growth of other microbes and are known as antibiotics. Antibiosis has a major role in plant defense against diseases and frequently acts in concert with parasitism and competition. The antibiotic activity of selected endophytic bacteria is evaluated by extracting and testing toxic effects of metabolites produced by the isolates. The percentage inhibition of individual antibiotics produced by each endophytic bacterium is also calculated. The result obtained in the present study showed antibiotic production by both the isolates. Both isolates have clearly shown strong anti-pathogenic activity. The concentration of some metabolites such as phenolic compounds is directly proportional to the level of secondary metabolism and has a direct relationship with nutrient deficiency. Because the higher concentrations of secondary metabolites such as phenolic compounds resulted in reduced levels of important nutrient such as N, P, K, and S in stressed plants, higher concentrations of nitrogen generally diminish phenolic assimilation in the plant, which is efficiently modulated by such endophytic bacteria. The coordinated defense responses in plants are aided by such bacteria, the endophytic bacteria and the pathogens, in which both reside in the plant at different sites mediated by accumulating signaling molecules. So, microbial antagonism is excluded and the protective effect is plant mediated.

9.8 Programmed Cell Death

Plants are adversely affected by biotic and abiotic stress, which results in reduced plant growth and badly reduces crop yield because of the deleterious effect of altered biochemical and physiological processes, finally resulting in plant cell death. Programmed cell death (PCD) is a consequence of events that resulted in organized and controlled destruction of cells (Lockshin and Zakeri 2004). Programmed cell death is a regular event for appropriate development of the multicellular body plan, critical for defense responses to limit the spread of pathogens in all living organisms. Apoptosis and necrosis are two distinct forms of cell death in plants in which apoptosis is characterized by nuclear condensation, fragmentation, and cell shrinkage and finally the breakup of the cell into 'apoptotic bodies' (Jha 2018b). Necrosis is characterized by uncontrolled cell death, caused by irresistible cellular stress and initiated in the cell unable to activate its apoptotic pathways. In necrosis, swelling is the common feature in morphological change in place of shrinkage. The production of phenolic compounds is initiated in the plant exposed to abiotic or biotic stress for the activation of defensive pathways and defense. The phenolic compound forms an insoluble complex with pathogen toxins or proteins, and inhibits pathogen enzymes to protect the plant cell against pathogenesis. The plants establish a multilevel process to protect themselves from numerous damaging environmental conditions. Reactive oxygen species (ROS)-induced programmed cell death is responsible for cellular proteins and membrane damage as well as obliteration of defending enzymes such as β-1,3-glucanase and catalase activity (Jha 2019). In multicellular organisms, programmed cell death is a critical phenomenon accompanying the normal growth of the organism and its immune responses for the destruction of its own harmful cells and for pathogen clearance (Wang et al. 2016). Host cell lysis is one of the common mechanisms of many plant pathogens to fulfill their nutrient requirement. So, activation of programmed cell death remains associated with plant pathogen interaction. Such interaction results in the production of flavonoid at the injured site and activation of the hypersensitivity reaction. The infected plants activate the defense tool to trigger programmed cell death as one mechanism for pathogen clearance (Jha and Subramanian 2015). Under stress, intensive programmed cell death has an adverse effect on the plant that exaggerates cell death responses.

In our study, phytopathogen-infected non-bacterized plant cells quickly lost cell viability as compared to plants inoculated with bacteria. More intense programmed cell death is recorded in control plants after pathogen infection, but endophytic bacteria-inoculated plants reduced the effect of programmed cell death (Table 9.4). In infected plants, among all defense responses, programmed cell death is the core and final-stage progression, otherwise a common mechanism for specific devastation of self-cell constituents for effective growth in a healthy plant, although under stress its induced self-mechanism protects the cell from stress effectors. So, the endophytic bacteria residing in a plant cell are one of the eco-friendly alternatives as a biological mechanism, compared to chemical pesticides, that is more encouraging for environmental preservation.

Table 9.4 Effect of endophytic bacteria on cell viability, catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA) in maize under salinity

Treatment	Cell viability (Evan blue conc mg cell^{-1})	CAT (mmol min^{-1} g^{-1} FW)	SOD (mmol min^{-1} g^{-1} FW)	MDA (nmol g^{-1} FW)
Normal				
Control	21.9^{d}	10.8^{d}	13.2^{cd}	338^{d}
Control + *P. pseudoalcaligenes*	18.4^{ab}	12.1^{b}	14.9^{c}	282^{c}
Control + *P. aeruginosa*	17.8^{c}	17.3^{bc}	15.3^{b}	253^{b}
Control + *P. pseudoalcaligenes* + *P. aeruginosa*	17.2^{a}	21.2^{a}	15.9^{a}	212^{a}
Stressed				
Control	32.3^{d}	17.2^{d}	11.5^{cd}	451^{d}
Control + *P. pseudoalcaligenes*	22.2^{ab}	15.3^{b}	12.8^{c}	347^{b}
Control + *P. aeruginosa*	24.6^{a}	14.4^{a}	13.3^{b}	384^{bc}
Control + *P. pseudoalcaligenes* + *P. aeruginosa*	25.3^{c}	13.5^{bc}	14.1^{a}	353^{a}

Values are the means of replicates. For each Parameter, values in columns followed by the same letter are not significantly different at ($P \leq 0.05$). Values with different alphabets are significantly different at $P < 0.05$ (Duncan's test)

9.9 Abiotic Stress Management

High salinity, which is increasing worldwide from poor irrigation systems in agricultural practices or related natural phenomena, widely affects agricultural lands (Munns and Tester 2008). Oxidative stress and osmotic stress are two main threats to plant growth under salinity. Salinity causes reduced leaf area, decreased internode length, abscission of leaves, and necrosis of plant parts and succulence. Such harmful effects on the plant result from the altered metabolic and physiological processes of plants under salinity stress. Altered metabolic and physiological processes result from production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), and superoxide anion (O^{2-}), under saline conditions (Mallik et al. 2011). Such reactive molecules actively react with biomolecules such as deoxyribonucleic acid, lipids, proteins, and enzymes, and impair the normal functions of the plant cell. Plants develop antioxidant protective systems to overcome the adverse effects of salinity, including both nonenzymatic (glutathione, ascorbic acid, cysteine) and enzymatic mechanisms [catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and ascorbate peroxidase (APX)], to prevent accumulation of ROS and assuage the oxidative damage from drought stress (Kaushal and Wani 2015). The over-generation of ROS under abiotic stress can also damage

lipids, which causes lipid peroxidation. Lipid peroxidation can be accessed on the basis of malondialdehyde (MDA) content.

To prevent cell death under abiotic stress, plants must develop the ability to scavenge ROS, with enhanced ability to check cell death and oxidation of important biomolecules. Plants in their usual atmosphere are populated with both intercellular and intracellular microorganisms. Beneficial bacteria such as endophytic bacteria can increase plant performance under environmental stress (Table 9.4) and directly and indirectly influence enhanced yield (Dimkpa et al. 2008). Such bacteria facilitate plants with higher fixed nitrogen, iron, phytohormones, soluble phosphate, and bacterial siderophores, which directly motivate plant growth and development, while indirectly protecting plants against soil-borne plant pathogens, most commonly pathogenic fungi (Lutgtenberg and Kamilova 2009), although a diverse group of microorganisms naturally remain associated with plants in various ways. One group of these microorganisms, endophytic bacteria, is colonized in plant tissues and seeds, including underground and aboveground parts, without damaging host cells (Reinhold-Hurek and Hurek 2011). Abiotic stress tolerance may be achieved by at least two mechanisms: (1) production of anti-stress biomolecules by endophytes, and (2) induction of host stress response systems just after exposure to stress, permitting the plants to mitigate or avoid the effects of the stress.

9.10 Induction and Accumulation of Chaperones Under Abiotic Stress

Global crop yields are reduced, with limited availability of water the only important factor. The overall costs of crop production will definitely increase with future scarcity of available water and initiate the need for crops that use water economically. There are several serious threats to agriculture, such as extreme temperatures, oxidative stress, salinity; chemical toxicity, and drought, which in combination cause deterioration of the environment for crop production. Such abiotic stresses are mainly responsible for worldwide crop loss, causing a loss of more than 50% of the yields for most major crop plants. The great importance and basic practice now is to activate plant responses to stress and aid in acquiring tolerance. The tolerance mechanisms include accumulation of osmo-protectants, production of late embryogenesis abundant proteins, transcriptional control, free radical scavengers, ion transporters, and factors involved in signaling cascades (Wang et al. 2004).

The widely used transcriptional profiling methodology is a logical continuation of proteomics. Proteomics is the study of complete protein complement of a genome or of the multi-protein systems of an organism (Karpievitch et al. 2010). Proteomics analysis is aimed to understand the roles of distinct proteins as a part of a larger networked system of the organism. The modern systems biology approaches include the vital component with the goal to characterize the system components rather than the behavior of a single component. Information about the protein is not possible to analyse by measuring messenger ribonucleic acid (mRNA) levels alone in a cell and

the regulatory behavior of the protein, as proteins are subjected to many posttranslational modifications and other modifications by environmental agents. A comprehensive understanding of systems biology requires proteomics, as proteins are responsible for cellular communications, structure components, the movement and division of cells, energy production, and defense. Multiple components of action are required to enhance the stress tolerance ability of the plant, and all living organisms, including viruses, have abundant molecular chaperones and ubiquitous proteins. Small proteins tend to be kinetically stuck in misfolded forms. Molecular binding proteins are molecular chaperones that help functional proteins, ensuring accessibility for biological function and acquisition of the specific structures required for activity. Such sets of low molecular weight small proteins act as molecular chaperones that quickly accumulate in the plant cell under stressed conditions (Horn et al. 2007). The molecular chaperones become associated with denatured proteins to maintain them in that specific state required for refolding. During our SDS analysis, protein isolated from the endophytic bacteria-inoculated plant under adverse conditions produced a few new low molecular weight bands of protein in the gel, which may be responsible for specific functions in plant survival by protecting/maintaining functional protein (Fig. 9.4). The dysfunction and maintaining proteins in their functional forms or protecting the accumulation of nonnative proteins are necessary for survival of the plant cell under stress that has usually resulted from abiotic stresses (Jha et al. 2014a). Molecular chaperones are necessary for the assembly, degradation, folding, and translocation of important enzymes for numerous normal cellular functions and are required for protein refolding, protein stability, and membrane transportation of protein under stress. In the plant, however, chaperones are articulated in several conditions such as water stress, cold stress, oxidative stress, salinity, and osmotic stress as well as when they experience high temperature stress (Wang et al. 2004). So, chaperones have a crucial role in plants against stress by maintaining normal protein conformation and overall cellular homeostasis.

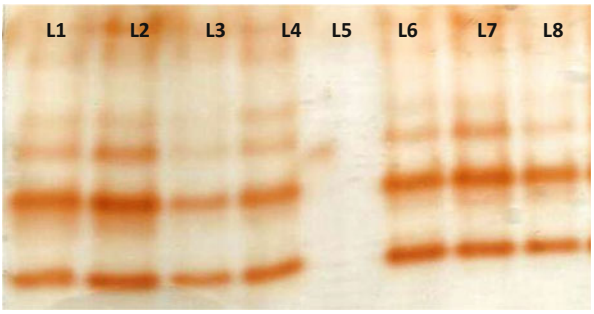

Fig. 9.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of total soluble proteins from inoculated plants in presence of biotic and abiotic stress. Lane 1, control; Lane 2, control + *P. pseudoalcaligenes*; Lane 3, control + *P. aeruginosa*; Lane 4, control + *P. aeruginosa* + *P. pseudoalcaligenes*, all under abiotic stress pathogen. Lane 5, infected; Lane 6, infected + *P. aeruginosa*; Lane 7, infected + *P. pseudoalcaligenes*; Lane 8, infected + *P. aeruginosa* + *P. pseudoalcaligenes*; all under biotic stress

9.11 Effect on Differential Gene Expression

Plants have the capability to rapidly sense surrounding environmental signals and have evolved mechanisms to respond accordingly. Coordinated signals produced by the abiotic and biotic stress tissue act in the harmonization to execute plant stress responses by modulating its metabolic and developmental activities. Primarily, such responses are initiated by osmotic stress signals (Chaves et al. 2008) that normally augment or reduce temporarily by secondary signal metabolites, which finally induce hormones (e.g., ethylene, ABA, cytokinins), or generate ROS and other intercellular/intracellular secondary messengers (e.g., sugars, phospholipids). Many bio-molecules have important roles in plant growth and development and are required for coordinating many stress-related signals, and by regulating gene expression modulate stress response through various biochemical reactions as well as transporters/pumps. Such bio-molecules include polyphosphoinositides, jasmonates (JA), abscisic acid (ABA), salicylic acid (SA), cyclic nucleotides, polyamines, nitric oxide (NO), calcium (Ca^{2+}), and sugars. Coordination between such chemical signaling pathways is a common mechanism in plants toward biotic and abiotic factors (Jha et al. 2014b).

To manage in the changing environment, plants have developed many ways, such as adaptive stress responses of the plant which are directly regulated by biological skill and inherent ability and can change with gene expression. The molecular changes concerned in plant stress responses will permit developing plants with superior confrontation against abiotic and biotic stress, by gene manipulation. Plants activate the manifestation of different PR genes in response to pathogens to recover their defensive ability (Jiang et al. 2014). There are also several reports on overexpressing PR genes, resulting in improved tolerance in the plant to biotic stress.

Pathogen infection results in a huge repertoire of defense responses in the plant, which results in the production of novel proteins having direct or indirect action against pathogenesis, which is the main mechanism. These proteins comprise different groups of extracellular and intracellular proteins including enzymes such as β-1,3-glucanases, peroxidase, and catalase, collectively known as PR proteins. The enzymes β-1,3-glucanases are mainly attractive as these are developmentally and hormonally controlled in healthy plants, among all other PR proteins (Gupta et al. 2013). These protect plants from fungal infection as β-1,3-glucans are mandatory structural components of fungal cell walls. An in vitro study showed catalase with β-1,3-glucanase has a straight fungicidal activity on phyto-pathogenic fungi. Such enzymes could, consequently, act directly by inhibiting the growth of invading fungal hyphae.

Because this effect most likely is associated with changes in plant gene expression, total RNA has been isolated 1 week after inoculation, from endophytic bacteria and pathogen co-inoculated plants and plants under abiotic stress, respectively, to analyze induction of genes by endophytic bacteria in plants under stress (Fig. 9.5). cDNA has been constructed by using mRNA and subsequently a gene amplified by PCR with specific primers. To amplify β-1,3 glucanase genes, two degenerate primers for β-1,3 glucanase, forward 5′-GTGTCTGCTATGGCGTTGTCG-3′ and

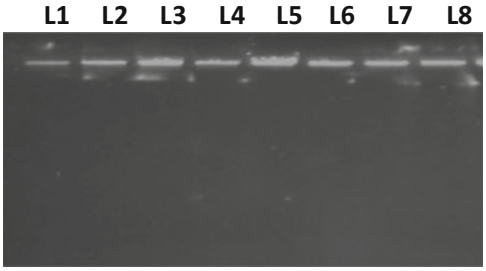

Fig. 9.5 Agarose gel showing bands of total RNA from inoculated plants in presence of biotic and abiotic stress. Lane 1, control; Lane 2, control + *P. pseudoalcaligenes*; Lane 3, control + *P. aeruginosa*; Lane 4, control + *P. aeruginosa* + *P. pseudoalcaligenes*; under abiotic stress pathogen. Lane 5, infected; Lane 6, infected + *P. aeruginosa*; Lane 7, infected + *P. pseudoalcaligenes*; Lane 8, infected + *P. aeruginosa* + *P. pseudoalcaligenes*; under biotic stress

reverse 5′-GGTTCTCGTTGAACATGGCGA-3′, have been designed. Accordingly, a 1.05 kb DNA segment has been amplified for β-1,3 glucanases (data communicated), with accession no. HM569719.1.

Similarly, the catalase gene is amplified by using forward prime TTAATCAGCCATGGATCCT′ and reverse primer AGCAGATTGCAACGCTGATC′. A band of 2 kb has been obtained that has been sequenced and submitted to the NCBI databank having accession no. JX875103. This study reports changes in gene expression induced by endophytic bacteria in the plant. It is therefore surprising that a stress-related gene is stimulated earlier to abiotic and biotic stress simply by inoculation of the plant with endophytic bacteria. The diverse plant stress response pathways can be activated in concert as an expected way under biotic stress conditions, which cause physical damage to plant tissue to facilitate access of pathogens (Jha and Subramanian 2016). Defense against the stress are coordinated mechanisms, and activation of biotic stress also cause induction of abiotic defense, but induction of abiotic stress does not result in activation of the biotic defense mechanism. Stress-related proteins need to be screened for their biochemical activities to analyze their function and are important for both types of stress. However, some abiotic and biotic stress conditions cause induction of similar molecular and physiological effects, and therefore co-regulation of selected defense genes may have evolutionary importance. For establishment of itself in the host plant and for host plant protection under adverse environmental condition, several diverse small protein molecules are induced by endophytic bacteria. So, to find more potential rhizobacterial strains for diverse agro-ecological conditions, endophytic bacteria-mediated phytostimulation can be encouraging for the researcher. Endophytic bacteria with a high aptitude to work efficiently under different agro-ecological conditions for sustainable agriculture are mechanisms of choice (Jha and Subramanian 2018).

9.12 Conclusion

Environmental stresses are always responsible for the limited agricultural productivity of crop plants. For increasing crop production to meet food demand, biological approaches are gaining more popularity among farmers, ecologists, and environmentalists for coordinated plant mineral management and environmental protection. Among biological approaches, endophytic bacteria have a major role in providing resistance against pathogens and the adaptation of plants in different stress environments. Endophytic bacterial interaction with plants not only can change plant physiology but also can modify soil properties and take a critical role in solving future food security issues. Such bacteria can induce osmotic response and new genes in the host plant to confirm plant survival under stress. Plant breeding is one possible means for the production of tolerant varieties, but nowadays development of crops through genetic engineering is gaining interest. At the same time, use of endophytic bacteria to assuage stress in plants is a new economic option for agricultural practice. So, a new chapter for future research is needed for the identification of the right types of potential microbes to address the current issues of field evaluation and delivery systems under stress. In this context, rigorous research is ongoing worldwide with greater impetus to explore a wide range of endobacteria possessing novel traits.

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Chapter 10
Biofertilizers in Argentina

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Abstract The increase in the use of chemical fertilizers in extensive agriculture and the associated environmental consequences encourage the use of biofertilizers, formulations with beneficial viable microorganisms, selected to favor nutrition and/or promote the growth of plants. The biofertilizers marketed in Argentina are strains of rhizobia (*Bradyrhizobium*, *Sinorhizobium*), *Azospirillum*, and in a lesser proportion *Pseudomonas* and mycorrhizal fungi. The investigations are focused on biofertilizers for the main crops of Argentina such as soybeans, wheat, corn, alfalfa, and rice. The effect of biofertilizers on the productivity of crops presents variable results since it depends on numerous biotic and abiotic environmental factors. The quality of biofertilizers and the understanding of the multiple biological interactions that occur between introduced microorganisms, native organisms, and plants are essential to achieve an efficient and appropriate use in each crop and ecosystem.

10.1 Introduction

About 90% (33,189,747 ha) of Argentine agriculture is carried out with direct sowing, known as conservation agriculture (AAPRESID 2018), with the consequent greater consumption of agrochemicals, new seeds, and fertilizers. Seventy percent of fertilizers in Argentina are used in soybean crops (*Glycine max* (L.) Merr.), maize (*Zea mays* L.), and wheat (*Triticum aestivum* L.), which currently exceed 3.5 million tons (MINAGRI 2018). The increase in the price of chemical fertilizers and the environmental consequences of their use encourage the use of biofertilizers (Lagler 2017).

The formulations of the biological fertilizers marketed in Argentina are strains of rhizobia (*Bradyrhizobium*, *Sinorhizobium*), *Azospirillum*, and, in a lesser proportion,

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Pseudomonas and mycorrhizal fungi. These microorganisms known as plant growth-promoting rhizobacteria (PGPR) or plant growth-promoting microorganisms (PGPM) are associated with the roots of plants extracellularly or intracellularly, favoring the growth and yield of agricultural crops through various direct or indirect mechanisms. Among the direct mechanisms are the biological fixation of nitrogen, production of phytohormones, solubilization of nutrients P and Fe, induction of systemic resistance, and production of siderophores and enzymes; among the indirect mechanisms related to biological control are the production of antibiotics, chelation of Fe available in the rhizosphere, synthesis of extracellular enzymes, and competition (García et al. 2013).

10.2 Biofertilizer Need for Inoculation in Agronomy

The Argentine legislation denominates "Biological Fertilizers" to the products that contain one or several microorganisms as the main component on a determined solid, liquid, or oil carrier (Resol. 0264/2011 SENASA). However, the REDCAI (Inoculant Control Network) of the Argentine Association of Microbiology (REDCAI-AAM) calls "Inoculant" "products formulated with beneficial viable microorganisms, selected to favor nutrition and/or promote the growth of plants" (Albanesi et al. 2013). The BIOFAG Network (Iberoamerican Network of Microbial Biofertilizers for Agriculture) establishes that "inoculant is any product whose active principle is living, non-pathogenic microorganisms of humans, animals or plants, or non-opportunistic pathogens of man, which favor nutrition and/or development of plants," and excludes the so-called agents of biological control, biofungicides, and bionematicides (Toresani et al. 2013).

10.2.1 History of Biofertilizers in Argentina

The first industrialized biological fertilizer called NITRAGIN was a rhizobial culture patented by Nobbe and Hiltner in 1898 (British Patent No. 11460 and US Patent No. 570813); the specificity of the rhizobia was already known, and there were 17 different formulations on the market in bottles of 8–10 ounces in a substrate consisting of sugar, asparagine, gelatin, and aqueous extract of legumes. Since 1910, the formulations used substrates such as dry sand, soil, peat, coal, silica, calcium carbonate, and calcium phosphate.

The former Experimental Institute of Agriculture and Livestock Research and Development of Santa Fe, Argentina, in 1939, disseminated selected bacterial cultures, and in later years, the Ministry of Agrarian Affairs of the Province of Buenos Aires did so. The former General Directorate of Agricultural Research began producing inoculants in 1948, and the National Institute of Agricultural Technology

(INTA) since 1958. In 1957 began its activities in the country the first factory of inoculants.

In the 1970s, most of the inoculants were imported along with the soybean seed from the USA. Since 1980, the Institute of Microbiology and Agricultural Zoology (IMIZA-INTA) has led the national program for the selection and evaluation of rhizobia strains in soybeans. The inoculants were mostly peat-based, and the inoculation method used was wet; they were also lyophilized and granulated. The peat used was not sterilized at the beginning, and subsequently, it was sterilized. Since 1990, the inoculants with liquid carriers have been introduced: oily nonsterile with fungicide and the sterile aqueous ones that constitute 90% of the products present in the market (Albanesi et al. 2013).

In the 1980s, studies began on the *Azospirillum* genus in Argentina with the guidelines of Dr. Johanna Döbereiner of the Agrobiology Laboratory of EMBRAPA, Brazil, and Dr. Yaacov Okon of the Hebrew University of Jerusalem, Israel. Thus, IMIZA-INTA obtained a collection of 64 strains lyophilized between 1981 and 1995, with a program of selection of strains of *Azospirillum* in wheat and corn crops from experimental fields of the Province of Buenos Aires, to evaluate their capacity to promote the growth. From this collection, *Azospirillum brasilense* strain AZ39 is currently used in more than 60% of commercial products and is recommended for wheat and corn (García et al. 2013). There are other strains used in a lesser proportion in the formulation of inoculants (Az78, Az70, Abv5, and Abv6, among others) (Cassán and Diaz Zorita 2016).

The first records for *Pseudomonas* date from just over a decade (Puente and Garcia 2009; Rossi et al. 2013). At present, there are several commercially available biofertilizers based on *Pseudomonas*, with *P. fluorescens* and *P. chlororaphis* subsp. *aurantiaca* used as biofertilizers and phytostimulators of the main crops such as wheat, corn, and soybeans (Ferraris 2013; Rossi et al. 2013).

10.3 Types of Inocula (Formulations) and Inoculation Techniques

The biofertilizers are classified based on the characteristics of the carriers, which constitute the largest proportion of the inoculant and nourish and protect the microorganism against adverse factors from development to use, being:

(a) Inoculants in liquid carriers: aqueous liquid; with peat in suspension; no peat in suspension; oily liquid with or without fungicide
(b) Inoculants in solid carriers: peat or bentonite

The liquid carriers are the most used for the formulation of biofertilizers (82%) (Cassán and Diaz Zorita 2016). Biofertilizers in Argentina can also be combined with other microorganisms (co-inoculation), with bioinducers or signal molecules to encourage early nodulation, and with micronutrients.

The most used inoculation technique is based on the addition of the inoculant to the seed prior to sowing; Another technique available to the producer is pre-inoculation, which allows up to 30 days of sowing to be inoculated in advance, combining a "long life" inoculant with seed-therapies that protect against fungi and insects and a covering polymer. Another alternative is to apply inoculants diluted in water in the sowing line (Lagler 2017).

10.3.1 Registration and Quality Control of Inoculants

The quality control of inoculants, of obligatory way, framed in the law 14.244/1953 and the decree 23960/953 established that the commercialization could be carried out by means of certification of an Agronomist Engineer. This decree was in accordance with resolution 1396/954 of the Ministry of Agriculture and Livestock.

Currently, the Resolution SENASA No. 0264/2011 regulates the control of inoculants and indicates that biological fertilizers, manufacturing laboratories, and natural or legal persons who import, export, distribute, elaborate, and/or fractionate biological fertilizers must be registered in the National Fertilizer Registry, Amendments, Substrates, Conditioners, Protectors and Raw Materials in the Argentine Republic, of the Directorate of Agrochemicals and Biologicals of SENASA. It is the responsibility of the Directorate of agrochemicals and biologicals of SENASA to control the establishments and compliance with the technical-administrative norms related to the preparation and/or formulation of biofertilizers. In addition, it carries out the technical evaluation of the documentation presented for the approval and registration of the active principles and/or formulated products, proposes the restriction or prohibition of the same, and intervenes in the import and export procedures.

10.3.2 Quality of Biofertilizers

The REDCAI-AAM, in accordance with the Resolution SENASA No. 0264/2011, considers that the microbiological criteria to be used in the control of inoculants by the inspection bodies, companies, and third-party service laboratories to define the aptitude or rejection of a finished product or lot are the type of inoculant to which it is applied (solid, aqueous, oily liquid); the target microorganism(s) selected; the analytical methods for its detection and/or its quantification; a plan for the number and sampling, as well as the size of the analysis unit; the microbiological limits of importance to define the quality of the product; harmlessness for man and the environment; the agronomic efficacy of the finished product; and the load of contaminating microorganisms that do not compromise the stability of the finished product (Albanesi et al. 2013; Toresani et al. 2013).

The period of validity of the inoculants for registration and marketing is conditioned by the characteristics of the carrier, ranging from 6 to 18 months, and is set by

the manufacturer under a sworn statement (SENASA Resolution No. 0264/2011). The minimum concentration of viable microorganisms that an inoculant must contain to demonstrate fitness is 1×10^8 and 1×10^7 CFU g^{-1} or mL^{-1} of product formulated on the basis of rhizobia and azospirilla, respectively. For other microorganisms, the law does not specify a minimum concentration (Resol SENASA No. 310/94 and No. 0264/2011).

The suggested concentration for inoculants with *Azospirillum brasilense* is 1×10^8–1×10^9 CFU mL g^{-1} inoculant (Puente and García 2009). Concentrations used for inoculants with *Pseudomonas* are 1×10^9–1×10^{10} CFU g^{-1} (Pérez et al. 2000), which are high concentrations since they lose viability during storage (Valverde and Ferraris 2009).

10.4 Microorganisms Used as Biological Fertilizers in Argentina

In Argentina, 693 commercial products were registered under the denomination of biological fertilizers in different formulations and from different companies (SENASA 2018). A total of 94.8% of the products are of national origin, and the rest come from the USA, Brazil, Spain, Colombia, Australia, and Canada. There were 94 companies registered, with most of them located in the central zone of the country, where there is a greater area sown with commodity crops that use large volumes of biological fertilizers. It is noteworthy that the companies that produce lower volumes of inoculants are from national capitals and those with large volumes are from foreign capital or Argentine-foreign companies.

The inoculant companies in Argentina use, mostly, strains selected by IMIZA-INTA, but some of them also have their own national or foreign selection programs (Anlló et al. 2011). About 49% of the national biological fertilizers that are produced are for soybeans, 23% for other legumes (alfalfa, melilotus, beans, chickpeas, peanuts, peas, vetches, lotus), 9% for cereals (wheat, corn, barley), and 2% for other crops (sunflower, cotton, rice) (SENASA 2018).

Biological fertilizers are used in 70% of the area cultivated with soybean, which implies around 15 million doses (inoculant required for 50 kg of soybean seed) (Izaguirre-Mayoral et al. 2007). In the case of other legumes such as clover, lotus, pea, peanut, and bean, less than 30% of the cultivated area is inoculated. For alfalfa, more than 60% of the planting is inoculated since there is a lot of seed pelleted by industrial processes (Perticari and Medana 2006).

The use of biofertilizers in non-legumes is more reduced; for example, about 200,000 doses are used in wheat (inoculant for 100 kg of seed) representing 4–5% of the total sowing area. For the rest of the crops such as corn, rice, horticultural species, etc., they are used to a lesser extent. There is a growing interest in these inputs from various components of the Argentine agricultural sector (Izaguirre-Mayoral et al. 2007).

10.4.1 Bradyrhizobium japonicum

In Argentina, biofertilizers for soybeans are formulated, for the most part, with strain E109 of *Bradyrhizobium japonicum* (Grageda-Cabrera et al. 2012; Piccinetti et al. 2013), selected by IMYZA-INTA for its ability to form nodules and set N (Cassán et al. 2013a, b). *Bradyrhizobium japonicum*, in symbiosis with soybean, converts N_2 into NH^{4+} through the action of the nitrogenase enzyme complex, a process called biological fixation of N (BNF) (Mohammadi and Sohrabi 2012). BNF occurs in root nodules, representing approximately 2/3 of the nitrogen fixed worldwide and is an economically and environmentally beneficial alternative (Ahemad and Kibret 2014). Soybean has a high protein accumulation in the seed, and it is estimated that between 70 and 80 kg N mg^{-1} of grain is required (Grageda-Cabrera et al. 2012).

Argentina is one of the main producers of soybeans (more than 50 Mt in recent years) and the main exporter of oil and flour derived from them (MINAGRI 2018). Much of the demand for N is covered by the BNF inoculating with *B. japonicum*, obtaining higher yield and grain quality, especially in lots with no crop history (Grageda-Cabrera et al. 2012). There are records of N contributions by BNF of 26–71% (Collino et al. 2007) and 50–80% of N requirements depending on the production system (Salvagiotti et al. 2008). Piccinetti et al. (2013) consider that inoculation increases 50% of yield in soils without soybean history and without nutritional or water limitations, and in soils with soy history, yield increases by 6–10%. In northern Argentina, yield increases of up to 16% were recorded (Brandan de Weht et al. 2013).

The use of biological fertilizers in soybeans is very high (94%), in all environments cultivated in Argentina, even in soils with soybean tradition that present a naturalized population of rhizobia, which is less efficient than the selected strains introduced by inoculation (Piccinetti et al. 2013). Thus, inoculation is a recommended practice that allows raising yields, contributing to the nitrogen reserves of the soil (Piatti and Ferreyra 2018) and reflects the greater competitiveness of the strains of the inoculants and the higher quality of the commercial formulations currently available. However, Althabegoiti et al. (2013) indicated that the efficiency of biofertilizers to nodulate soybeans should be improved from the point of view of the competitiveness of rhizobia to nodulate.

Bradyrhizobium sp. can grow efficiently in seeds of grasses or other legumes during germination, stimulating the development of the root in a similar way to that of free-living rhizobacteria (Cassán et al. 2013a). *B. japonicum* promotes the growth of wheat because the genome of the strain E109 of *B. japonicum* consists of a single chromosome of 9.22 Mbp containing several genes related to nitrogen fixation, phytohormone biosynthesis, and a rhizospheric lifestyle (Torres et al. 2015). Most species of rhizobia produce indole acetic acid (IAA) that contributes to cell division and to the differentiation and formation of vascular bundles, essential for the formation of nodules (Ahemad and Kibret 2014).

10.4.2 Sinorhizobium meliloti

Argentina exceeds 50 million heads of cattle (SENASA 2018), which is fed on the basis of natural or introduced pastures of grasses and legumes. The BNF contributes to approximately 235 kg N ha^{-1} $year^{-1}$ for alfalfa (*Medicago sativa*), 132 kg N ha^{-1} $year^{-1}$ for clovers (*Trifolium* spp.), 85 kg N ha^{-1} $year^{-1}$ for lotus (*Lotus* spp.), and 125 kg N ha^{-1} $year^{-1}$ for *Melilotus* spp. (Racca et al. 2001 in Izaguirre-Mayoral et al. 2007).

In Argentina, there are 4 million ha planted with alfalfa that produce 15 Mt of dry matter (DM), with a high content of total N in the form of proteins and amines. To produce between 21.3 and 47.5 t of dry matter (DM) in irrigation, ha^{-1} $year^{-1}$ requires between 784 and 1120 kg of N ha^{-1}, and to produce in dry land, 15 t MS ha^{-1} $year^{-1}$ requires 450 kg of N ha^{-1} (Basigalup 2014). The biofertilizers for alfalfa are formulated with *Sinorhizobium meliloti* and satisfy 43–64% of the nitrogen requirements of alfalfa, fixed between 50 and 740 kg of N_2 ha^{-1} $year^{-1}$, with an average of 200 kg of N_2 ha^{-1} $year^{-1}$. The amount of N fixed is conditioned by factors related to the strain, the environment, the genotype of the plant, and the management of the crop.

Likewise, the productivity and accumulation of nitrogen in the plant and the proportion of N from the BNF are a consequence of the competitive interaction of the naturalized and introduced strains of *Sinorhizobium meliloti* with Argentinean alfalfa. The competitiveness of the introduced strains varies between 30% and 75% of nodular occupation in the 0–30 cm stratum and 14% and 53% at greater depths and decreases in time up to 31% and 23%, respectively (Racca and González 2007).

10.4.3 Azospirillum *sp.*

Biofertilizers formulated with *Azospirillum* are used to increase the yield of nonleguminous crops such as wheat and corn, reduce the amount applied of chemical fertilizers, increase the efficiency of their use, and maximize the use of soil nutrients (Hungria et al. 2010). They are able to colonize more than 100 plant species and significantly improve their growth, development, and productivity under agronomic conditions, which indicates the versatility to adapt to diverse edaphic conditions (Puente et al. 2009; Bashan and de-Bashan 2010 in Cassán et al. 2013b).

In Argentina, the area sown with wheat and corn is 6.2 Mha and 5.8 Mha, respectively, with average productions of 18 Mt of wheat and 30.2 Mt of corn (Calzada and Rozadilla 2018; PAS 2018). To produce a ton of grain, wheat requires 19.06 kg of N and 3.74 kg of P and maize 14.29 kg of N and 2.88 kg of P. This implies a high extraction of nutrients not replaced in equal magnitude, which generates a reduction in fertility of soils (Cruzate and Casas 2012). *Azospirillum brasilense* strain AZ39, selected by IMIZA-INTA, is found in more than 60% of commercial products and is recommended for wheat and corn (García et al. 2013).

There are other strains used in a lesser proportion in the formulation of inoculants (Az78, Az70, Abv5, and Abv6, among others) (Cassán and Diaz-Zorita 2016).

Azospirillum fixes N and produces and releases growth-promoting substances such as phytohormones (indole-3-acetic acid, gibberellic acid, zeatin, and ethylene), plant growth regulators (abscisic acid and diamine cadaverine), and enzymes (e.g., pectinolytics) that distort the root cell functionality and contribute to the increase in the production of exudates. Indirectly, the inoculation with *Azospirillum* promotes the proliferation and establishment in the rhizosphere of other favorable microorganisms for the culture (Perrig et al. 2007; Cassán et al. 2013a, b). Increases in root length and volume were observed in plants inoculated with *Azospirillum* under controlled conditions, as well as in total dry weight; nitrogen concentration in foliage and grain; total number of spikes, fertile spikes, and cobs; height of the plant; size of leaves; germination rate; flowering; and appearance of spikes (García et al. 2013). However, the effectiveness of the inoculation under field conditions has been recorded in vegetative stages (Abril et al. 2006) but not in harvest (García et al. 2013), because the colonization by rhizospheric organisms is strongly affected by the conditions of the soil and the complex interaction between it and the modes of action of *Azospirillum* sp. (Lagler 2017).

Puente et al. (2009) reviewed fieldwork in the Pampas region of Argentina and reported 53% positive responses in the yield of wheat and corn, inoculated with *Azospirillum*, with increases in average yield of 18% in wheat and 11.5% in corn. Ferraris and Faggioli (2011) conducted field evaluations for 6 years and recorded an average yield increase of 7.8% in wheat inoculated with *Azospirillum*, demonstrating that the highest response to inoculation occurs under medium to high doses of nutrients.

Abril et al. (2006), in field experiments in semi-arid environments of Argentina for 15 years, found a 34% increase in yield in different cultures inoculated with *Azospirillum*, due to water stress conditions and competition with native rhizosphere populations with greater adaptation. In coincidence, Ferraris and Faggioli (2013) indicated that in environments with lower rainfall, there is a greater response to biofertilization due to the competitive advantage for the acquisition of water and nutrients from the inoculated plants, which have higher initial aerial and root biomass, due to modifications in the distribution pattern of phospholipids of the roots (Pereyra et al. 2006). Cassán and Diaz Zorita (2016) showed that *Azospirillum* under conditions of water stress resists and promotes greater growth and productivity of plants. They also reported responses on grain yield in winter cereals (14%), in summer cereals (9.5%), and also in legumes (6.6%) under severe drought conditions.

Currently, evaluations of foliar inoculation with *Azospirillum* are being carried out in wheat, but positive responses in grain production have not yet been reported (Zanettini and Puente 2017).

10.4.4 Pseudomonas *sp.*

Phosphorus, the second most important nutrient, limits the growth of plants due to the low number of available forms in the soil. The use of biofertilizers formulated with phosphate-solubilizing microorganisms is a reasonable economic and ecological option to improve the production of crops in soils with low P (Ahemad and Kibret 2014). Species of the genus *Pseudomonas* are suitable for developing biofertilizers because they have a broad spectrum of properties that promote plant growth, such as the following: (1) they produce phosphatases enzymes, organic acids (e.g., gluconic acid, citric acid), and inorganic acids (e.g., sulfhydric acid, nitric acid, carbonic acid) that break links and acidify the environment recovering the native phosphorus from the soil and that contributed by fertilization (biofertilizers); (2) they produce plant growth regulators (phytohormones) such as auxins, gibberellins, AIA, etc. and reduce the levels of ethylene produced by water stress (phytostimulators); and (3) they produce antibiotics [i.e., pyrrolnitrin, pioluteorin, 2,4-diacetyl fluoroglucinol (DAPG)], induce systemic resistance in the plant, and deplete the essential elements for the growth of fungi and pathogenic bacteria by the release of fluorescent pigments that act as chelating agents (biocontrollers) (Rossi et al. 2013).

In Argentina, there are no reference or recommended strains for the formulation of inoculants; it is recommended that *Pseudomonas* isolates that are currently used be subjected to a process of microbiological, genetic, effectiveness, and safety characterization and conveniently registered by control authorities (Valverde and Ferraris 2009). There are no commercial products for biocontrol formulation because the registration before SENASA is more complex, and the trials are longer than those of biological fertilizers (Valverde and Ferraris 2009; Lagler 2017), but there are several commercially available biofertilizers based on *P. fluorescens* and *P. chlororaphis* subsp. *aurantiaca* used as biofertilizers and phytostimulators of the main crops such as wheat, corn, and soybean (Ferraris 2013; Rossi et al. 2013). About 55% of the biofertilizers formulated with *Pseudomonas* that are commercialized in Argentina are in combination with other microorganisms (Rossi et al. 2013).

Valverde and Ferraris (2009) reported average yield increases between 286 and 310 kg ha^{-1} in wheat and between 622 and 690 kg ha^{-1} in corn inoculated with *Pseudomonas* and with an adequate fertilization with N and P; there was an increase of 7% in the efficiency of use of these chemical fertilizers. Ferraris and Faggioli (2011) indicated that the inoculation with *Pseudomonas* in wheat made it possible to maintain greater productivity due to an early and greater aerial and root development, without increasing the water requirement which increased the efficiency in the use of water (EUA) of 11.9–13 kg wheat mm^{-1} rain, representing a potential increase of 500 kg ha^{-1} grain.

At present, field evaluations are carried out on the ability of *Pseudomonas* and *Azospirillum* to degrade xenobiotic compounds in different stages of growth in corn plants grown with glyphosate. The inoculated plants increase the biomass of roots and shoots and the foliar area, the photosynthetic pigments and the phytohormone

content, the grain yield and decrease the accumulation of herbicides in leaves and grains. This would reduce the persistence of xenobiotic compounds in the environment (Travaglia et al. 2015).

10.4.5 Soil Fungi

Soil fungi used as biological inoculants alone or in association are arbuscular mycorrhizal fungi (AMF) in agricultural crops and ectomycorrhizal fungi in forest species. AMF belong to the phylum *Glomeromycota*, class *Glomeromycetes*, and establish mutualistic symbiotic relationships by colonizing the roots of 90% of terrestrial plants; improve plant nutrition, water absorption, and metabolic functions; increase the resistance or recovery of plants in stress; contribute to the stability of soil aggregates (Consolo et al. 2014) because they increase the root area for nutrient intake; increase the amount of mycelium and secretion of glomalin that favor soil aggregation (Rillig et al. 2002); improve aeration and water dynamics; allow the use of insoluble P sources (Smith and Read 1997); and grant greater tolerance to contamination by heavy metals or drought and a lower susceptibility to root pathogens or herbivores (Gentili and Jumpponen 2006).

The increase in yield in agricultural crops can be easily demonstrated in laboratory and greenhouse experiments and with much difficulty in the field, although combinations of host-fungus species and environmental factors can cause a variation in host response to fungal inoculation mycorrhizal fungi (Johnson et al. 1997; Thougnon et al. 2014). The use of mycorrhizal inoculants increased production and decreased the need for P fertilization in wheat, potato, and soybean crops, among others (Adholeya et al. 2005; Godeas 2007; Covacevich et al. 2008).

In the Pampas region of Argentina, in soybeans co-inoculated with *Glomus mosseae* and *B. japonicum*, there were increases of 40% in the growth parameters, and around 20% in the grain yield with respect to the control (Clua et al. 2013). High natural biodiversity of HFMA associated with wheat crops, pastures of grasses, and legumes and forage grasses (Thougnon et al. 2014) was determined to grain crops, under different modalities of tillage and application of fertilizers, with promising results to mycorrhization levels (Lagler 2017).

The isolation and multiplication of AMF propagules for commercialization is very complex (obligate symbionts), and quality standards are still being studied. Currently, inoculants with AMF are produced in inoculated plots, in containers with different substrates and plants, hydroponic, or in vitro systems. Basically, the development of the formulation consists of placing fungal propagules (root fragments colonized with AMF, fragments of fungal mycelium, and/or spores) on the carriers such as perlite, peat, inorganic clay, zeolite, vermiculite, sand) (Cabello et al. 2013).

Another group of fungi includes those of free life, i.e., *Trichoderma*, which act as biological control agents against pathogenic fungi, are able to solubilize soil

nutrients and produce factors that contribute to the promotion of plant growth (Consolo et al. 2014).

10.4.6 Cyanobacteria

In the Argentine coast, 235,000 ha of rice are grown, producing 1.6 million tons per year, with an average yield of 6.7 t ha^{-1} (MINAGRI 2018). The diazotrophic cyanobacteria fix photo-dependent nitrogen in rice, and, within them, the heterocysts forming are the most ubiquitous (Vaishampayan et al. 2000; Irisarri et al. 2008). The nitrogen fixed by the cyanobacteria can be released and made available to the rice plant from the decomposition of the cells and, in some species, excreted in the form of ammonium or small polypeptides in the vegetative stage.

Cyanobacteria have a positive influence on soil physicochemical properties: pH, electrical conductivity, availability of phosphorus, and protein quality of the grain (Vaishampayan et al. 2001; Kaushik 2014). The cyanobacteria of the *Nostoc* and *Anabaena* genera are selected as biofertilizers and as symbionts with other organisms (Monteros and Iglesias 2005; Singh 2014).

The *Azolla-Anabaena* complex has been used as a naturally competent biofertilizer and established in many rice-growing countries (Singh and Gupta 2016), providing between 8 and 30 kg of N ha^{-1}. It is a common practice in Asia (Singh 1979), but in Argentina, only one product is registered.

10.5 Co-inoculants

The microorganisms are organized in communities and release different metabolites related to their interaction with plants and other microorganisms involved in defense processes and/or competition of natural origin by space and nutrients. However, the application of mixed cultures is complicated because the ecological interactions between the autochthonous microorganisms of the soil and the microorganisms of the biofertilizers are unknown (Vassilev et al. 2015).

In Argentina, co-inoculation is a little used technology; the benefits that this contributes seem to be greater in comparison with the simple inoculations; 20 years ago, co-inoculation studies were already reported with PGPR mixtures (Fischer and Jofré 2009). In the following decade, studies continued and evidence was reported that highlighted the importance of co-inoculate commodities crops such as soy, cotton, wheat, and corn (Table 10.1). The co-inoculation with rhizobia focuses on the improvement of production by increasing the N fixed by the rhizobia by increasing the capacity of infection by the non-rhizobial PGPR and increasing the competitiveness of the rhizobia. Worldwide, some PGPR cited as co-inoculants of *B. japonicum* include *Bacillus subtilis*, *Bacillus thuringiensis*, *A. brasilense*, and *Pseudomonas aureofaciens*. However, the ability to promote the plant growth of

Table 10.1 Results published on inoculation with microbial consortium in different crops

Culture	Consortium	Effects	References
Alfalfa	*Sinorhizobium meliloti* + *Pseudomonas putida*	Modification of shoot and root system dry weights occurred in soybean but not in alfalfa in presence of *Pseudomonas* strains. The presence of *P. putida* strains did not negatively affect the rhizobia symbiosis	Rosas et al. (2006)
Algodón	*A. brasiliense Az39* + *Saccharomyces* sp.	Co-inoculation produces a greater number of buds per plant	Iglesias et al. (2000)
Maíze	*P. fluorescens* + *A. brasiliense*	7% increase in performance	Faggioli et al. (2007)
Soja	*A. brasiliense* + *B. japonicum*	There is an effect of stimulation of the growth of the soybean crop by co-inoculation	Benintende et al. (2010)
	A. brasiliense + *B. japonicum*	Favorable advantages for the number of nodules and vegetative development, not being so at the time of harvest	Marko and Iglesias (2003)
	B. japonicum + *P. putida*	Modification of shoot and root system dry weights occurred in soybean but not in alfalfa in presence of *Pseudomonas* strains. The presence of *P. putida* strains did not negatively affect the rhizobia symbiosis	Rosas et al. (2006)
	B. japonicum + *cyanobacteria*	The benefit of co-inoculation is observed in the first stages	Sotelo et al. (2006)
	B. japonicum + G. *mosseae*	Greater performance in treatments with double inoculation and seed phytotherapics (IBMC) and in those with simple inoculation with *B. japonicum*, independently of the application of seed phytotherapics	Clua et al. (2013)
	B. japonicum + *A. brasiliense*	Advantages of co-inoculation with respect to simple inoculation with *B. japonicum* in the fresh weight of plants	Puente et al. (2013)
Trigo	*A. brasiliense* + *P. ferruginosa*	Co-inoculation does not outperform simple inoculation with Az	Cracogna et al. (2003)

(continued)

Table 10.1 (continued)

Culture	Consortium	Effects	References
	A. brasiliense + *R. leguminosarum*	Increase of 33% and 22% of the dry matter of the aerial part and grain, respectively	Galal et al. (2001) cited by Fischer and Jofré (2009)
	A. lipoferum + *B. megaterium*	Increase of 27% and 100% in the height and dry matter of the aerial part, respectively	El-Komy et al. (2005) cited by Fischer and Jofré (2009)

these non-rhizobial PGPR is little known (Pérez Montaño et al. 2014). In Argentina, there is evidence that the co-inoculation of *B. japonicum* with different bacteria (*A. brasilense*, *Pseudomonas putida*) and fungi (*Glomus* sp., *Saccharomyces* sp.) is a promising technology (Rosas et al. 2006; Sotelo et al. 2006; Benintende et al. 2010).

The co-inoculation of soybean with *B. japonicum* and *Pseudomonas* (solubilizer of phosphorus) increased the vegetative growth (Rosas et al. 2006) through the interaction between two PGPR mechanisms (BNF and solubilization of P). Soybean co-inoculation with *B. japonicum* and *A. brasilense* stimulates growth (Marko and Iglesias 2003; Benintende et al. 2010). Hungria et al. (2013) reported that the inoculation with *B. japonicum* increased the average yield of soybeans by 222 kg ha^{-1} (8.4%) and the co-inoculation with *A. brasilense* increased by 427 kg ha^{-1} (16.1%). It has not yet been experimented in Argentina with the *B. japonicum* + *Bacillus* sp. consortium that could have significant positive effects on nodulation and nitrogen fixation (Masciarelli et al. 2014; Prakamhang et al. 2015). There are still barriers to the co-inoculation in soybeans becoming a frequent practice that contributes to the development of a sustainable agriculture (Atieno et al. 2012).

In Argentina, the co-inoculation of wheat with *A. brasilense* and other microbial species such as *Rhizobium leguminosarum* and *B. megaterium* has shown benefits on growth and yield (Fischer and Jofré 2009). In corn, co-inoculation with *A. brasilense* and *Pseudomonas fluorescens* increased yield by 7% increasing the P content in the plant (Faggioli et al. 2007).

10.6 New Technologies and Future Perspectives

The effectiveness of biofertilizers depends on several factors: cultivation, soil, interactions in the rhizosphere, management practices, knowledge of farmers, and formulation of inoculants (Creus 2017). The companies that manufacture biofertilizers focus on technological improvement with the aim of offering products that increase the productivity of crops without generating adverse impacts on the environment. The new technology focuses on the induced selection of new strains, especially for environments with restrictions. In this regard, inoculants based on

Table 10.2 Diversity of PGPM, rhizobia, and AMF in saline soils

	Taxa	
Bacteria	*Bacillus patagoniensis*	Olivera et al. (2005)
	Mesorhizobium	Estrella et al. (2009)
	Rhizobium	Estrella et al. (2009)
AMF	*Acaulospora aff. undulata*	Soteras et al. (2012), Becerra et al. (2014)
	Acaulospora bireticulata	Soteras et al. (2012), Becerra et al. (2014)
	Acaulospora scrobiculata	Soteras et al. (2012), Becerra et al. (2014)
	Acaulospora sp.	Becerra et al. (2014)
	Ambispora leptoticha	Soteras et al. (2012), Becerra et al. (2014)
	Claroideoglomus claroideum	Soteras et al. (2012), Becerra et al. (2014)
	Claroideoglomus etunicatum	Becerra et al. (2014)
	Claroideoglomus luteum	Soteras et al. (2012), Becerra et al. (2014)
	Diversispora spurca	Soteras et al. (2012), Becerra et al. (2014)
	Funneliformis geosporum	Soteras et al. (2012), Becerra et al. (2014)
	Funneliformis mosseae	Soteras et al. (2012), Becerra et al. (2014)
	Funneliformis sp.	Soteras et al. (2012)
	Glomus brohultii	Soteras et al. (2012), Becerra et al. (2014)
	Glomus clarum	Soteras et al. (2012)
	Glomus magnicaule	Soteras et al. (2012), Becerra et al. (2014)
	Glomus sp.	Soteras et al. (2012), Becerra et al. (2014)
	Rhizophagus clarus	Becerra et al. (2014)
	Rhizophagus intraradices	Becerra et al. (2014)
	Scutellospora sp.	Soteras et al. (2012)
	Septoglomus aff. constrictum	Soteras et al. (2012), Becerra et al. (2014)

B. japonicum capable of establishing a symbiotic association with soybean in environments with restrictions due to high temperatures, water stress, water logging, and soil acidity, i.e., the LPU83T strain (*Rhizobium favelukesii* sp. nov), isolated from alfalfa root nodules in acid soils of Argentina (Torres Tejerizo et al. 2016).

Saline-tolerant PGPR native microorganisms inhabit saline soils (Covacevich et al. 2017). One of the first works was carried out in saline soils of the Province of Buenos Aires, where they reported the prevalence of *Bacillus* sp. (Arias et al. 1998). This genus has very interesting PGPR characteristics, as it is demonstrated that the co-inoculation of soybean with *Bacillus* sp. and *Pseudomonas* sp. (isolated from soybean rhizosphere, India) had a higher tolerance to saline stress showing higher biomass and photosynthetic activity and lower osmotic stress injuries. The increase in proline content and lipoxygenase activity in inoculated plants contributed to the increase in tolerance to salinity (Kumari et al. 2015). However, in Argentina, there is no evidence in this regard.

Several works in Argentina studied the diversity of PGPM, rhizobia, and AMF in saline soils and highlighted their potential to be used as inoculants (Covacevich et al. 2017) (Table 10.2). However, these investigations only remained in merely descriptive stages. Currently, with the advent of soybeans resistant to water and salt stress, it will possibly be investigated in biofertilizers for saline environments.

Other technological advances are focused on the development of bacterial protectors that allow improving the survival of the rhizobia on the seeds of soybean inoculated with prolonged anticipation to the sowing. These bacterial protectors allow reducing the bacterial mortality when fungicides and insecticides "cureseeds" are used. In the north of Argentina with water limitations, there were increases of 6% with the use of protectors (Brandán de Weht et al. 2013).

In the market, there are products with additional quantities of Nod factors called bioinductors, which improve the nodular capacity of the plant; the osmoprotective additives improve the tolerance of the bacteria with the anticipated inoculation and even the protection after sowing in situations of stress (desiccation); and alternatives such as inoculation in the furrow can be done when the use of insecticides and/or fungicides is very aggressive for rhizobia (Piccinetti et al. 2013). There are formulations with higher concentration of bacteria, in order to reduce the volume of application, decrease the detachment of the products applied to the seed, decrease the drying time of the inoculated seed, and improve the operation in the field.

10.7 Conclusions

Biofertilization is a sustainable and easily accessible technology for farmers, decreases dependence on agricultural chemicals, and helps improve soil quality. However, the effect of biofertilizers on the productivity of crops presents variable results that depend on numerous biological and abiotic environmental factors. It should be considered that a biofertilizer is a complex biological formulation resulting from the combination of microorganisms with the products of their metabolism that also influence the plants. Understanding the multiple biological interactions that occur between introduced microorganisms, native organisms, and plants is essential to be able to achieve efficient and adequate use of biofertilizers and achieve the most appropriate for each crop and ecosystem in particular.

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Chapter 11
Rhizobial Inoculants for Sustainable Agriculture: Prospects and Applications

Iqra Naseer, Maqshoof Ahmad, Sajid Mahmood Nadeem, Iqra Ahmad, Najm-ul-Seher, and Zahir Ahmad Zahir

Abstract Due to continuous growth of world population, there is dire need of serious efforts and innovative approaches to meet food demands through sustainable production practices, improvement in supply chain, and control of food wastage. All these efforts should ensure the access to nutritious food to all suffering from hunger and malnutrition. Due to intensive crop cultivation and use of synthetic fertilizers, soil health is seriously deteriorating. However, soil fertility can be improved by incorporating legumes in the cropping system and/or use of rhizobial inoculants, which not only increase nitrogen fixation but also improve soil fertility and crop production through several other attributes such as phosphate solubilization, siderophores production, phytohormones production, enzymes synthesis, and exopolysaccharides production. Moreover, these bacteria can be helpful for improvement in crop production on marginal lands due to their tolerance against various biotic and abiotic stresses. All these characteristics make rhizobia equally important for non-legumes as for legumes. The use of rhizobial inoculants can ensure improvement in crop productivity and environment sustainability by enhancing soil fertility and reduction in use of synthetic chemical fertilizers. Present review focuses on important plant growth-promoting mechanisms of rhizobia and the use of these rhizobia for sustainable crop production through improvement in crop nutrition, physiology, productivity, and stress tolerance of crop plants. The potential of the synergistic use of rhizobia with other soil microorganisms for sustainable agriculture has also been elucidated with examples, followed by their future prospects.

Keywords Rhizobium · Plant growth promotion · Sustainable agriculture · Soil health and fertility

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11.1 Introduction

Decline in soil fertility and organic matter contents is one of the major constraints of crop production in arid and semiarid regions that is attributed to low rainfall, high temperature, and increase in calcareousness of these soils. As the demand for increase in crop production is rising due to expansion of colonization on agricultural lands, farmers try to use more chemical fertilizer which deteriorates soil biology and environmental quality. The governments all over the globe are prioritizing the development of eco-friendly alternate strategies for crop production. Beneficial soil bacteria have significant impact on the growth and productivity of crop plants (Uren 2007). Among these, rhizobia are a group of bacteria which fix atmospheric nitrogen by developing symbiotic association with legumes (Wang et al. 2018). Rhizobia fix about 50% of the total annually fixed nitrogen in the world (Hatice et al. 2008). They develop special structures within the plant cells, called nodules (Beneduzi et al. 2013; Wang et al. 2018). Soil fertility can be improved by incorporating legumes in the cropping system and/or use of rhizobial inoculants which not only increase nitrogen fixation but also improve soil fertility and crop production through several other attributes (Zahir et al. 2018).

The incorporation of grain legumes in cropping system can also be helpful to improve the productivity of the following cereal crops. Moreover, the rhizobia in root nodules of these crops not only fix atmospheric nitrogen in the presence of legume host (Bhattacharyya and Jha 2012) but also help cereal crops through other growth-promoting characteristics such as phosphate solubilization (Khan et al. 2010), siderophores production (Chandra et al. 2007), phytohormones production (Chi et al. 2010), enzymes synthesis (Duan et al. 2009), and exopolysaccharides production (Monteiro et al. 2012). Rhizobia are ubiquitous microorganisms in soil; however, their diversity and population depend upon different factors including crop species, crop rotation, soil properties, agricultural practices, and the extent and distribution of wild species of leguminous plants (Sadowsky 2005; Roberts et al. 2017).

The efficiency of rhizobia varies greatly among different strains depending upon plant host variety, soil and environmental factors, and their interaction (Allito et al. 2014), so efficient host-cultivar-specific combination is recommended in diverse agro-ecological zones and soils with different fertility status. Although *Rhizobium* inoculation increases the nodulation, nitrogen uptake, physiology, shoot and root growth, and yield of legume crops (Sogut 2006; Ahmad et al. 2013a, b), the effectiveness of these inoculants for nodulation and nitrogen fixation is reduced in the presence of high dose of nitrogen-containing chemical fertilizers (Ogutcu et al. 2008). For example, nitrogen application rates greater than 40 kg N ha^{-1} decreased the nodulation and nitrogen fixation in field pea (Clayton et al. 2004), an initial dose of nitrogen is however, required for establishment of root system at early stages of crop growth (Simonsen et al. 2015). The organic amendments on the other hand increase the nodulation and yield of peanut (Agegnehu et al. 2015) and thus can be used in integration with rhizobial inoculants (Argaw and Mnalku 2017).

Rhizobial inoculants are cheaper than inorganic fertilizers, so less financial risks are present in using them as source to improve productivity of legume crops (Ronner et al. 2016). Rhizobial inoculation is considered to be effective for symbiotic nitrogen fixation (SNF) and is being advocated to be used in the absence of effective rhizobia for a specific crop, in low population of effective indigenous rhizobia that really slows down the nodulation process, and/or when more effective rhizobial inoculants are available for a specific crop variety to be grown than the indigenous rhizobial species (Giller 2001). The selection of native rhizobia is imperative for the development of effective and affordable rhizobial inoculants to improve productivity of agro-ecosystems (Koskey et al. 2017). Moreover, the compatibility of rhizobial strain and host plant species/variety must be taken into account along with plant growth-promoting characteristics. In the case of the combined use of rhizobia with other beneficial soil microbes, the compatibility of strains should be tested before their use as inoculants.

Under field conditions, the inoculated bacterial strains have survival disadvantage as compared to indigenous microbial populations. In addition to strong plant growth-promoting abilities, the bacterial strains in developed rhizobial inoculants should have the ability to effectively colonize plant roots and capability to compete for nutrients and space with indigenous microorganisms in the soil and rhizosphere (Stephens and Rask 2000). Genetic engineering and strain selection can be helpful in improving the survival competency of rhizobial inoculants (Geetha and Joshi 2013).

The application of rhizobial inoculants to improve crop productivity has potential for sustainability of agriculture systems. The integrated use of these rhizobial inoculants with other soil microbes can be more beneficial to improve plant growth (Figs. 11.1 and 11.2) and for sustainable crop production by meeting the climate

Fig. 11.1 Effect of *Rhizobium* and plant growth-promoting rhizobacteria on *Cicer arietinum* under wire house conditions in pot experiment

Fig. 11.2 Effect of *Rhizobium* and plant growth-promoting rhizobacteria on root growth of *Cicer arietinum* under wire house conditions in pot experiment

change challenges and nutrient depletions and biocontrol of plant pathogens. The combined use helps to increase the efficiency of rhizobial inoculants through synergistic effects and combination of various mechanisms of actions in legumes (Pierson and Weller 1994) and non-legumes.

11.2 Plant Growth-Promoting Mechanisms of Rhizobia

Rhizobia are a diverse group of bacteria which are ubiquitous in all types of soils in different agro-ecological zones. In addition to symbiotic nitrogen fixation in legumes, they can improve soil fertility and crop productivity through a number of growth-promoting characteristics. These characteristics have been summarized in Table 11.1. Moreover, these bacteria can help in improving crop productivity on marginal lands due to their tolerance against various biotic and abiotic stresses.

11.3 Nodulation and Symbiotic Nitrogen Fixation

Legumes are considered as important component of cropping systems for maintaining the soil fertility and productivity. These crops have the ability to fix atmospheric nitrogen by forming symbiotic associations with rhizobia present in root nodules. The symbiotic nitrogen fixation (SNF) accounts for major share of globally fixed nitrogen through all means that can meet about 50–60% of crop nitrogen requirements as reported in the case of soybean (Salvagiotti et al. 2008). The SNF in legumes is a complex process, mediated by chemical signals between legume host

Table 11.1 Plant growth-promoting characteristics of rhizobial strains

Bacterial species	Plant growth-promoting characteristics	References
Rhizobium sp.	IAA production, P solubilization, N fixation	Shengepallu et al. (2018)
Rhizobium sp.	Improved enzymatic activities, N fixation	Mouradi et al. (2018)
Rhizobium sp.	Antagonistic activity, P solubilization, IAA production, ammonia production, siderophores production, HCN production	Manasa et al. (2017)
Rhizobium hainanense	Nitrogen fixation, IAA production, exopolysaccharides production	Mujahidy et al. (2013)
Rhizobium sp.	IAA production, siderophores production, exopolysaccharides production, HCN production, ammonia production	Ahemad and Khan (2010)
Rhizobium sp.	P solubilization	Sridevi and Mallaiah (2009)
Rhizobium sp.	Exopolysaccharides production	Santaella et al. (2008)
Rhizobium leguminosarum	Exopolysaccharides production	Janczarek et al. (2015)
Rhizobium leguminosarum	P solubilization, IAA production, ACC deaminase activity, siderophores production	Prabha et al. (2013)
Rhizobium leguminosarum	Siderophores production, IAA production, P solubilization, N fixation	Flores-Felix et al. (2012)
Rhizobium sp.	Antimicrobial activity	Bhattacharya et al. (2013)
Rhizobium phaseoli	IAA production	Zahir et al. (2010)
Sinorhizobium sp.	Exopolysaccharides production	Castellane et al. (2015)
Sinorhizobium sp.	Chitinase activity, glucanase activity, IAA production, siderophores production, P solubilization	Kumar et al. (2010)
Sinorhizobium meliloti	IAA production, nitrogen fixation, P solubilization	Bianco and Defez (2010)
Mesorhizobium sp.	IAA production, siderophores production, benzoic acid production, exopolysaccharides production, HCN and ammonia production	Ahemad and Khan (2012)
Mesorhizobium sp.	Siderophores, IAA, ammonia, and HCN production, P solubilization, antifungal activity	Ahmad et al. (2008)
Mesorhizobium ciceri	Siderophores, HCN, and ammonia production	Wani et al. (2007b)
Mesorhizobium loti	Siderophores and IAA production, antagonistic activity, P solubilization	Maheshwari et al. (2007)
Bradyrhizobium sp.	P solubilization, IAA, siderophores, and HCN production	Badawi et al. (2011)
Rhizobium	P solubilization, K solubilization, IAA production	Patel et al. (2017)

(continued)

Table 11.1 (continued)

Bacterial species	Plant growth-promoting characteristics	References
Bradyrhizobium japonicum	ACC deaminase activity, IAA production	Shaharoona et al. (2006)
Azorhizobium sp.	ACC deaminase activity; IAA, ammonia, and siderophores production; P solubilization; Zn solubilization; S oxidation	Islam et al. (2009)
Rhizobium sp.	Exopolysaccharides production	Marczak et al. (2017)
Bradyrhizobium, Rhizobium	Nitrogen fixation, P solubilization, IAA and siderophores production, production of hydrolyzing enzymes (cellulase and pectinase)	Shamsuddin et al. (2014)
Rhizobium cellulosilyticum, *Rhizobium radiobacter*,	P solubilization, Zn solubilization, IAA production	Gontijo et al. (2018)
Rhizobium sp.	Production of IAA, GA, flavonoid, and siderophores, Zn and P solubilization	Routray and Khanna (2018)
Rhizobium nepotum Rhizobium tibeticum	P solubilization	Rfaki et al. (2015)
Rhizobium sp.	IAA production	Abrar (2017)
Rhizobium sp.	Nitrogen fixation	Malisorn and Prasarn (2014)
Rhizobium sp.	P solubilization	Karpagam and Nagalakshmi (2014)
Rhizobium leguminosarum, *Bradyrhizobium japonicum*, *Mesorhizobium thiogangeticum*	P solubilization, IAA production	Singha et al. (2016)

and rhizobia that facilitate nodulation and nitrogen fixation. Complex oxidation and reduction reactions occur during the process of nodulation which consume high amount of metabolic energy, thus reducing atmospheric dinitrogen to ammonia. During the nodulation process, the flavones are released by host plant in the rhizosphere where they trigger the *nod* (nodulation) genes in rhizobia (Subramanian et al. 2006). The activated *nod* genes mediate the production of *nod* (nodulation) factors by rhizobia (D'Haeze and Holsters 2002) which signal the host plant for curling and deformation of root hairs, thus trapping the rhizobia within these special structures (Gage 2004). Infection threads are developed in root hairs through which rhizobia enter in to the inner cortex of plant roots (Jones et al. 2007). Once bacteria enter into the cortical cells of nodule primordium (Mylona et al. 1995), they differentiate into nitrogen-fixing forms "the bacteroids." The bacteroids multiply in the root nodules and fix nitrogen. On nodule senescence, some of these bacteria may enter back into the soil (Denison and Kiers 2011). Bacteria live in the root

nodules, supply fixed nitrogen to plant, and get carbon compounds from plant in return (Lodwig and Poole 2003; Andrews et al. 2009) which are being utilized by these rhizobia as carbon and energy source for respiration and nitrogen fixation, in the form of adenosine triphosphate (ATP) (Lodwig et al. 2003; Hungria and Kaschuk 2014). The SNF can contribute significantly to sustainable crop production.

Rhizobia are very specific to their host plants where they can form nodules and fix atmospheric nitrogen. For decades, scientists were of the opinion that each legume can make symbiotic association with only one rhizobial strain. For example, for decades *Bradyrhizobium japonicum* has been thought to be the only strain that can make symbiotic association with soybean (Rodriguez-Navarro et al. 2010). Later literature reports that there are a number of strains from different genera such as *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium* which can also develop successful symbiosis with soybean, thus fixing atmospheric nitrogen in soybean crop (Biate et al. 2014). Beijerinck, a Dutch microbiologist and botanist, in 1901, reported the process of biological nitrogen fixation (BNF) for the first time (Wagner 2011). The SNF is the major process that contributes plant-available nitrogen; however, nitrogen-fixing efficiency of different crops varies with soil physicochemical conditions (Thies et al. 1992; Giller 2001), the mineral nitrogen status of soil (Thies et al. 1991), indigenous rhizobial population, soil organisms, and environmental factors (Al-Falih 2002; Liu et al. 2011).

11.4 Phosphate Solubilization

Phosphorus (P) is the second most limiting plant nutrient after nitrogen that has a major role in plant metabolic processes such as photosynthesis, respiration, energy transfer, transmission of phosphorus-associated heredity material, cell division and development, and synthesis of nucleic acid and phospholipids (Fernandez et al. 2007; Richardson and Simpson 2011). Farmers use synthetic chemical fertilizer for meeting the crop P requirements (Turan et al. 2006). Plants absorb P in the form of primary and secondary orthophosphates (Bhattacharyya and Jha 2012). When P fertilizer is applied in the soil, it becomes unavailable to plants due to complexation with calcium carbonate in alkaline calcareous soils under arid and semiarid climate (Leytem and Mikkelson 2005) and with sesquioxide in acidic soils (McLaughlin et al. 2011).

Soil microbes play an important role in the availability of phosphorus in soils (Sharma et al. 2013) which use different P-solubilizing mechanisms such as lowering of soil pH by production of low molecular weight organic acids, siderophores production, and release of hydroxyl ions (OH^-) and enzymes (Barroso et al. 2006; Rodriguez et al. 2006; Glick 2012). The microorganisms are also involved in the mineralization of phosphorus through decomposition of organic compounds, thus making P available to plants (Rodriguez et al. 2006) through the production of phosphatases (Aseri et al. 2009) and phytases (Maougal et al. 2014).

Rhizobia have the ability to make available the fixed inorganic P through solubilization and organic P through decomposition (Tao et al. 2008) by above-described mechanisms. A number of rhizobial strains have been documented which solubilize inorganic and mineralize organic P compounds in soil (Afzal and Bano 2008; Khan et al. 2010). Rhizobial species from the genera *Rhizobium* (Egamberdiyeva et al. 2004), *Bradyrhizobium* (Egamberdiyeva et al. 2004; Afzal and Bano 2008), *Sinorhizobium* (Bianco and Defez 2010), and *Mesorhizobium* (Rodrigues et al. 2006; Chandra et al. 2007) have been reported to solubilize P through production of low molecular weight organic acids.

11.5 Siderophores Production

Siderophores are low molecular weight organic compounds which have high affinity for Fe and other metals. These compounds are released by soil microbes especially bacteria in iron-deficient soils, make complexes with Fe, and make it available to plants (Raymond and Dertz 2004; Skaar 2010). Siderophores may chelate with ferric iron, making it available to crop plants and microorganisms (Ahmed and Holmstrom 2014); however, pathogenic fungi are unable to use chelated iron. Iron plays an important role in chlorophyll synthesis and respiration (Kobayashi and Nishizawa 2012). It is also essential for ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), metabolism of oxygen, transfer of electron, and catalysis/enzymatic processes in plants (Aguado-Santacruz et al. 2012). Iron is an important component of nitrogenase complex ferredoxin and leghemoglobin thus helps in nitrogen fixation (Raychaudhuri et al. 2005).

Iron converts into oxyhydroxides and hydroxides; the insoluble forms, under aerobic conditions, thus become unavailable to plants and microorganisms (Rajkumar et al. 2010). Soil pH also affects Fe availability to plants and microorganisms (Masalha et al. 2000). So, under such conditions, siderophores help the microorganisms and plants to meet their Fe needs. Siderophores also make complexes with other essential elements such as molybdenum, cobalt, nickel, and manganese, thus enhancing their availability to microorganisms and plants (Bellenger et al. 2008; Braud et al. 2009). Siderophores complex with heavy metals such as cadmium, copper, and aluminum and radioactive elements like neptunium and uranium (Neubauer et al. 2000) and thus alleviate the heavy metal stress.

It is a well-established fact that rhizobial strains from the genera *Azorhizobium* (Islam et al. 2009), *Rhizobium* (Carson et al. 2000; Arora et al. 2001; Mehboob et al. 2011; Prabha et al. 2013; Manasa et al. 2017; Routray and Khanna 2018), *Bradyrhizobium* (Badawi et al. 2011; Shamsuddin et al. 2014), *Mesorhizobium* (Chandra et al. 2007; Ahmad et al. 2008), and *Sinorhizobium* (Carson et al. 2000; Ahmad et al. 2008) can produce siderophores which chelate with ferric ion under iron-limiting soil conditions (Ahemad and Khan 2011a) and make it available to crop plants.

11.6 Phytohormones Production

Phytohormones are organic molecules, involved in important physiological processes of plants, and thus improve their growth and development. They are synthesized within the plant body at one point and transport to some other place for performing physiological functions (Saharan and Nehra 2011). Phytohormones when applied exogenously are termed as plant growth regulators, due to their involvement in plant growth regulation. They are classified in five major classes as cytokinins, gibberellins, auxins, abscisic acid, and ethylene (Khalid et al. 2006; Saharan and Nehra 2011).

Auxins are involved in root and shoot growth especially at seedling stage (Patten and Glick 1996). Indole-3-acetic acid (IAA), one of the important auxins, is involved in cell division, cell differentiation, gene regulation (Ryu and Patten 2008), apical dominance, cell enlargement, root development (Khan et al. 2014), and nodulation (Remans et al. 2007). It has been well documented that most of the rhizobial strains isolated from root nodules produce indole-3-acetic acid in the presence and absence of L-tryptophan, the immediate precursor of auxins (Ahmad 2011). A number of studies report the production of IAA by rhizobial strains from the genera *Azorhizobium* (Naidu et al. 2004), *Rhizobium* (Dazzo et al. 2005; Weyens et al. 2009; Abrar 2017; Shengepallu et al. 2018), *Mesorhizobium* (Ahemad and Khan 2012), *Bradyrhizobium* (Badawi et al. 2011), and *Sinorhizobium* (Bianco and Defez 2010). The auxins produced by bacteria are involved in production of more nodules and induce root morphogenesis (by improving its size, weight, number of branches, and the surface area of roots) and more adventitious roots (Dazzo and Yanni 2006; Solano et al. 2010).

Cytokinins are involved in plant cell division, development of roots, formation of root hairs, shoot and branching, chloroplast development, and leaf senescence. It also controls cell division in embryonic as well as mature plants (Srivastava 2002; Oldroyd 2007). Cytokinin is important for regulating the number of nodules in a symbiotic relationship between *Rhizobium* and legume crops. It is reported to play a critical role in the activation of nodule primordial, thus, a positive regulator of nodulation (Kisiala et al. 2013).

Cytokinins produced by bacteria stimulate shoot growth and reduce root/shoot ratio in drought-stressed plants (Arkhipova et al. 2007). Different rhizobial species such as *Rhizobium leguminosarum* (Zahir et al. 2010), *Sinorhizobium meliloti*, *Sinorhizobium fredii*, *Sinorhizobium medicae*, and *Mesorhizobium loti* (Kisiala et al. 2013) have the ability to produce cytokinins. Moreover, *Rhizobium* regulates the expression of signaling pathway and activates cortical cells to divide in plants and enhances the endogenous cytokinin production in plants (Oldroyd 2007).

Gibberellins (GA) play a role in leaf expansion and stem elongation of plants. Exogenous application of gibberellins helps to promote bolting of the plants and parthenocarpy in fruits, increases the number of buds and fruit size, and is involved in breaking of tuber dormancy. Soil microorganisms have been studied to produce gibberellins which help to improve plant growth. Bacterially produced gibberellins

affect plant growth and nodulation positively as well as negatively. They induce nodule organogenesis however and inhibit the nodulation at infection stage (McAdam et al. 2018). A number of rhizobial strains from the genera *Rhizobium* (Bottini et al. 2004), *Bradyrhizobium* (Morrone et al. 2009; Afzal et al. 2010), and *Sinorhizobium* (Boiero et al. 2007) have been reported to produce the gibberellins (Mirza et al. 2007).

Abscisic acid (ABA) plays an important role in seed germination, leaf development, root growth, and stomatal closure (De Smet et al. 2006). Its production is mostly prominent in stress conditions like drought stress, where it is in guard cells and stimulates stomatal closure and prevents water loss through transpiration. Its role is also reported during salt stress, resistance against pathogen, and developmental processes, such as seed dormancy and germination (Goggin et al. 2009; Rodriguez-Gacio et al. 2009). The ABA also regulates nodulation in legumes (Suzuki et al. 2004). Rhizobial species from different genera including *Rhizobium* and *Bradyrhizobium* have been reported to produce abscisic acid (Dobbelaere et al. 2003; Boiero et al. 2007) and help in plant growth regulation.

11.7 Enzyme Synthesis

Enzyme production is an important attribute of soil bacteria including rhizobia. During recent years, a number of rhizobial strains have been reported to produce extracellular enzymes. Important rhizobial enzymes include chitinase, phosphatase, cellulase, catalase, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Prabha et al. 2013; Mouradi et al. 2018) which help plants to cope with biotic and abiotic stresses (Ahmad 2011). It has been well documented that ACC deaminase produced by bacteria in soil lowers the ethylene levels in plant body by cleaving the ACC, the immediate precursor of ethylene (Shaharoona et al. 2007). The lower concentration of ethylene is required for regulation of physiological processes in plants (Arshad and Frankenberger 2002; Owino et al. 2006); its higher concentration, however, under stress negatively affects plant growth (Zahir et al. 2008).

The bacterial ACC deaminase converts ACC into ammonia and α-ketobutyrate for use by bacteria as carbon and nitrogen source (Saleem et al. 2007; Singh et al. 2015). Inoculation of crop plants with bacteria containing ACC deaminase enzyme increases mineral uptake, nodulation, and seedling growth of plants (Ahmad et al. 2011, 2013b) leading to improved growth and productivity (Glick 2012; Ahmad et al. 2014). The ACC deaminase-containing bacteria help plants to cope with damaging effects of stresses such as salinity (Nadeem et al. 2007; Ahmad et al. 2011), heavy metals (Khan et al. 2013), flooding (Grichko and Glick 2001), drought (Zahir et al. 2008), and pathogenic stress (Wang et al. 2000).

A number of rhizobial strains having ACC deaminase activity from the genera *Azorhizobium* (Islam et al. 2009), *Rhizobium* (Mirza et al. 2007; Hafeez et al. 2008; Duan et al. 2009), *Bradyrhizobium* (Shaharoona et al. 2006), and *Sinorhizobium* (Ma et al. 2004) have been reported. Rhizobia also produce some other enzymes

such as catalase (Bumunang and Babalola 2014), urease (Deshwal and Chaubey 2014; Nosheen and Bano 2014), and chitinase (Saha et al. 2012) and protect plants under stresses along with enhancing nutrient availability. Rhizobial strains also produce lipase, cellulase, protease, β-1,3-glucanase (Gopalakrishnan et al. 2014), and oxidase (Gauri et al. 2011). All these enzymes are important in nutrient availability and induction of tolerance against biotic and abiotic stresses.

11.8 Exopolysaccharides Production

Exopolysaccharides (EPSs) are complex polymers of high molecular weight which are released by soil microbes including rhizobia (Vijayabaskar et al. 2011; Rao et al. 2013). The bacterial EPSs include humic acids, nucleic acids, phospholipids, proteins, glycoproteins, and polysaccharides (Flemming et al. 2007). Exopolysaccharides are involved in biofilms formation (Sutherland 2001) and protect microorganisms against toxic effects of osmotic stress, desiccation (Sandhya et al. 2009), salinity (Ashraf et al. 2004; Qurashi and Sabri 2012), bacteriophage attacks, and poisonous compounds (Sutherland 2001). Exopolysaccharides improve root and shoot growth and increase fertilizer use efficiency through better water use (Alami et al. 2000).

Rhizobia have the ability to produce exopolysaccharides which help in biofilm formation. The EPSs-producing bacteria can better survive against environmental extremities and can efficiently utilize water and nutrients. Rhizobial exopolysaccharides increase soil aggregation, help plant roots to adhere with soil, and improve water holding capacity of soil and nutrient availability in the root zone (Donot et al. 2012; Hussain et al. 2014). The EPSs also help in the establishment of symbiotic association between plants and rhizobia (Skorupska et al. 2006). The EPS-producing species from different rhizobial genera including *Rhizobium* (Zafar-ul-Hye et al. 2013; Janczarek et al. 2015; Marczak et al. 2017), *Sinorhizobium* (Castellane et al. 2015), *Mesorhizobium* (Castellane et al. 2015), and *Bradyrhizobium* (Ahemad and Khan 2011b) have been reported.

11.9 Production of Other Compounds

Nitrogen is an essential element for plant and microbial growth that is involved in the synthesis of a number of compounds including nucleic acids, amino acids, and proteins. Certain rhizobial strains have the ability to produce ammonia and thus help plants in mineral nutrition (Goswami et al. 2014) and improve plant growth and biomass (Mia et al. 2005). The ammonia-producing bacteria also help in biological control of fungi (Al-Mughrabi 2010; Jha et al. 2012) and reduce the growth of competing microflora, thus increasing the growth of nitrifying bacteria in soil (Angus et al. 1999). Rhizobial species from the genera *Rhizobium* (Zafar-ul-Hye

et al. 2013), *Bradyrhizobium* (Wani et al. 2007a, b; Ahemad and Khan 2011c), and *Mesorhizobium* (Ahmad et al. 2008; Ahemad and Khan 2012) have been reported as ammonia producers.

Lumichrome helps in plant growth (Zhang et al. 2002; Dakora 2003) by improving net carbon assimilation especially under water-stressed conditions (Matiru and Dakora 2005). Inoculation of plants with lumichrome-producing rhizobial strains induces water stress tolerance in plants through minimizing the stomatal conductance of water and transpiration losses in leaves (Mehboob et al. 2009). Riboflavin is a component of bacterial flavin coenzymes which are the typical cofactors of flavoproteins. These flavoproteins are important for various cellular processes, such as for energy production, DNA repairing, redox reactions, biosynthesis, and light emission (Burgess et al. 2009). Riboflavin also affects the rhizobial symbiotic relationship, rhizobial survival in the rhizosphere, and their ability to colonize plant roots (Victor et al. 2013).

Several strains of rhizobia including species from the genera *Rhizobium* and *Sinorhizobium* have been recognized as riboflavin producing which act as plant growth promoter (Yang et al. 2002). Riboflavin produced by bacteria can reduce Fe^{+3} into its more soluble Fe^{+2} forms where it acts as electron donor (Crossley et al. 2007). Rhizobia can also produce zeatin (Boiero et al. 2007), hydrogen cyanide, tensin, viscoinamide, pyrrolnitrin (Bhattacharyya and Jha 2012), and antibiotics (Chandra et al. 2007) such as phenazines (Krishnan et al. 2007) and thus help plants in biocontrol of pathogenic bacteria (Triplett et al. 1994). Rhizobia have the ability to produce bio-stimulatory agents which induce systemic resistance in the plant body (Yanni et al. 2001; Singh et al. 2006).

11.10 Rhizobial Inoculants for Sustainable Crop Production

Using rhizobial inoculants is an emerging technology not only for the improvement of leguminous crops but also for non-legumes due to their cost-effectiveness and environment-friendly nature. The specific group of rhizobia makes symbiotic relation with specific legume plant but may improve plant growth without making symbiotic association in non-legumes. Therefore, the inoculation with rhizobia improves plant growth and productivity in the most significant manner under both normal and stressed conditions as summarized in below sections and Table 11.2.

11.10.1 Crop Nutrition

Rhizobia have positive influence on soil nutrients and thus improve nutrient uptake (Allito et al. 2014) through phosphate solubilization (Khan et al. 2010), siderophores

Table 11.2 Effect of rhizobial inoculants on growth, nutrient uptake, and yield of different crops under in vitro, pot, and field conditions

Crop	Rhizobial strain	Growth conditions	Effects on plants	References
Soybean	*Bradyrhizobium* sp.	Field experiment	Increased N, P, and S contents and improved seed and straw yield	Raja and Takankhar (2018)
Soybean	*Bradyrhizobium* sp.	Field study	Increased number of pods, pods weight, and grain yield	Galindo et al. (2018)
Soybean	*Bradyrhizobium* sp.	Field experiment	Increased phosphorus use efficiency and plant N and P uptake	Fituma et al. (2018)
Soybean	*Bradyrhizobium japonicum*	Field experiment	Increased nodulation, dry matter production, and nitrogen uptake	Solomon et al. (2012)
Soybean	*Bradyrhizobium*	Field experiment	Increased nodulation, shoot nitrogen accumulation, and improved plant growth	Cerezini et al. (2016)
Peanut	*Bradyrhizobium* sp.	Field experiment	Increased plant N and P uptake and nodulation	Argaw (2018)
Peanut	*Rhizobium* sp.	Field conditions	Improved shoot growth and nodulation under saline conditions	Akhal et al. (2013)
Groundnut	*Rhizobium* sp.	Field study	Increased growth, oil contents, protein contents, and yield parameters	Mohammed and Sahid (2016)
Chickpea	*Rhizobium* sp.	Field study	Improved plant growth and yield	Laabas et al. (2017)
Wheat	*Rhizobium* sp.	Pot study	Improved shoot length, shoot and root dry weight	Kamran et al. (2017)
Maize	*Azospirillum brasilense Rhizobium tropici*	Greenhouse	Enhanced plant height, stem diameter, dry biomass of shoots and roots, and N accumulation in shoots	Picazevicz et al. (2017)
Legumes	*Rhizobium* sp.	In vitro	Improved plant growth, enhanced plant defense mechanisms, and resistance against herbivores	Thamer et al. (2011)
Chickpea	*Rhizobium* sp.	Field study	Increased growth and yield parameters and concentration of nitrogen and organic matter in soil	Zaman et al. (2011)
Common bean	*Rhizobium* sp.	Greenhouse Field study	Significant effect on chlorophyll contents, photosynthesis, intercellular CO_2 concentration, and the transpiration rate	Bambara and Ndakidemi (2009)
Pepper Tomato	*Rhizobium phaseoli*	In vitro Pot study	Promoted growth at different stages, increased yield and quality of seedlings and fruits	Garcia-Fraile et al. (2012)
Mung bean Mash bean	*Rhizobium japonicum*	Greenhouse Field study	Increased height, root and shoot growth, pod number, pod length, nodulation, and seed weight	Ravikumar (2012)

(continued)

Table 11.2 (continued)

Crop	Rhizobial strain	Growth conditions	Effects on plants	References
Carrot Lettuce	*Rhizobium leguminosarum*	In vitro Pot study	Promoted plant growth by increasing dry matter of shoots and roots and increased N, P, and Ca uptake	Flores-Felix et al. (2012)
Pea	*Rhizobium leguminosarum*	Pot study	Decreased disease severity, increased seed fresh and dry weights, and better seed filling in pods	Wienkoop et al. (2017)
Pea Lentil	*Rhizobium leguminosarum*	Field study	Increased seed yield and effective in disease control	Huang and Erickson (2007)
Kidney bean	*Rhizobium etli*	Pot study	More nodules with increased nitrogenase activity and higher biomass	Suarez et al. (2008)
Lettuce	*Bradyrhizobium japonicum*	Axenic conditions	Reduced heavy metal stress and increased shoot and root length	Seneviratne et al. (2016)
Cowpea	*Bradyrhizobium* sp.	Greenhouse	Increased biological nitrogen fixation, plant growth, and crop productivity	Rodrigues et al. (2015)
Cowpea	*Bradyrhizobium japonicum*	Field study	Increased plant height and chlorophyll contents	Nyoki and Ndakidemi (2014)
Peanut	*Bradyrhizobium* sp.	Axenic conditions	Improved plant growth, nodule number, and nitrogen contents	Castro et al. (2012)
Wheat	*Azorhizobium caulinodans*	Axenic conditions	Increased number and weight of leaves and roots	Liu et al. (2017)
Black medic	*Sinorhizobium meliloti*	Pot study	Increased biomass production under metal stress	Fan et al. (2011)
Chickpea	*Mesorhizobium* sp.	Field conditions	Improvement in symbiotic parameters leading to enhanced growth and yield	Kaur et al. (2015)
Chickpea	*Mesorhizobium mediterraneum*	Field study	Capable to nodulate in stress conditions and increased nodule number and grain yield	Romdhane et al. (2009)
Bean	*Rhizobium*	Field conditions	Increased growth and yield parameters and protein contents	Yadegari et al. (2010)
Bean	*Rhizobium* sp.	Hydroponic culture	Higher nodulation and increased phosphatase and phytase activity	Mandri et al. (2012)
Soybean	*Bradyrhizobium japonicum*	Field study Glasshouse experiment	Enhanced plant height, number of leaves, leaf chlorophyll content, stem girth, leaf area, and leaf area index	Tairo and Ndakidemi (2013)
Soybean	*Bradyrhizobium japonicum*		Increased N content of inoculated plants and increased root nodulation and yield	Dhami and Prasad (2009)
Kidney vetch	*Mesorhizobium metallidurans*	In vitro	Enhanced tolerance to high concentrations of heavy metals	Vidal et al. (2009)

production (Chandra et al. 2007), and phytohormones production (Chi et al. 2010), in addition to improvement in nitrogen uptake through SNF of atmospheric nitrogen. Rhizobial inoculation can minimize the dependence on chemical fertilizers as it enhances the nutrient uptake of crop plants. For example, Soumaya et al. (2016) conducted an experiment to study the effect of *Rhizobium* inoculation on mineral contents of sulla (*Hedysarum coronarium* L.) crop grown on calcareous soil and reported a significant increase in nutrients uptake leading to improved performance of crop in terms of growth and nodulation.

Rhizobium inoculation improves the nutrient (P, K, Ca, and Mg) uptake in different plant parts such as leaves, shoots, roots, and pods (Makoi et al. 2013), enhances the availability of macro- and micronutrients, and thus improves the nutritional quality of different plant components (Tairo and Ndakidemi 2014). Nyoki and Ndakidemi (2014) observed that inoculation of *Bradyrhizobium japonicum* in cowpea resulted in greater uptake of macronutrients such as N, P, K, Mg, Ca, and Na as compared to control. Similar results were obtained by Tairo and Ndakidemi (2014) where they reported that *B. japonicum* inoculation significantly enhanced the uptake of N, P, K, and Na within the roots, pods, shoots, and whole plant of cowpea (*Vigna unguiculata* (L.). In another study, rhizobial inoculation increased nitrogen fixation which resulted in increased root growth enabling it to acquire more nutrients (Rokhzadi and Toashih 2011; Das et al. 2012). It has been reported that *Mesorhizobium* inoculation not only improves growth and nutrient uptake, but it also positively affected the yield attributes, symbiotic relationship, and enhanced quality of chickpea grains (Singh and Singh 2018). The increased nitrogen content resulted in higher protein content which was also due to *Rhizobium* inoculation (Kumar et al. 2014). In another study, it was observed that *Mesorhizobium* sp. enhanced N and P uptake in both grain and shoot in chickpea as compared to uninoculated control (Sahai and Chandra 2011). Similarly, Chandra and Pareek (2015) reported 0.6%, 6.5%, and 4.3% increase in organic carbon, available N, and available P, in chickpea plant after *Rhizobium* inoculation. Further, Kaur et al. (2015) reported higher protein contents and increase in N and P contents after *Mesorhizobium* inoculation in chickpea. The application of *Rhizobium* improves the N and P content of soil which can be utilized by the next crop after harvesting of crop (Abdalla et al. 2013; Tagore et al. 2013). Studies revealed that *Mesorhizobium* inoculation increased the soil microbial biomass carbon (Bhattacharjya and Chandra 2013) that resulted in more crop biomass and subsequently higher return of organic matter into the soil, thus increasing microbial biomass and activities (Babu et al. 2015).

It has been well documented that rhizobial inoculation separately and in combination with other bacterial strains can improve the nodulation and nutrient uptake in crop plants (Ahmad et al. 2013a). For example, Elkoca et al. (2010) studied the effect of *Rhizobium leguminosarum* bv. *phaseoli* separately and in combination with *Bacillus subtilis* and *Bacillus megaterium* on nitrogen fixation and nutrient uptake of the common bean (*Phaseolus vulgaris* L. cv. "elkoca-05") and reported that the triple inoculation of *Rhizobium leguminosarum*, *Bacillus subtilis*, and *Bacillus megaterium* increased the plant N (52.1%), K (25.6%), Mg (97.6%), and sulfur

(282.4%) as compared to uninoculated control. Similarly, it also improved the seed protein (30.1%), K (25.8%), Mg (95.5%), and S (282.8%) contents in seed of the common bean when compared with uninoculated control. The improvement in micronutrient contents (Zn and Cu in plant and seed) was also observed by inoculation with *Rhizobium leguminosarum* in combination with *Bacillus subtilis* and *Bacillus megaterium*.

11.10.2 Crop Physiology

Nitrogen is an essential nutrient that needs to be applied as a fertilizer for plant growth and development. Chlorophyll also contains nitrogen which is an integral component of photosynthesis. The biological nitrogen fixation (BNF) accounts for about 60% of the total fixed nitrogen (Bano and Iqbal 2016). In BNF, nodulating bacteria gain carbon and other energy resources from photosynthesis and in turn provide nitrogen. This mechanism depends on the activity of chloroplasts which is a structural component of photosynthesis (White et al. 2009). Besides nitrogen fixation, rhizobia that make symbiotic association with plants may also improve physiological status of plants by improving nutrient bioavailability and uptake (Afzal and Bano 2008), phytohormones production (Chandra et al. 2007), siderophores and osmolytes production (Grover et al. 2010; Saidi et al. 2013), and regulation of ACC deaminase (Duan et al. 2009). Rhizobial inoculation has the ability to improve chlorophyll contents of crop plants (Elkoca et al. 2010) and thus can improve the photosynthetic activity and productivity of crop plants. Hussain et al. (2018) found that *Rhizobium phaseoli*-RS-1 and *Mesorhizobium ciceri*-RS-8 improved the transpiration rate, photosynthetic rate, stomatal conductance of water, intrinsic water use efficiency, relative water contents, chlorophyll contents, and nutrients uptake of maize crop under normal and stressed conditions.

Rhizobium inoculation improves physiological characters of plants which direct toward maximum growth and yield. In a study rhizobium alone as well as in combination with *Pseudomonas* strains reduced the adverse effects of salinity by significantly improving the transpiration rate, photosynthetic rate, stomatal conductance of water, C assimilation rate, relative water contents, and chlorophyll contents in mung bean (Ahmad et al. 2013b). They improved the physiology, growth, and quality of plant by adapting several mechanisms mainly by lowering endogenous level of ethylene (Ahmad et al. 2011). The rhizobial inoculation enhanced leaf chlorophyll contents in both glasshouse and field conditions when compared with control treatment (Bambara and Ndakidemi 2009).

Literature reports the increased photosynthetic leaf area, chlorophyll content, and relative water contents due to inoculation of ACC deaminase- and IAA-producing or phosphate-solubilizing rhizobium strains (Saghafi et al. 2018). In another study, Jimenez-Gomez et al. (2018) observed that *Rhizobium laguerreae* possessing several plant growth-promoting abilities showed positive results for vegetative parameters of leafy vegetable which include leaf number, size and weight, as well as

chlorophyll and nitrogen contents as compared to uninoculated control. *Rhizobium* inoculation in legumes enhances the leaf chlorophyll contents of crops (Tairo and Ndakidemi 2013). For example, in the case of soybean and cowpea, it was observed that *B. japonicum* inoculation and phosphorus supplementation significantly increased leaf chlorophyll contents both in field and glasshouse experiments (Makoi et al. 2013; Nyoki and Ndakidemi 2014). The increase in chlorophyll contents results in increased photosynthetic processes (Sylvie and Patrick 2009), and as a result plant produces more sugars for its growth and development.

11.10.3 Crop Productivity

Rhizobial inoculation has been well documented to improve productivity of legume crops under normal (Anjum 2011; Shurigin et al. 2015; Khaitov et al. 2016; Woldemeskel et al. 2018) and stressed conditions (Aamir et al. 2013; Ahmad et al. 2014; Sistani et al. 2017). For example, it has been observed that rhizobial inoculation improved growth, yield, and nitrogen fixation in chickpea (Kyei-Boahen et al. 2002; Fatima et al. 2008), pea (Huang and Erickson 2007), and the common bean (Argaw 2016). Similarly, Sharma et al. (2011) observed significant improvement in nitrate reductase activity, number of effective nodules, and leghemoglobin contents in groundnut due to inoculation with *Rhizobium* strains. The improvement in plant height, straw yield, and grain yield was observed in lentil in response to rhizobial inoculation (Haque et al. 2014).

Indigenous population and cropping history affect the performance of crop-specific symbionts under field conditions. Higher population of indigenous rhizobia in soil where the same legume is being grown in previous years suppresses the influence of inoculated rhizobial strains, while, in the case of low indigenous population, rhizobial inoculants have the ability to improve production of legume crops. For example, about 57% higher seed yield of inoculated plots of soybean was observed by Martyniuk et al. (2018) when compared with uninoculated control. They studied the influence of rhizobial inoculation on productivity of soybean, pea, and yellow lupine in a soil with higher populations of indigenous pea and lupine symbionts and low population of soybean rhizobia. The improvement in grain yield of soybean might be due to higher nodulation in inoculated plots (169%) as compared to uninoculated plots. In the case of soil with relatively high populations of indigenous rhizobia of pea and yellow lupine, no response of inoculation was observed on yield or yield contributing parameters of these crops.

It has been observed that inoculation of *Bradyrhizobium japonicum* improved the root and shoot growth, grain yield and yield-related parameters, and grain nitrogen contents of mash bean (Hussain et al. 2011). Similarly, the inoculation with *Mesorhizobium* strains improved the grain yield in *Cicer arietinum* (Wolde-meskel et al. 2018). In another study, Bhatt and Chandra (2014) also observed that the inoculation with *Mesorhizobium* improved the straw yield, grain yield, nodulation, and phosphorus and nitrogen uptake in chickpea. Alam et al. (2015) found that in

soybean plant, inoculation with *Rhizobium* sp. BARIRGm901 increased the nodule weight, nodule number, plant height, root biomass, shoot biomass, nitrogenase activity, nitrogen fixation and assimilation, strove yield, and seed yield as compared to uninoculated control. Argaw and Muleta (2017) reported that rhizobial inoculation improved the number of nodules, dry mass of nodules, and total biomass yield and grain yield of *Phaseolus vulgaris*.

The use of the most efficient rhizobial strain for specific host variety can maximize the profitability of inoculants, thus capitalizing the maximum productivity of crops (Allito et al. 2014). For example, Kulasooriya et al. (2017) conducted an experiment on *Trifolium repens* L. with the objective to develop cost-effective and eco-friendly technology for crops to minimize the use of nitrogenous fertilizers. They prepared inoculants by using efficient rhizobial strains. They observed significant improvement in biomass of inoculated *Trifolium* plants as compared to plants which were fertilized with urea, under field conditions. They attributed the increased biomass with significant increase in root nodulation of inoculated plants. In another study, Tena et al. (2016) studied the efficiency of different rhizobial strains on nodulation in lentil (*Lens culinaris* Medik.) under field conditions. They evaluated six rhizobial strains and reported a significant increase in nitrogen fixation and grain yield as compared to uninoculated control; however, these strains varied in their ability to improve grain yield of lentil under field conditions.

11.10.4 Stress Tolerance in Crop Plants

Heavy metals are among the main inorganic soil pollutants that are added from agrochemicals, industrial wastes, and mining (Marchiol et al. 2004). The persistence and non-degradable nature of heavy metals pose enormous harmful impacts on microorganisms (Broos et al. 2005; Krujatz et al. 2011), plants (Wani et al. 2008; Wani and Khan 2010), and ecosystem (Cheung and Gu 2007). For example, cadmium (Cd) negatively affects nitrogenase activity of rhizobia and photosynthesis activity of legume host, thus reducing nodulation efficiency (Ahmad et al. 2012). In another study, zinc toxicity adversely affected the symbiotic association between *Rhizobium leguminosarum* bv. *viciae* and pea by decreasing rhizobial population, thus reducing the nodulation and plant growth (Chaudri et al. 2000).

Using rhizobia under stress is not only beneficial for legume crops but can also improve growth of non-legumes (Fig. 11.3) and help in phytoremediation of contaminated soils. The use of rhizobia in combination with legumes is useful in phytoremediation and is recommended as eco-friendly, cost-effective, and easy-to-use approach under adverse soil conditions (Kang et al. 2018). They used *Sinorhizobium saheli* YH1 for reducing the uptake of metal by *Leucaena leucocephala* in mine tailings and metal-polluted soils. It was observed that *S. saheli* YH1 improved plant health of *L. leucocephala* by reduction in metal uptake by plants under heavy metal-polluted soils and recommended to use the approach for phytoremediation of Cd- or Mn-polluted soils.

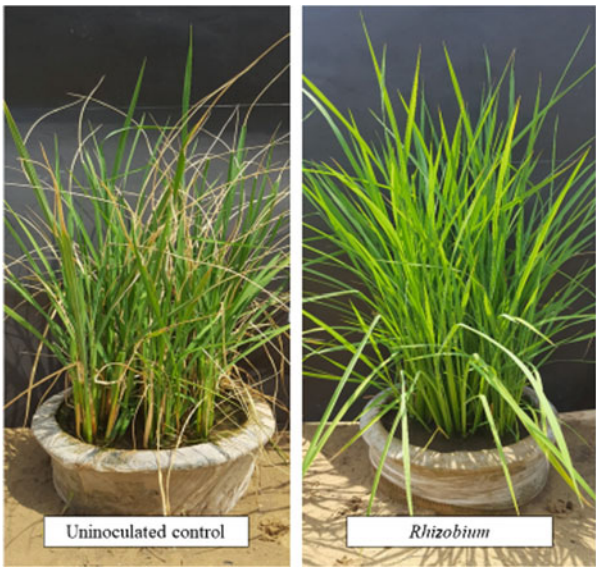

Fig. 11.3 Effect of *Rhizobium* inoculation on rice growth under water-stressed conditions

Rhizobial growth, survival, and distribution in soil are affected by environmental stresses including salinity (Tate 1995). Indigenous population can easily adapt to the local environmental conditions, so they are comparatively more efficient and competitive (Mrabet et al. 2005); however, inoculated rhizobial strains have been well documented to improve plant growth under normal as well as stressed conditions (Ahmad et al. 2014; Allito 2015; Khaitov et al. 2016). The strains vary in their growth under stressed environment with some strains showing more growth even at higher levels of stress that might be owing to stress tolerance ability of these rhizobial strains (Sgroy et al. 2009; Ahmad et al. 2011). Rhizobial strains use different mechanisms to deal with salinity stresses. Inoculation of crop plants with salt-tolerant rhizobia has the ability to improve crop productivity under salt stress (Ahmad et al. 2012, 2014).

Beneficial soil bacteria including several species of *Pseudomonas*, *Rhizobium*, and *Bacillus* have been reported to improve disease resistance in crop plants (Kang et al. 2006; El-Batanony et al. 2007; Samavat et al. 2011) through production of different antimicrobial compounds and hydrolytic enzymes and inducing plant defense mechanisms (Duan et al. 2009). For instance, El-Batanony et al. (2007) reported that *Rhizobium leguminosarum* in combination with AM fungi was effective in biocontrol of *Fusarium solani*, *F. oxysporum*, and *Rhizoctonia solani* in faba bean. In another study, Gao et al. (2012) reported that inoculation with AM fungi and rhizobia directly inhibited the growth and reproduction of pathogen and activated the overall defense system of plant by enhancing PR gene expressions and recommended it for controlling soybean red crown rot in acid soils.

11.11 Synergistic Effects of Rhizobial Inoculation with Other Soil Microbes and Organic Sources

Rhizobial strains can be used in combination with other soil microbes to develop inoculants having two or more strains: the co-inoculation or consortium inoculants. It has been observed that AM fungi in combination with *Rhizobium* improved the mineral nutrition of legume crops (Tavasolee et al. 2011). Similarly, Guo et al. (2010) conducted a study on udorthent to evaluate the efficacy of *Sinorhizobium meliloti* separately and in combination with arbuscular mycorrhiza and lime on growth, nodulation, and nutrient uptake of lucern. It was observed that integrated use was better in improving the nodulation and growth of lucern, as compared to alone application of rhizobial strain. The combined use also improved the nitrogen and phosphorus uptake in lucern crop as compared to uninoculated plants. In another study, the combined use of AMF fungi and *Rhizobium* enhanced productivity, nutrient use efficiency, and profitability of pea crop in addition to saving of about 25% N and P fertilizers in Himalayan acid Alfisol (Bai et al. 2016). The integrated use of *Rhizobium* and AM fungi can also be effective to enhance symbiotic nitrogen fixation under stressed conditions (Chalk et al. 2006). For instance, the integrated use of *Rhizobium* and AM fungi has been well documented to improve plant growth and control of pathogens under field conditions (Akhtar et al. 2011).

The use of *Rhizobium* in combination with plant growth-promoting bacteria can better improve the crop productivity under normal as well as marginal soil conditions. For example, use of consortium developed from *Rhizobium tropici* (CIAT 899), *Paenibacillus polymyxa* Loutit (L), and *P. polymyxa* (DSM 36) improved growth, phytohormone levels, nitrogen content, and nodulation in the common bean (*Phaseolus vulgaris* L.) under drought-stressed conditions, thus having the ability to induce drought stress tolerance in crop plants (Figueiredo et al. 2008).

Rhizobial inoculation in combination with other organisms has also been found beneficial for agriculture ecosystem. Co-inoculation of *Rhizobium* and *Pseudomonas fluorescens* in the common bean increased root and shoot growth, nitrogenase activity, nodulation, and chlorophyll contents in leaves. It also increased the nitrogen and phosphorus uptake by crop plants (Samavat et al. 2012). Similarly, the increase in plant growth and nodulation was observed due to the combined use of *Bradyrhizobium* and ACC deaminase-containing PGPR in mung bean (Shaharoona et al. 2006). The co-inoculation of *Cicer arietinum* with rhizobium and phosphate-solubilizing bacteria significantly improved the seed yield, strove yield, nodule number, and protein content in grain as well as in straw. This co-inoculation also improved the uptake of nitrogen and phosphorus in seed and straw (Singh et al. 2018). Similarly, *Rhizobium* in combination with phosphate-solubilizing bacterial inoculants increased the grain and straw yield, thousand-seed weight, pod number plant^{-1}, seed number pod^{-1}, nodule leghemoglobin content and its number, and fresh and dry biomass (Tagore et al. 2013).

The integrated use of rhizobial inoculants with organic sources can be helpful to increase the productivity of crop plants in soils with poor nutrient contents. The

Table 11.3 Synergistic effect of rhizobial inoculants with other soil microbes on growth, nutrient uptake, and yield of different crops

Crop	Rhizobial sp.	Synergizing organism	Effects on plant growth	References
Wheat	*Rhizobium* sp.	*Azospirillum* and *Pseudomonas*	Increased zinc contents in plant at different growth stages	Shah et al. (2016)
Wheat and soybean	*Bradyrhizobium*	*Azotobacter*	Increased nitrogen contents	Rawat et al. (2013)
Maize	*Rhizobium tropici*	*Azospirillum* sp.	Improved shoot dry weight, total N contents, and grain yield	Mark et al. (2015)
Rice	*Rhizobium* sp.	*Azospirillum brasilense*	Increased plant growth	Hahn et al. (2016)
Rice	*Bradyrhizobium*, *Rhizobium*	*Lysinibacillus*, *Alcaligenes*, and *Bacillus*	Early growth and vigor of rice	Shamsuddin et al. (2014)
Soybean	*Rhizobium japonicum*	*Azotobacter chroococcum* and *Azospirillum*	Improved membrane stability and chlorophyll contents	Zahedi et al. (2013)
Chickpea, pea, and lentil	*Rhizobium*	*Pseudomonas fluorescens*, *Anabaena laxa*	Enhances soil polysaccharide content and plant dry weight	Babu et al. (2015)
Black gram	*Rhizobium*	*Azotobacter* sp.	Increased shoot length, root length, fresh and dry biomass, number of leaves, root nodules per plant, chlorophyll contents, and reducing and non-reducing sugar contents	Gaur et al. (2017)
Chickpea	*Rhizobium* sp.	*Pseudomonas fluorescens*, *Azotobacter chroococcum*, and *Bacillus megaterium*	Significant increase in nodule number, dry weight of nodules, root and shoot growth, nitrogen and phosphorus contents, and grain and straw yield	Verma et al. (2010)
Chickpea	*Sinorhizobium ciceri*	*Pseudomonas* sp.	Increased nodulation and plant dry matter	Messele and Pant (2012)
Chickpea	*Mesorhizobium ciceri*	*Bacillus* sp.	Increased seed yield and grain protein contents	Wani et al. (2007b)
Chickpea	*Mesorhizobium* sp.	*Pseudomonas fluorescens*, *Azotobacter chroococcum*, and *Bacillus megaterium*	Increased root and shoot dry weight and nodulation	Werma et al. (2012)

(continued)

Table 11.3 (continued)

Crop	Rhizobial sp.	Synergizing organism	Effects on plant growth	References
Cowpea	*Bradyrhizobium* sp.	*Paenibacillus graminis*	Increased plant growth, enhanced efficiency of symbiotic association	Rodrigues et al. (2015)
Rajmash	*Rhizobium leguminosarum*	*Pseudomonas lurida*, *Pseudomonas putida*	Enhanced plant biomass and increased uptake of N, P, K, Zn, and Fe contents	Mishra et al. (2014)
Lentil	*Rhizobium* sp.	*Rhizobacteria*	Increased shoot length, root length and total biomass, and nodulation	Zafar-ul-Hye et al. (2013)
Lentil	*R. leguminosarum*	*Pseudomonas fluorescens*	Improved plant growth and nodulation	Khanna et al. (2011)
Pea	*Rhizobium leguminosarum*	Arbuscular mycorrhizal fungi	Increased plant biomass, photosynthetic rate, and N fixation activity	Geneva et al. (2006)
Common bean	*Rhizobium* sp.	*Paenibacillus polymyxa* and *Bacillus megaterium*	Enhanced shoot and nodule weight	Korir et al. (2017)
Common bean	*Rhizobium* sp.	*Paenibacillus polymyxa* and *Bacillus megaterium*	Increased plant growth and nodulation	Korir et al. (2017)
Pigeon pea	*Rhizobium* sp.	Arbuscular mycorrhizal fungi	Increased growth, nutrition, and chlorophyll contents	Havugimana et al. (2016)
Pigeon pea	*Sinorhizobium fredii*	*Pseudomonas fluorescens*	Enhanced growth and yield and potential biocontrol agent against *Fusarium* wilt	Kumar et al. (2010)
Soybean	*Bradyrhizobium* sp.	*Azospirillum* sp.	Increased grain yield and nodulation and enhanced nitrogen contents	Ferri et al. (2017)
Soybean	*Bradyrhizobium elkanii*	*Streptomyces griseoflavus*	Significantly increased plant growth, nodulation, N_2 fixation, N uptake, and yield	Htwe et al. (2018)
Soybean	*Bradyrhizobium* sp.	*Rhizobium*	Enhanced drought tolerance IAA production, EPS production, nodulation, and nodule N contents of plants	Uma et al. (2013)

(continued)

Table 11.3 (continued)

Crop	Rhizobial sp.	Synergizing organism	Effects on plant growth	References
Soybean	*Rhizobium* sp.	Arbuscular mycorrhizal fungi	Enhanced shoot dry weight and increased plant N and P contents	Wang et al. (2011)
Peanut	*Bradyrhizobium* sp.	Fungal endophyte, *Phomopsis liquidambar*	Increased nodule number, shoot nitrogen contents, and flavonoid synthesis	Zhang et al. (2016)
Peanut	*Bradyrhizobium* sp.	*Serratia marcescens* and *Trichoderma harzianum*	Increased number and mass of root nodules	Badawi et al. (2011)
Peanut	*Bradyrhizobium* sp.	*Ochrobactrum intermedium*	Promoted growth and tolerance against high temperature and salinity stress	Paulucci et al. (2015)
Corn and Soybean	*Bradyrhizobium japonicum*	*Azospirillum brasilense*	Promoted seed germination, nodule formation, and early seedling development	Cassan et al. (2009)
Soybean	*Bradyrhizobium japonicum*	*Bacillus amyloliquefaciens*	Better root colonization and increased number of nodules	Masciarelli et al. (2014)

effectiveness of the combined use of *Rhizobium* and different levels of vermicompost to improve the growth and productivity of faba bean was investigated by Argaw and Mnalku (2017) under field conditions. The integrated use of *Rhizobium* and vermicompost significantly improved all parameters of faba bean including number of nodules plant^{-1}, nodule dry weight plant^{-1}, and grain yield as compared to uninoculated control. They recommended using *Rhizobium* inoculant in combination with 8 tons ha^{-1} of vermicompost to boost the productivity of faba bean under field conditions in Haramaya, Ethiopia. More examples on the effectiveness of rhizobia in combination with other soil microbes for improving the productivity of different crops have been summarized in Table 11.3.

11.12 Conclusion and Future Prospects

It is evident from the above literature that rhizobia improve the productivity of cropping systems which not only increase nitrogen fixation but also improve soil fertility and crop production through several other attributes such as phosphate solubilization, siderophores production, phytohormones production, enzymes synthesis, and exopolysaccharides production. Moreover, these bacteria can be helpful

for improvement in crop production on marginal lands due to their tolerance against various biotic and abiotic stresses. Their sole application and co-application with other plant growth-promoting rhizobacteria have the synergistic effects on crop plants both under normal and stressed environmental conditions.

Integrating legumes in the existing cropping systems and/or use of rhizobial inoculants can give better economic returns to farmers and contribute in maintaining soil fertility status for future use. Keeping in view the importance of rhizobia in sustainability of cropping systems, future research should focus on understanding the mechanisms involved in rhizobial-induced growth promotion. Strategies for improvement in plant-rhizobia interactions through molecular genetics, bioinformatics, and modeling tools should also be developed for sustainable crop production.

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Chapter 12
Biofertilizers and Their Role in Sustainable Agriculture

Pinderpal Kaur and Sukhvinder Singh Purewal

Abstract The designing of strategies/protocols for the improvement and enhancement of agricultural output is of utmost importance. Green revolution brings tremendous changes in the field of agriculture and farmer's life. However, green revolution, similar to other scientific methods, has some drawbacks on sustainability of agriculture. Excessive uses of chemical fertilizers and pesticides in the crop field not only deteriorate the quality of soil but also largely degrade the quality of groundwater and thereby the available mineral nutrients. Biofertilizer being a mixture of growth-specific nutrients could be a boon for the agro-industry which could be helpful in enhanced crop production, while on other side it either protects or maintains the environmental conditions. Commercial production of biofertilizers and their easy availability in the market could change the life of farmers as well as agricultural sectors. Scientific advancement for the production of biofertilizer brought impressive attractions because of their involvement in food production and maintaining environmental protection. The government should motivate farmers to use fertilizers of natural origin instead of synthetic ones that could have beneficial impact on the society, environment, and lands. The present chapter focuses on the agricultural as well as societal benefits of using biofertilizers and intervenes to set efforts at the commercial level for the production of biofertilizers with applied functions.

Keywords Biofertilizer · Sustainability · Agriculture · Farmer's life

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12.1 Introduction

Agriculture and agricultural resources have been the sole factor of subsistence for humans since their evolution. The majority of the world's population rely on agriculture for food, feed, and other important things (fiber, wood, gums, and secondary products of medicinal values) to sustain lifestyle in a healthy way (Herve et al. 2016; Kaur et al. 2018a, b). As per the increasing population trend, to meet the hunger requirements of the growing population, researchers/agricultural scientists/agro-industries have to develop suitable methods for sustainable agriculture (Bharadwaj et al. 2014; Singh et al. 2014). The main purpose of any civilized society is to manage agricultural practices up to a level that can sufficiently banish the hunger requirements. The traditional methods of farming involve the production of food and feed at domestic levels only. Traditional methods are limited only up to the farmer's families and their local village communities (Jehangir et al. 2017; Pandey 2018). With the advancement in scientific techniques, the increase in per hectare production could be achieved. Sustainable agriculture concepts are not just a way to grow crops to their maximal limit, but at the same time maintenance of ecology is a determinant factor for the success of sustainability in agriculture (Barragan-Ocana and Rivera 2016). Nowadays, the way of agricultural practices is changing in a mysterious manner as people have focused on the maintenance of environmental resources and are just concerned with the agriculture system for maximal agro-productions. Agricultural practices involve the use of various hormones, chemical fertilizers, and other synthetic minerals to enhance crop production. Synthetic chemicals and minerals have their own effects on soil health as well as plant system (Campos et al. 2018; Umesha et al. 2018a). Although production may increase with increased use of chemicals, however, sometimes depletion of important mineral and other nutritional factors with increased production acts as a barrier.

Sustainability in agriculture system could be achieved without compromising the environmental resources and capabilities of forthcoming generations to meet their own requirements (Wang et al. 2015; Umesha et al. 2018b; Calabi-Floody et al. 2018). Excess use of chemical fertilizers results in depletion of favorable living conditions as the residues that act as secondary pollutant could enter the food chains/food web and finally enter into human beings. With the health hazard effects, secondary pollutant may persist in the surrounding environment for a relatively longer period (Uosif et al. 2014). Use of biofertilizers instead of chemicals in the agriculture system may open up a new era of industrialization. Biofertilizers could be helpful in providing required nutrients to crop plants without deteriorating natural climate (Mishra and Dash 2014). This chapter may serve as a friendly approach for the design of biofertilizers and their use to achieve sustainability in agriculture.

12.2 Biofertilizers

Biofertilizers are organic in nature and possess secondary metabolites of microbial origin or microorganisms itself (Mishra and Dash 2014). For the production of biofertilizers, microorganisms are isolated from soil, water, air, or the rhizosphere which are further processed to a concentrated form for use in field. Microorganisms in response to certain specific conditions start producing metabolites of agricultural importance, and they could be utilized by plants to sustain various biochemical reactions (Salar et al. 2017a). Microorganisms and microbial metabolites ease the release of complex minerals of soil to a simpler form which acts as growth stimulant for a specific crop. Indeed, biofertilizers could be used for various functions (Fig. 12.1).

12.3 Types of Biofertilizers

Biofertilizers can be categorized into different forms based on their type, action, and availability. Figure 12.2 displays the types of biofertilizers that are available for enhanced crop production.

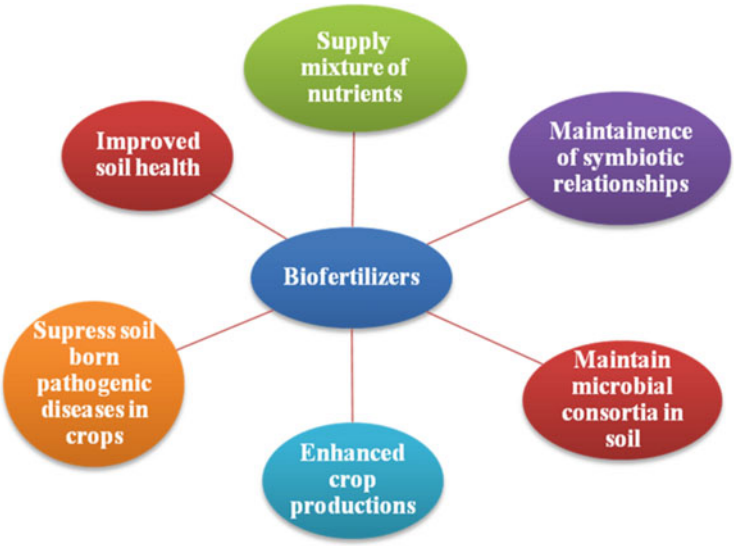

Fig. 12.1 Various uses of biofertilizers

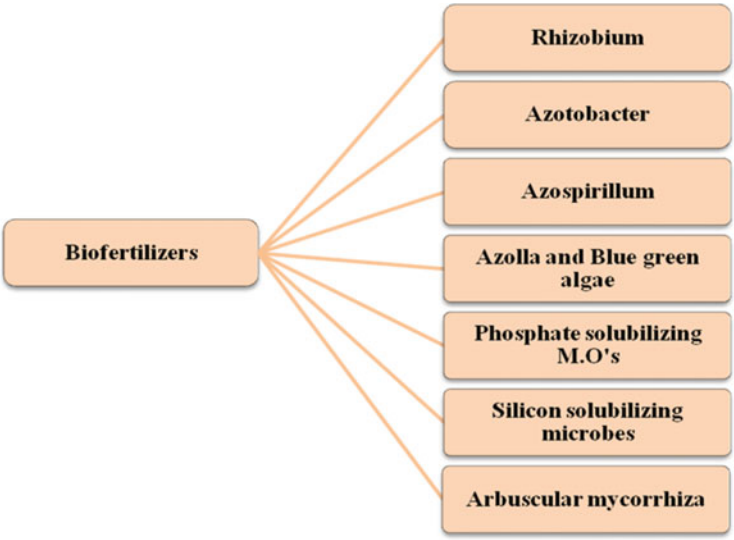

Fig. 12.2 Different types of biofertilizers

12.3.1 Rhizobium *Biofertilizer*

Deficiency of important nutrients in food crop is more challenging in developing countries (Burchi et al. 2011; Kumari et al. 2018). For the solution of these challenging tasks, there is a need of technologies with more emphasis on the use of microbial consortia especially plant growth-promoting rhizobacteria for the sustainable growth of crops and to meet the future demands related to food. *Rhizobium* is a nitrogen-fixing endosymbiont that belongs to the family *Rhizobiaceae*. Rhizobium infects the roots of plants and leads to the formation of specific root nodules (Gouda et al. 2018). Kumari et al. (2018) reported that the isolates BHU B13-398 and BHU M strains were more frequent in mung bean crop. These strains are plant growth boosters found in the rhizosphere region, and their activity results in increased shoot and root length, increased plant height, and increased biomass on dry weight basis. Chen et al. (2018) reported that inoculation of *Rhizobium* in *Medicago sativa* regulates phytochelatin biosynthesis and MT-related gene expression to protect the crops from excess Cu stress. Their results showed that *Rhizobium* inoculation alleviated inhibition of Cu-induced growth which further resulted in increased nitrogen concentration in seedlings of *Medicago sativa*. A significant increase in Cu uptake was observed in *Rhizobium*-inoculated *Medicago sativa* plants as compared to untreated counterparts. Several scientific reports suggested that inoculation of effective microbial strains at planting time results in an increased grain production and overall yield of chickpea (Funga et al. 2016; Tena et al. 2016; Wolde-meskel et al. 2018).

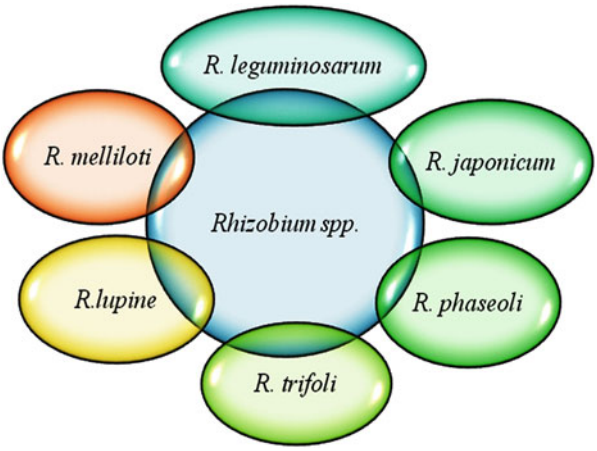

Fig. 12.3 Diversity in *Rhizobium*

Microorganisms within root nodules reduce the molecular nitrogen in the form of ammonia which is further utilized by the plant system for the synthesis of proteins, vitamins, and other important nitrogen-containing compounds (Belhadi et al. 2018; Nyoki and Ndakidemi 2018). They are further categorized into diverse forms by the amount of nitrogen they fix (Fig. 12.3). Application of *Rhizobium* in the specific legumes and other host plants is helpful for sustaining important benefits in the field of agriculture (Sahu et al. 2018). These microbes are nontoxic and have been proved to be free from adverse effects on human health (Singh et al. 2011). Despite their presence in the nodules of leguminous plants, some artificially manufactured formulations of *Rhizobium* are also available in the market under the name Reap N4, Krishi Bio-Nitrex, Shakti Rhizo, Sharad Rhizo, Rhizo Cyll, Tarumitra, and many other names.

12.3.2 Azotobacter *Biofertilizer*

Being nonsymbiont, gram-positive diazotrophs, *Azotobacter* provides various benefits to the crops. The relationship of *Azotobacter* with growing plants helps them to sustain their healthy lifestyle along with maximal production. *Azotobacter* is aerobic in nature and belongs to the family *Azotobacteraceae* (Sethi and Adhikary 2012). Several scientific reports suggest the use of *Azotobacter* in the field for obtaining maximal crop production. The application of *Azotobacter* and related strains enhances plant's dry matter as well as secondary metabolite production (Paul et al. 2002; Nagananda et al. 2010; Damir et al. 2011). Important functional properties (improving soil fertility and nitrogen fixation, enhancement of yield, improving plant growth, helping plants during drought resistance, and being anti-pathogenic)

present in *Azotobacter* strains could be a boon for sustainable agricultural practice (Jnawali et al. 2015; Mahato and Kafle 2018; Shirinbayan et al. 2019).

Azotobacter and related strains (*Azotobacter chroococum*, *Azotobacter salinestris* and *Azotobacter vinelandii*) under specific circumstances start forming cysts—a natural defense mechanism against environmental factors (UV, drying, ultrasound, gamma and solar radiations) (Socolofsky and Wyss 1962). During the process of nitrogen fixation in the field, the strains start producing specific pigments varying from dark-brown to yellowish green and purple colors. The main purpose of pigment production by the strains during the nitrogen fixation cycle is to protect nitrogenase from the damaging effects of oxygen (Jensen 1954; Johnstone 1955; Shivprasad and Page 1989).

Fermenter and shaker are currently being used for the production of *Azotobacter* at the commercial scale. Use of fermenter is an automatic and scientific way for the multiplication of microbial consortia. Specific nutrient media required to sustain the growth of microorganisms are prepared and sterilized, and the pH of the medium may be stabilized to initiate proper growth of microorganisms. Mother culture (1–2%) may be used to boost up the growth. Regular supply of oxygen and maintenance of temperature are other important requirements. Depending on the customers' demand, growth may be enhanced using shaker as it improves the rate of consumption of nutrients within a short time. *Azotobacter* is available in the market under the brand names GROTOP, Nitro-Shakti, Azobiofer, Orga-Azoto, Nitrogreen, and many more.

12.3.3 Azospirillum *Biofertilizer*

Azospirillum is another category of biofertilizer which helps crop to sustain various biochemical reaction required for food production (Llorente et al. 2016). Basically, *Azospirillum* is an important member of the order *Rhodospirillales* and showed close relationships with grasses sometimes with monocots especially corn and rice (Ruiz-Sanchez et al. 2011). The relationship between them is directly associated with nitrogen fixation, secretion of specific fungicides, and phytohormones (Gonzalez et al. 2015; Cassan and Diaz-Zorita 2016). *Azospirillum* possesses distinct capability to produce phytohormones especially indole-3-acetic acid (IAA) (Fukami et al. 2018), salicylic acid (Sahoo et al. 2014), and auxins (Spaepen and Vanderleyden 2015). *Azospirillum* protects crops from biotic and abiotic stress conditions and improves moisture and nutrient uptake, thus enhancing overall yield (Okon et al. 2015; Pereg et al. 2016; Fukami et al. 2018). Inoculation of *Azospirillum* in plant results in remarkable morphological and physiological changes which include shoots and grains with enhanced nitrogen content (Kapulnik et al. 1981; Cassan and Diaz-Zorita 2016). Application of *Azospirillum* in field results in lesser requirement of chemical fertilizer as compared to untreated field (Cassan and Diaz-Zorita 2016; Gassman and Appel 2016).

Azospirillum is available in the market under the brand names Nitromax azos, Azospi, Nitrospirillum 36, Asia green, Nitro booster, and many more. Their stage may vary from powder to liquid, and the color may range from blue to dull white.

12.3.4 Azolla *and Blue Green Algae Biofertilizer*

Azolla belongs to *Salviniaceae* family commonly known as duckweed phototrophic fern with seven diversified species (Roger and Ladha 1992). *Azolla* could grow to form huge biomass with 10 days depending on several factors including soil conditions (pH, nutrients, type, and moisture). *Azolla* is a small free-floating super plant with specific scaly leaves and floating roots. Azolla is well known for its symbiotic nitrogen fixing nature with *Anabaena azollae*. *Azolla* is in routine use for nitrogen fixation purpose in developing as well as developed countries (Emrooz et al. 2018). Rice crop is well known for higher water consumption, and *Azolla* is used by farmers to avoid excessive weed growth. Despite their free-floating nature, *Azolla* provides up to 10 tons of protein and other important minerals to growing rice plants (Yao et al. 2018). Blue green algae (BGA) are nitrogen-fixing microbes that are filamentous in nature and possess specific creature of cells known as heterocysts (micronodules). Heterocysts show functionality in nitrogen fixation mechanism. These microbes establish symbiotic relationships for the purpose of nitrogen fixation in conjunction with fungal strains, ferns, and flowering plants (Soma et al. 2018; Sarker et al. 2018; Islam and Shamsuddoha 2018). Blue green algae are quite important for the agriculture sector, as they show quick action and efficient nitrogen fixation. Despite nitrogen fixation, they are also involved in fixation of phosphorous, zinc, potassium, sulfur, and other micronutrients (Chatterjee et al. 2017; Renneberg et al. 2017; Adeniyi et al. 2018).

Azolla is available in the market under the brand names Azolla biofertilizer, urban farm, and Hasiru green manure. Blue green algae are available in the market in powder form under the brand names Klamath Blue Green Algae, natural blue green algae, bulk supplement pure, and blue green algae pure crystals.

12.3.5 Phosphate-Solubilizing Microbe Biofertilizer

Among macronutrients, phosphorus has its own importance as it regulates signal transduction, protein synthesis, respiration, and nitrogen fixation in plants (Khan et al. 2010; Pande et al. 2017). Phosphorus is available in soil as an insoluble ingredient; hence, plants fail to utilize it. For regular consumption, it needs to be converted from bound complex form to free form (Corona et al. 1996). Certain bacterial strains have the capability to convert phosphorus in the simplest form so

that it can be easily absorbed by plant roots. However, phosphate-solubilizing bacteria are ubiquitous in nature; their number may vary depending on the types of soils and the region from where they are isolated (Chen et al. 2006; Vessey 2003; Awais et al. 2017). Phosphate-solubilizing bacteria used in conjunction with low-quality rock phosphate may be an alternative to costly phosphate fertilizers in developing nations (Mahanta et al. 2018).

Research is being carried out throughout the world to discover microorganisms that could be useful to maintain sustainability in the agricultural sector. Some of the scientific reports suggest that bacterial strains like *Achromobacter*, *Micrococcus*, *Aerobacter*, *Erwinia*, *and Pseudomonas* could have potential to solubilize insoluble forms of phosphate compounds (Chen et al. 2006; Rodriguez and Fraga 1999; Mishra and Dash 2014). Both aerobic and anaerobic strains of microorganisms are present in the rhizosphere region of crop plants and soil. Comparable to other regions, bacterial strains isolated from the rhizosphere region possess maximal phosphate-solubilizing potential. Phosphorus is a highly reactive macronutrient which could bind with iron, aluminum, potassium, and oxygen and result in the formation of complex derivatives. The whole conversion process consists of a series of biochemical reactions which involve the action of various enzymes produced by microbial strains. The initial step results in conversion of complex phosphates into organic and inorganic acids which lowers down the pH of the medium resulting in the maximal availability of the simplest phosphorus to growing plants. Artificially manufactured formulations are also available in the market under the brand names Phosphoz, Phosco, and Green phospho.

12.3.6 Silicon-Solubilizing Microbe Biofertilizer

Natural processes like weathering of silica and silica-based-derivative-containing rocks could result in modification of soil profile (Kang et al. 2017; Vasanthi et al. 2018). Some sorts of microbial consortia play an important role in decomposition, conversion, and modulation of silicon and its derivatives. The action of microbial consortia is dependent on the availability of moisture, pH conditions, and growth factors in the surrounding soil. These are required for the production of specific enzymes and metabolites that could be helpful in the mineralization process (Webley et al. 1963; Lauwers 1974; Northup and Lavoie 2010; Gadd 2010). Conversion of tough silicon derivatives into the simplest consumable forms by biological means gained more importance rather than chemical methods. Biological methods include activities of microbial consortia that are self-controllable and cheap and could result in conversion and transformation within a short span of time. Maximum silicon leaching capability was observed in *Thiobacillus thiooxidans* (Friedrich et al. 1991) and *Bacillus globisporus* (Sheng et al. 2008).

12.3.7 Arbuscular Mycorrhizal Biofertilizer

Natural resources are continuously confronted with abiotic stresses at their different stages of growth and development. Under stressed conditions, plants start producing certain specific category of secondary metabolites to combat overproduction of reactive oxygen species (ROS) (Ahanger et al. 2014; Dhull et al. 2016; Salar et al. 2017b; Kaur et al. 2018a, b; Singh et al. 2018). The production of specific constituents up to certain extent helps the plant to survive under harsher conditions. Symbiotic relationship is one of the most important factors that contribute to sustaining healthy lifestyle of crop plants. Arbuscular mycorrhizal fungi (AMF) are an important symbiont that helps the majority of the plants in efficient nutrient uptake and various enzymatic reactions (Yang et al. 2018). AMF associations with the rhizosphere region of plants provide a range of growth-promoting benefits which include improved nutrition, enhanced resistance, drought tolerance, and modulated soil structure (Gosling et al. 2006; Berruti et al. 2015). Organic farming excludes the use of water-soluble fertilizers and generally has diverse rotations. Scientific evidence suggests that this leads to enhanced inoculation of AMF in soils with maximal nutrient uptake. AMF might therefore be an alternate for chemical fertilizers.

12.4 Scale-Up and Quality Control

Availability of quality-grade biofertilizers in the market is one of the major constraints for enhanced crop production. However, grading of biofertilizers varies from unit to unit and their mode of action. The steps requisite for the production of biofertilizers in the market are summarized in Fig. 12.4. Before the production of

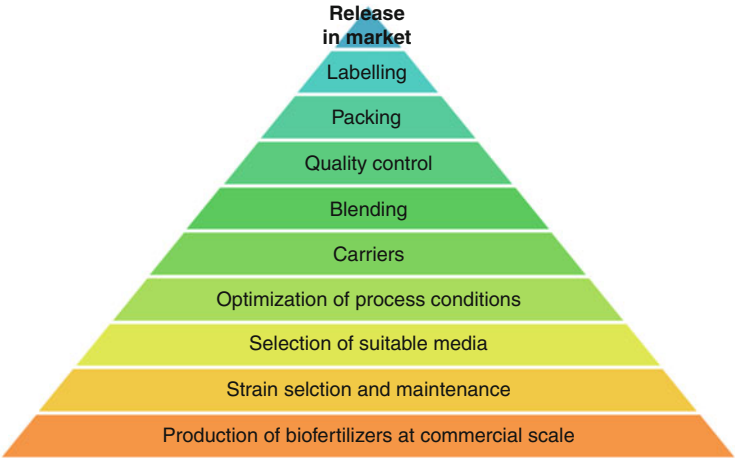

Fig. 12.4 Flowchart starting from production to release of biofertilizers in the market

biofertilizers at commercial scale, the production unit must have the following prerequisites:

- Determination of inoculum needed and suitable field design.
- If the production is considered at economical level and feasible, planning of facilities and organization should commence.
- Adequate training of staff working at technical aspects of production and quality control.
- Provision of required microbiological facilities.
- Uninterrupted supply of microbial consortia and availability of required equipment to sustain healthy life cycle of strain with maximal biomass production.

12.5 Characteristics Necessary for the Release of Biofertilizer in the Market

One of the major constraints in the agricultural sector is the use of biofertilizers for enhanced crop production by the farmers. Although nowadays a number of biofertilizers are available in the market, their quantity and quality may vary depending on the production unit. Before releasing in the market, a biofertilizer should possess the following prerequisite qualities (Fig. 12.5):

Fig. 12.5 Characteristic features required for biofertilizers

1. *Availability*: Biofertilizers should be easily available in market. Easy availability reduces the transport cost and saves time for farmers.
2. *Storage stability*: Biofertilizer formulations should be stable under a wide range of atmospheric conditions. The quality of formulation should remain the same with the duration of time.
3. *Effectiveness*: Biofertilizers should be required in minimal amount for their application in field, and they should be effective in providing mixture of nutrients required for crops.
4. *Solubility and action*: The formulation should be soluble in water as it reduces the overall cost and could be applied by spray method in broader areas of the field.

The formulation should provide immediate supply of nutrients without causing any side effects on plants. It should be user-friendly and should not have any side effect on the farmer's health. It should be available for the farmers at low cost as it also affects the crop price. It should be season independent and remain available for the farmers throughout the year.

12.6 Future Prospects

Consumer perceptions toward the use of biofertilizers and acceptability of food produced and safety of production for human welfare are of utmost importance. Chemical fertilizers have deteriorating effects on the health of consumers, soil, and environment. Biofertilizers can solve agro-industrial problems in a much-specified way; however, development, promotion, and their method of application are under the control of large corporations and genetic committees. In addition, biotech industries in developing countries have achieved remarkable success for the development and distribution of biofertilizers. Similarities in the functionality of biofertilizers and chemical fertilizers are almost similar for the plant kingdom although their health hazard effects create a significant difference among them. Slow release of mineral elements during the use of biofertilizers in the fields limits their adaptability in current agriculture practices. The majority of farm and farm-hold practices are based on overall benefits without knowing their effects on the environment. Farmers should be educated about the environmental and other important beneficial effects of biofertilizers on the agriculture system so that they could be more popularized among farmers.

12.7 Conclusions

In-depth knowledge of the production and use of biofertilizers is required for the economic growth of a country. The design, method of production, utilization, and storage conditions are important to understand the basic principle of sustainability in

agriculture. Sustainability in agriculture is quite helpful for the removal of actual agricultural problems related to crop production. In addition, marginal farmers in developing countries need to be trained for planning their agriculture system based on biotechnological and environmental aspects of biofertilizers. This chapter is an elaborative study on the effectiveness of biofertilizers for attaining sustainable agriculture. Biofertilizers can meet the challenges in agro-industries and open up new opportunities in rural areas for the betterment of farmers in the agriculture sector, business, academia, and other important governmental sectors.

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Chapter 13
The Use of Microorganisms for the Biodegradation of Sewage Sludge and the Production of Biocompost for Sustainable Agriculture

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Abstract Recycling by composting presents a sustainable and a cost-effective approach to reduce the high quantity of sewage sludge. In addition, the relationship between compost stability and functional microflora is reflected in the evolution of

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several parameters as C/N and NH_4^+/NO_3^- ratios. However, the microorganisms that populate the substrates during composting reflect the evolution and the performance of structural stability of amended soils, the water retention capacity, and the biodegradation-humification process in compost and soil. Therefore, monitoring humic substance variation during composting is one of the methods used to estimate microorganism activity. In this study, two different mixtures were prepared. The physicochemical indices of maturity changed during composting to reach C/N around 10 and $NH_4^+/NO_3^- < 1$. These two physicochemical indices of maturity presented a linear correlation with mesophilic actinobacteria with $R^2 = 0.3$ and 0.29 for C/N and $R^2 = 0.29$ and 0.41 for NH_4^+/NO_3, respectively, for mixtures A and B. However, for thermophilic actinobacteria, $R^2 = 0.78$ and 0.25 for C/N, and $R^2 = 0.73$ and 0.37 for NH_4^+/NO_3^-, respectively, for mixtures A and B. The progress of physicochemical and microbial parameters is justified by the germination of turnip seeds, which exceed 100% by using final composting products. These findings clearly demonstrate that exploitation of treated sewage sludge as a soil amendment could regulate the carbon, nitrogen, phosphorus, and organic matter requirements for a sustainable agriculture in Morocco where, for example, more than 6.25 million tons of organic matter is needed.

Keywords Sewage sludge · Composting process · Aerobic microbes · Actinobacteria · Agronomic value

13.1 Introduction

The treatment of sewage sludge is a major concern in developing countries. Nowadays, about 200,000 tons per year of sludge is produced in Morocco. Therefore, finding a suitable strategy to reduce its impact on the environment has become of great interest. Recycling by composting presents a sustainable and a cost-effective approach to reduce the huge quantity of sewage sludge.

Composting process is a suitable way of transforming organic wastes into valuable organic amendments (Said-Pullicino et al. 2007). The compost is produced after biological degradation of organic materials under aerobic conditions. The process is characterized by a succession of various microbial populations during successive composting stages: (1) the mesophilic phase that occurs for a few days is characterized by the activity and growth of mesophilic organisms, which lead to a rapid increase in temperature, followed by (2) the thermophilic phase which is characterized by high temperature from a few days to several months in which thermophilic organisms dominate the decomposition process. The third phase is cooling and maturation that occurs for a several months and is characterized by the development of new mesophilic communities that are characterized by the reorganization of the organic matter in stable molecules and the formation of humic substances (El Fels et al. 2014, 2015).

Compost can be produced from several types of biowaste including industrial organic waste, municipal solid waste, agricultural waste, etc. with the addition of other compounds as bulking agents or amendments to improve the substrate structure and the composting conditions. Several kinds of waste organic matter are rich in macro- and micronutrients and contain organic and inorganic materials as well as trace elements that are essential for plant growth (Dzulkurnain et al. 2017). Despite the use of the traditional application of the compost-based solid waste as amendments to improve long-term soil fertility and productivity, the process has been found very effective (Goyal et al. 2005). Nevertheless, the application of undecomposed wastes or immature composts can lead to the immobilization of plant nutrients and cause phytotoxicity due to insufficient biodegradation of the organic matter (Butler et al. 2001).

Sewage sludge is still posing a significant problem worldwide with regard to human health and environmental pollution (El Fels et al. 2015; Dzulkurnain et al. 2017). Therefore, it is critical to find ways to effectively reuse the wastes and reduce their impact on the environment (Lu and Guo 2009).

The objective of this study was to investigate, on a pilot scale, the characteristics of sludge composting with green waste and the changes of physical, chemical, and microbial parameters during the composting process. The obtained results could provide a guide for the application on a larger scale.

13.2 Composting Characteristics

13.2.1 Physicochemical Parameters

Published composting parameters show clearly the need for more information on the several composting parameters to assess the compost quality (Azim et al. 2018). There are various composting conditions for decomposers: carbon/nitrogen ratio (C/N), humic substance content, concentration of water soluble carbon (WSC), ratios of NH_4^+ and NO_3^-, microbial activity, and germination index (Azim et al. 2018). However, single parameters are not accepted; hence, a combination of several tests is likely suitable to evaluate the compost maturity.

13.2.1.1 Porosity

In order to allow degradation in the compost pile, the porosity of the pile is important. Azim et al. (2018) showed that the density of compost influenced the mechanical properties such as strength, porosity, and ease of compaction. Whereas, the porosity is positively correlated to the airflow, which is required for the pile in composting to allow degradation under aerobic conditions. The oxygen content depends on the compost pile porosity since small particles having small pores increase the diffusion of oxygen. The shape, the size, and the structure of particles

affect the settling conditions. For instance, tight packing arrangements increase the bulk density and reduce the porosity (Azim et al. 2018). Sludge has a high water content and a dense structure, which results in the need for large quantities of bulking agent during the sludge composting to provide enough pores for air flowing to ensure enough oxygen for microorganisms. Azim et al. (2018) reported that the porosity is negatively correlated to bulk density ($R^2 = 0.93$) and positively correlated to the compost moisture content ($R^2 = 0.60$). Ahn et al. (2008) suggested that the high porosity makes the water flux available both as a water vapor and a liquid form.

13.2.1.2 Temperature

Temperature is an important and a simple parameter that indicates the compost maturity (Bari and Koenig 2012) and affects the microbial metabolism. This parameter is used to evaluate the evolution of the process. Monitoring the temperature is highly required to insure the removal of pathogens. It has been demonstrated that high temperature is necessary to sanitize the final product (El Fels et al. 2015).

The temperature is usually controlled as follows: >55 °C for sanitation, 45–55 °C to maximize the biodegradation rate, and 35–40 °C to maximize microbial diversity (Stentiford 1996). As it is mentioned above, the aerobic composting process can be divided into three major phases: a mesophilic-heating phase, a thermophilic phase, and a cooling phase (Alberti 1984; Mustin 1987, Leton and Stentiford 1990). De Bertoldi et al. (1983) showed that optimum temperatures vary from 45 to 55 °C. Nevertheless, high temperature should be avoided since it slows down the biological activity and causes undesirable chemical modifications of the organic matter. Khalil et al. (2001) and Liang et al. (2003) have shown that a temperature above 80 °C inhibits bacterial activity and, therefore, negatively affects the composting process.

13.2.1.3 Moisture Content

The water is a necessary parameter to (1) start composting, (2) secure the microorganisms' lives, and (3) transport nutrients and energy elements through the cell membrane (Roman et al. 2015). Nevertheless, the humidity during composting varies according to the nature and structure of the substrates and the evolution of the composting process. Razmjoo et al. (2015) found that a moisture content ranging from 45 to 50% is optimum for the composting process. Nevertheless, moisture values, less than 30%, can lead to rapid dehydration of the compost, which pauses the biological process, and provide physically stable but biologically unstable compost (De Bertoldi et al. 1983). In contrast, high humidity values (more than 80%) generate anaerobic conditions in the compost. El Fels et al. (2014) reported that 60% is preferred to correctly start composting.

13.2.1.4 C/N Ratio

The nutrient expressed as carbon to nitrogen ratio (C/N ratio) is one of the important factors for the composting process, with carbon serving as a source of energy for microorganisms and nitrogen for the synthesis of amino acids, proteins, and nucleic acids. Mustin (1987) reported that the microorganisms use 15–30 times more carbon than nitrogen, and the time of composting is long at high initial C/N ratio. In general, the optimal value of C/N ratio in composting of most materials has been found to be between 25 and 30 (Choi 1999). If the initial C/N ratio is greater than 35, the microorganisms must pass through many life cycles to oxidize excess of carbon. In contrast, if the C/N ratio is too low, nitrogen losses to the atmosphere are relatively higher. This ratio tends to decrease during composting. Its evolution toward 10 is a crucial indicator of the process maturity, the absence of phytotoxicity, and toxic environment for plant growth.

13.2.2 Microbiology of Composting and Its Contribution to the Determination of the Composting Phases

Organic matter decomposition by microorganisms is the mainstay of organic waste processing during composting. Depending on the species of the available microorganisms, their evolution presents a certain profile during composting. This evolution is mainly related to the variation of the physicochemical parameters, the nature and the structure of the composted substrates (El Fels et al. 2016). Several theoretical phases succeed during composting. In the mesophilic phase during which the conditions are favorable (raw material, the physicochemical conditions such as moisture, aeration, and C/N), the native microorganisms of the substrates (mesophilic microorganisms) activate their metabolism on the substrates that are easy to metabolize (simple sugars and free amino acids) which raises the composting temperature. The heat released during this phase depends on the nature of the composted waste and the isolation conditions of the external environment (Ahn et al. 2008). The second phase is characterized by a change of mesophilic communities to thermotolerant and thermophilic communities. The new physicochemical conditions prepared by the micro-mesophilic organisms facilitate the installation of thermophilic species that resume the work of substrate degradation and continue the process. The temperature increases up to 50 °C. During this very active phase, a significant part of the organic matter is lost by mineralization of the organic carbon and release of CO_2. Drying of the compost due to the evaporation of water is often observed in this phase. In addition, the activity slows down and the temperature gradually decreases. New mesophilic microorganisms colonize the compost again, and the third phase of constructive maturation takes place, and the precursors of the humus appear slowly.

13.3 Methodology

13.3.1 Composting Assay

Different composting trials were conducted in a windrow on a purpose-built platform as follows:

– Mixture A: 1/3 sludge + date palm tree waste 2/3, total volume 4 m^3 (El Fels et al. 2014)
– Mixture B: 1/2 sludge + date palm tree waste 1/2, total volume 4 m^3 (El Fels et al. 2014)

The mixtures were prepared as windrow. To provide aerobic conditions, the mixtures were mechanically turned each week then sampling.

13.3.2 Physicochemical Analyses

The temperature during composting was measured every day using sensors with data memory (PH0700115 model 1.20, Ector-Traceability software, ECTOR France). The samples were dried out at 105 °C. Total organic carbon and ash content were calculated by calcination method in a muffle furnace at 600 °C for 6 h. Total Kjeldahl nitrogen (TKN) was assayed in 0.5 g samples using classical Kjeldahl procedure according to AFNOR T90-1110 standard.

13.3.3 Actinobacteria Enumeration

Growth standard nutrient agar and composting time extract agar (CTEA) media were used to enumerate the indigenous microflora according to El Fels et al. (2015). Samples of different composting times (0, 15, 22, 30, 60, and 180 days) were suspended in sterile distilled water (10 g in 100 ml), homogenized, and then treated 10–15 min by sonication according to Ouhdouch et al. (2001). For actinobacteria enumeration, samples were serially diluted up to 10^{-9}, and cultivable microbial flora was enumerated by plating and spreading 0.1 ml of CTEA prepared according to El Fels et al. (2015) as follows: One liter of distilled water and 35 g of compost were mixed overnight. After filtration and sterilization at 120 °C for 15 min, agar (15 g) was added to the collected filtrate, and the media were supplemented with 40 μg/ml of actidione in order to stop the development of fungi (Olson 1968) and 10 mg/ml of nalidixic acid to inhibit the Gram-negative bacteria (Bulina et al. 1997; Barakate et al. 2002). For each composting time, three replicates were made, and the plates were incubated at 28 °C for enumeration of total mesophilic microflora and 45 °C for total thermophilic.

13.3.4 Turnip Germination

The germination of 20 seeds was conducted in petri dishes with 5 ml of water-soluble extracts of compost in darkness at room temperature (Zucconi et al. 1981). Three replicates were made. The phytotoxicity test was computed as the product of the percentage of viable seeds. It was performed by monitoring the seedling emergence, the number of germinated seeds (tests 24 h), and growth of roots (after 72 h), using the following equation:

$$\mathrm{GI}\% = (\mathrm{NGext} \times \mathrm{LRext})/(\mathrm{NGwater} \times \mathrm{LRwater}) \times 100$$

where:

NGext and NGwater: number of seeds germinated in water-soluble extracts and distilled water, respectively

LRext and LRwater: the length of rootlets in soluble extracts and distilled water, respectively

13.3.5 Statistical Analysis

The results are presented as averages ± SEM. The comparison of the averages is made by ANOVA using SPSS Win version 20. The differences are considered significant at $p < 5\%$.

13.4 Results and Discussion

13.4.1 Monitoring of Physicochemical Parameters

Composting is essentially a microbiological phenomenon that depends highly on temperature variation within the windrows. As shown in Fig. 13.1a, the temperature increased to reach 65 °C at the 15th day of composting of sewage sludge with palm waste that lasted for about 1 month as a consequence of biodegradation of organic compounds (El Fels et al. 2014). El Mezouari El Glaoui et al. (2018) and El Hayany et al. (2018) showed that the temperature varies differently during composting and the temperature patterns were not similar for three composted mixtures. The maximum temperatures reached in mixture 1 (1/2 sludge + 1/2 green waste) and mixture 2 (1/3 sludge + 2/3 green waste) were 45 on day 15 and 50 °C on day 11, respectively, which correspond to the thermophilic stage that lasted for about 1 day in mixture 2 and 2 days in mixture 1. At the beginning of composting, the mesophilic microflora started vigorous oxidation of easily biodegradable compounds. This intense microbial activity leads to a rapid increase in temperature which improves

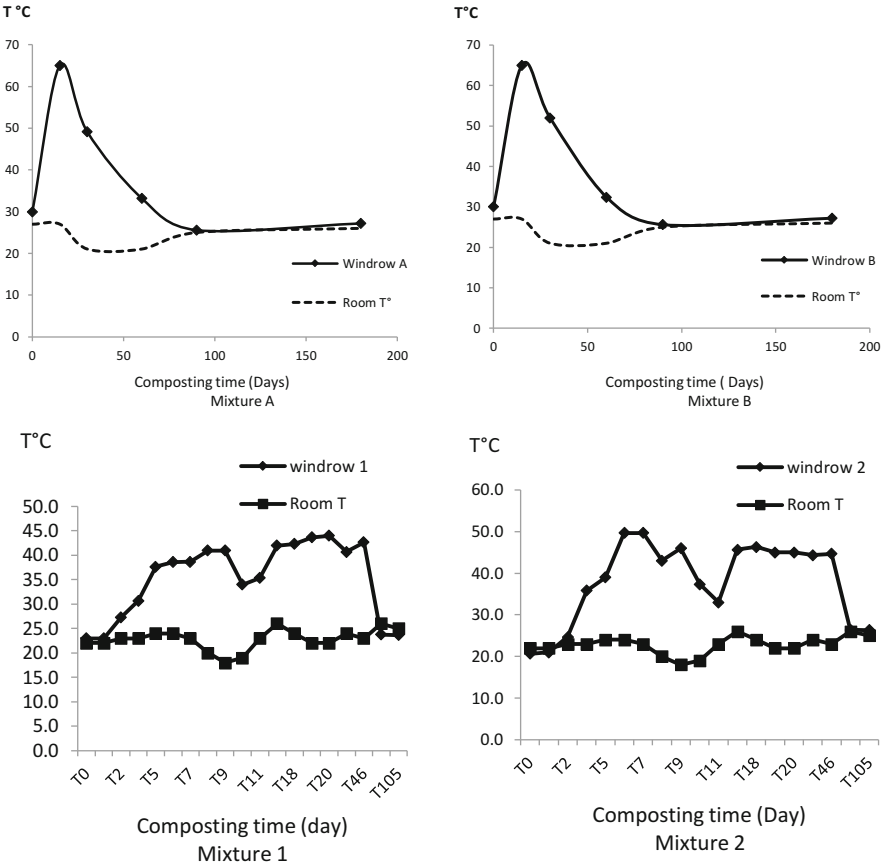

Fig. 13.1 Temperature evolution at versus time during composting of mixtures A and B (El Fels et al. 2014) and mixtures 1 and 2 (El Hayany et al. 2018)

the development and proliferation of total thermotolerant followed by thermophilic microflora (Fig. 13.1).

The final composts should not contain pathogens or viable seeds, and it should be stable and suitable for use as soil amendment (Epstein 1997; Tønner-Klank et al. 2007). De Bertoldi et al. (1988) reported that composting material should reach 55–65 °C in order to achieve hygienization. It has been demonstrated that after maturation phase the composting product is safe for agricultural use (Zucconi et al. 1985).

At the end of the composting, the C/N was about 10 and $NH_4^+/NO_3^- < 1$. The decomposition rate was around 40%, which explains the high biotransformation of organic matter by microbial activity, thereby showing a faster maturity for the composted substrates. During composting, the percentage of NH_4^+ and NO_3^- varied inversely due to the conversion of NH_4^+ to NO_3^- (El Fels et al. 2014; El Mezouari El Glaoui et al. 2018). The enrichment of the final compost by NO_3^- and NH_4^+/NO_3^-

Table 13.1 C/N and NH_4^+/NO_3^- variation during composting of mixture 1 and mixture 2 (El Mezouari El Glaoui et al. 2018) and mixture A and mixture B (El Fels et al. 2014)

Sludge mixed with green waste				Sludge mixed with palm waste			
	T_0	T_i	T_f		T_0	T_i	T_f
Mixture 1				**Mixture A**			
C/N	20.71	16.75	9.47	C/N	26.2	12.8	10.09
NH_4^+/NO_3^-	12	10.94	1.15	NH_4^+/NO_3^-	13.75	2.6	0.12
Mixture 2				**Mixture B**			
C/N	20.71	14.84	10.1	C/N	27.4	14.39	10.08
NH_4^+/NO_3^-	12.54	5.01	1.03	NH_4^+/NO_3^-	7.5	2.5	0.12

lower than 1.0 (Table 13.1) demonstrates the conversion of the substrate to free phytotoxic compost. The increase in nitrates during the maturation phase, causing the reduction in NH_4^+/NO_3^- ratio, was attributed to the good conditions during this phase allowing the development of the nitrifying bacteria (Table 13.1).

The final C/N ratio during composting reached a value close to 10 (reference value), indicating the maturity of the final compost. The C/N ratio is an important factor influencing compost quality (Michel et al. 1995) and one of the best indices to evaluate the maturity of compost (El Fels et al. 2014). El Mezouari El Glaoui et al. (2018) showed that the decrease of C/N ratio is mainly due to the carbon losses through organic carbon oxidation of organic matter. Carbon is used as energy source, while nitrogen is used for building cell structure (Iqbal et al. 2015). Carbon oxidation and CO_2 loss lead to an increase in the proportion of total nitrogen of the medium (El Fels et al. 2014).

13.4.2 Evolution of the Microbiological Parameter

As shown in Table 13.2, after the second month of composting for mixture A, mesophilic and thermophilic actinobacteria show a peak of about 80 and 90% and 70 and 80% after the third month in mixture B. These results could explain the

Table 13.2 Mesophilic and thermophilic actinobacteria in relation to the composting time of mixtures A and B (El Fels et al. 2015)

Composting time (months)	Mixture A		Mixture B	
	Mesophilic actinobacteria (%)	Thermophilic actinobacteria (%)	Mesophilic actinobacteria (%)	Thermophilic actinobacteria (%)
0	90	15	15	30
1	50	50	15	30
2	80	90	40	40
3	70	60	70	80
6	70	60	30	50

increase of actinobacteria activity because of the presence of lignocellulosic compounds at the maturation stage (Table 13.2).

Tuomela et al. (2002) reported that the enzymatic capacity of actinobacteria to attack recalcitrant molecules explains their activity and their proliferation during the maturation stage. Steger et al. (2007) and Xiao et al. (2011) demonstrated that actinomycetes contribute to the degradation of recalcitrant compounds and the formation of stable compounds of humic substance. In general, during composting, the microorganisms present at the beginning of the process are introduced with the raw material. It is known that composts typically contain very high numbers of microorganisms (about 10^{10}–10^{12} viable cells per g) (Beffa et al. 1996; Tiquia et al. 1996). El Fels et al. (2015) showed that the actinobacteria and fungal microflora are the most dominant microorganisms in sludge mixed with date palm waste with a dominance of mesophilic and thermotolerant microflora. Microbial properties of the compost play a significant role in the decomposition and the humification of organic waste materials. At the beginning of the composting process, a significant change of microbial community occurs. The indigenous microflora degrades the original substrate by producing different enzymes needed for the degradation of organic substrate, thereby producing metabolites and creating new physical and chemical conditions during composting. Changes in parameters, such as temperature, affect the succession of microbial communities (Tuomela et al. 2002). The rise in temperature at the thermophilic stage, due to various kinds of microbial activity, affects the fungal activity which is completely suppressed (Thambirajah et al. 1995; Guo et al. 2007). Gram-positive bacteria increase by increasing the temperature and decrease when the compost cools down (Klamer and Bååth 1998). Williams et al. (1983) reported that various actinobacteria are involved in the three compost stages which demonstrate a wide temperature range for their growth. Very often, the optimum temperature ranges between 25 and 30 °C for mesophiles and between 45 and 55 °C for thermophiles. El Fels et al. (2015) showed that all microorganisms especially thermophilic and thermotolerant microflora decrease significantly after the thermophilic stage.

13.4.3 Correlation Between Actinobacteria and Physicochemical Parameters at Various Periods of Time

The microbial activity is dominated by actinobacteria as a primary decomposer that consume the organic fractions in composted substrates. As shown in Table 13.1, the physicochemical parameters changed from the second month of composting of mixtures A and B to reach reference values of maturity C/N around 10 and $NH_4^+/NO_3^- < 1$. These two physicochemical indices of maturity (C/N and NH_4^+/NO_3^-) present a linear correlation with mesophilic actinobacteria [$R^2 = 0.3$ and 0.29 for C/N and $R^2 = 0.29$ and 0.41 for NH_4^+/NO_3^-, respectively, for mixtures A and B

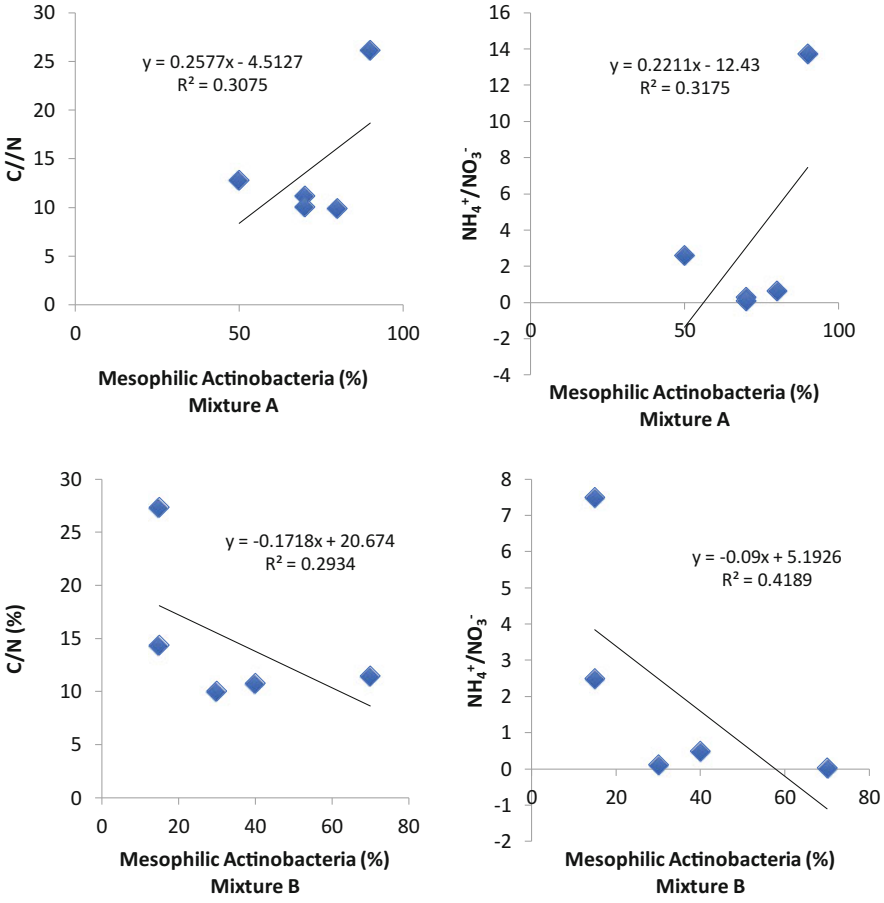

Fig. 13.2 Correlation between the physicochemical parameters (C/N, NH_4^+/NO_3^-) of compost maturity and mesophilic actinobacteria evolution during composting of mixtures A and B

(Fig. 13.2)]. However, for thermophilic actinobacteria, the correlation coefficients were as follows: $R^2 = 0.78$ and 0.25 for C/N and $R^2 = 0.73$ and 0.37 for NH_4^+/NO_3^-, respectively, for mixtures A and B (Fig. 13.3). A positive correlation explains that the evolution of physicochemical parameters during composting process is linked to a significant increase of microorganisms such as actinobacteria that can degrade lignin materials (Vicuña 1988) (Figs. 13.2 and 13.3).

At the end of the process, the actinobacteria group decreased in mixture B and slightly decreased in mixture A (Table 13.2). This could be related to the high amount of lignocellulosic substrate in mixture A. Cunha-Queda et al. (2007) showed that the highest enzymatic activity occurs during the thermophilic stage. During composting of mixture A, C/N and NH_4^+/NO_3^- are the main indicators of the decomposition of organic matter and the maturity of the composted substrate.

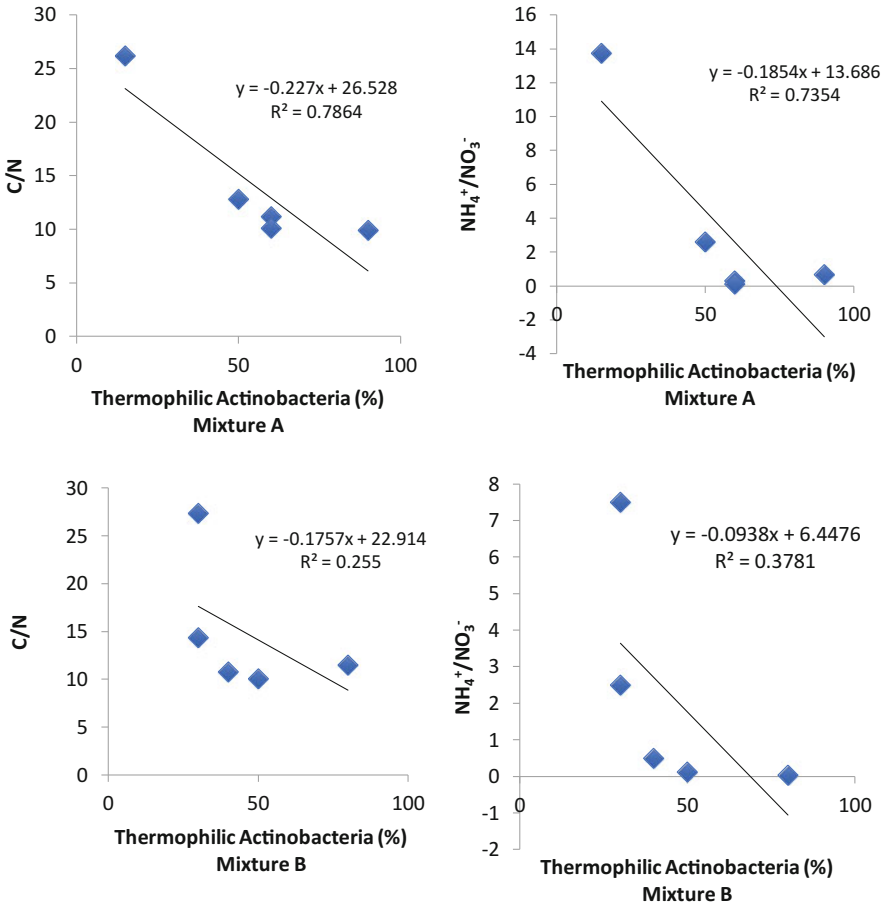

Fig. 13.3 Correlation between the physicochemical parameters (C/N, NH_4^+/NO_3^-) of compost maturity and thermophilic actinobacteria evolution during composting of mixtures A and B

They are highly and positively correlated with thermophilic actinobacteria. Contradictory results were found during the composting of mixture B. In mixtures A and B, the C/N and NH_4^+/NO_3^- variation with time was positively correlated with mesophilic actinobacteria. The difference of the evolution in the physicochemical analysis and actinobacteria between two mixtures is linked to the proportion of sludge and lignocellulosic matter in each mixture.

El Fels et al. (2015) showed that during composting, actinobacteria microflora was inversely correlated with temperature and highly correlated with pH (in the case of mixture B), which could explain their high evolution, especially during the maturation phase when pH increases. El Fels et al. (2016) showed that the mineralization of lignin by microorganisms is an established enzymatic process, which

occurs during the secondary phase of growth under starvation conditions of nitrogen or carbon. Most studies on processes involving lignin degradation have shown that the lignin is degraded by a complex microflora, which includes both eukaryotic and prokaryotic organisms such as white-rot fungi and actinomycetes, respectively.

13.4.4 Compost and Turnip Germinations

As shown in Table 13.3, low germination indices (GI) (32.89% and 16.23%, respectively, for mixtures A and B at the initial phase for turnip species) were observed during composting of sewage sludge with palm date. El Mezouari El Glaoui et al. (2018) showed that during composting of sludge mixed with green wastes, the germination index was low at the initial stage (Table 13.3). Thereafter, the GI has increased to reach 47.29% and 72%, respectively, for mixture 1 and mixture 2 at intermediate stage of composting and 58.73% and 34.1%, respectively, for mixtures A and B. In contrast to different composts during the maturation phase, there was a significant increase in the germination index, reaching a maximum over 100%.

The phytotoxicity is due to the presence of high levels of $NH4^+$, soluble salts, organic acids, or high pH (Wang et al. 2017).

- If GI $<$ 25, the product is considered as very phytotoxic.
- If 26 $<$ GI $<$ 65, the substrate is considered as phytotoxic.
- If 66 $<$ GI $<$ 100, the substrate is considered as non-phytotoxic.
- If GI $>$ 101, the substrate is stable and can be used as fertilizer and phytostimulant (Aggelis et al. 2002; Moharana and Biswas 2016).

El Fels et al. (2014) showed that the GI values that exceeded 100% can generally be explained by a great reduction of phytotoxic compounds. Meng et al. (2017) showed that the increase would be caused by the decomposition of toxic materials. The difference between the compositions of composted substrates could also explain the difference in the evolution of the process and consequently the evolution of organic matter and its decomposition, which influences the reduction of the toxic compounds.

Table 13.3 Turnip germination index (GI) during composting of mixture 1 and mixture 2 (El Mezouari El Glaoui et al. 2018) and mixture A and mixture B (El Fels et al. 2014)

	Sludge mixed with green waste (2018)				Sludge mixed with palm waste (2014)			
		T_0	T_i	T_f		T_0	T_i	T_f
Turnip (GI%)	**Mixture 1**	49.56	47.29	151.96	**Mixture A**	32.89	58.73	130.03
	Mixture 2	56.8	72	197.33	**Mixture B**	16.23	34.1	113.08

T_0 = initial stage
T_i = intermediate stage
T_f = final stage

13.5 Conclusion

Many tests have been proposed to assess the composting progress (e.g., empirical means such as the color, odor, texture, and temperature; physical techniques such as physicochemical analyses, C/N and NH_4^+/NO_3^- ratios, humification index, steroid, total lipids, fatty acid methyl esters; and biological testing such as germination index and microbial evolution). The outcome of this study demonstrated that the sewage sludge treated by composting can be characterized by using various physicochemical and microbiological parameters. Reference indices for maturity such as a temperature increase up to 50 °C, a C/N around 10, and a NH_4^+/NO_3^- below 1 were determined to reach the maturity. Besides the evolution of these physicochemical parameters, the starting biooxidation of organic compounds is the main factor of organic waste processing, and the phenomenon is closely related to the physicochemical conditions of composting substrates, which consequently affect the proliferation and the succession of indigenous microorganisms and the compost maturity level. The results were confirmed by the positive and linear correlation between the C/N and NH_4^+/NO_3^- ratios and the mesophilic actinobacteria (R^2 over 0.29). The high correlation (R^2 over 0.70) was noted between C/N and NH_4^+/NO_3^- ratios and thermophilic actinobacteria when the medium was rich in lignocellulosic waste.

The change of physicochemical, mesophilic, and thermophilic actinobacteria during composting showed that the composting process acts as biotechnological tools to transform the organic matter to a fertilizer for the soil without any contamination of the soil-plant system. This was confirmed by a germination index that exceeded 100% at the end of the composting.

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Chapter 14
Circadian Rhythms in Plant-Microbe Interaction: For Better Performance of Bioinoculants in the Agricultural Fields

Raghavendra Maddur Puttaswamy

Abstract Circadian rhythm (CR) is an important regulator of numerous basic functions of the living organisms such as carbon metabolism, gene expression and regulation, growth and reproduction. It is widely accepted, and several research activities prove its implication on health and disease especially in humans and plants including microbes associated with it. CR is reported to regulate circadian clock which is subjected to extensive natural variation during day and night, light intensity, availability of nutrients, stress and other factors. CR varies within and between species; this underlies the importance of understanding the phenomenon at the individual level to develop disease management strategies or production of microbial formulations used for growth promotion. In plants, rhizosphere microorganisms extensively depend on the root exudates, and its composition is reported to alter with CR in response to external stimuli including global warming and pollution. These microbes play an important role in plant growth and its environmental fitness and hence the concept of plant growth-promoting rhizobacteria (PGPR) came to existence. However, even today circadian clock regulating interaction of PGPR with plants is not extensively studied, and hence most of the time, microbes developed in the laboratory fail to perform in the field level. The world is awaiting another green revolution to feed the growing population with bitter experience of the previous revolution. It is the right time to understand the circadian clock at the species level and to develop suitable formulations to exploit the beneficial aspect of plant-microbe interaction to achieve high yield in the agricultural fields as a part of the sustainable agriculture. Understanding the CR in plant-pathogen interaction will also help to develop suitable treatment strategies to overcome the yield loss due to infection.

Keywords Plant growth-promoting rhizobacteria · Sustainable agriculture · Rhizosphere microflora · Circadian clock

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14.1 Introduction

Genes not only inherit the capacity of the organisms to clone but also the capacity of the generations to endure environmental changes referred to as chronon, which means the cyclical, irreversible, recursive and chronological expression of genes as a function of biological time. The stimulation of these constitutive biological rhythms of the living organisms defines its fitness to the environmental variations. Halberg et al. (1959) referred this rhythm as circadian (daily clock phenomenon) derived from the Latin word circa for "about" and dies for "day". It is defined as the biological activities with a frequency of one activity cycle every 24 h (Halberg et al. 1977).

Linnaeus (1770) is the pioneer in studying the plant behaviour in response to time. He observed the periodical movement of flowers in response to external conditions such as temperature and change in light. His observations on timely response of different varieties of flowers recorded using garden clock helped in developing a concept of unique rhythms in many species. He named it as sleep of plant analogous to that of animals. Even though these observations are connected with plant response to external stimuli in time scale, detailed research on this concept was taken up later to prove it.

Animals also respond to this clock and select the feed accordingly based on the variation in the plant metabolites. Related to this, an interesting study on feeding habit of olive baboon was reported by Adeola et al. (2014). These animals showed different choices of feeding during wet and dry season. Among the plants used for consumption, 7 plants, viz. *Andropogon gayanus*, *Strychnos spinosa*, *Nuclear larifiora*, *Vitellaria paradoxa*, *Ficus sycomorus*, *Annona senegalensis* and *Tamarindus indica*, were consumed in wet season with 303 feeding events, while other 10 plants *Detarium macrocarpum*, *Gardenia sotoemsis*, *Parkia biglobosa*, *Piliostigma thonningii*, *Pterocarpus erinaceus*, *Prosopis africana*, *Ficus sycomorus*, *Ximenia americana*, *Annona senegalensis* and *Vitex doniana* were consumed with 315 feeding events during dry season. It is a clear indication that the plant with higher nutritional quality was consumed by the animals. The change in feeding habit also indicates that the plants are subjected to seasonal variation due to which the nutritional composition also alters. It is a best example for how animals choose their feeding to satisfy the nutritional balance. This change in feeding habit also indicates change in plant metabolism in response to seasonal variation and provides clear evidence that plant physiology is altered with season and time.

Present-day advanced research is providing more insights into this concept, the broader understanding of this phenomenon and its widespread application in several aspects of plant growth and adaptability. The study on this behaviour needs accurate observations and mathematical interpretation of numerous experimental data recorded in different intervals of day and night. Recording the biological fluctuations or variability in measurements of hormone and pigment concentrations, membrane transport rates, growth, ion fluxes, protein production, etc. underlies the basic understanding of rhythms.

14.2 Rhizosphere Microflora and Root Exudates

Soil being a natural media supports plant-microbe interaction. Beneficial microorganisms such as asymbiotic and symbiotic nitrogen-fixing microorganisms, ecto- and endomycorrhizal fungi and plant growth-promoting rhizobacteria including K and P solubilizers play a vital role in plant growth. Soil microbes also exhibit antifungal activity, produce volatile organic compounds and induce systemic resistance in plants. To maintain these microorganisms in the vicinity of the root, plants release 5–10% of net photosynthate by roots, and this percentage increases when it is grown in nonsterile system (Barber and Martin 1976). This indicates that the structure and diversity of the rhizosphere microflora vary among plant species and over time (Baudoin et al. 2002). It is also interesting to note that different root zones of the same plant choose colonization of specific microbial communities by releasing specific substrates which varies from simple sugar to complex aromatic compounds (Kamilova et al. 2006). Composition of the root exudates hence is an important selection force for beneficial plant-microbe interaction. It comprises phenolics, sugars, amino acids and secondary metabolites of low molecular weight and polysaccharides, proteins and other biomolecules of high molecular weight (Abbot and Murphy 2003; Walker et al. 2003). These biomolecules are often less diverse but available in larger proportion in the exudates, and polysaccharides in general decide the association of heterotrophic rhizobacteria with rhizosphere and rhizoplane. Glycosides and hydrocyanic acid are considered as toxic metabolites of root origin which is known to inhibit the growth of pathogens (Rangaswami 1988).

Recent studies proved that rhizosphere microbiome associated with plant growth is also influenced by the type of soil, climate change and anthropogenic activities (Igiehon and Babalola 2018). Even plant cultivar which is having variations in single gene is reported to alter the microbiome. Bressan et al. (2009) observed change in rhizosphere microflora between wild-type and transgenic *Arabidopsis*, due to release of glucosinolates. They revealed that the presence of a single metabolite significantly affected alphaproteobacteria and fungi population in the rhizosphere.

Abiotic factors such as pH, type of soil, availability of oxygen, intensity of light, soil temperature, availability of proper nutrients and even presence of specific microorganisms govern the qualitative and quantitative composition of root exudates. It varies among the plant species, for example, differential exudation pattern was observed in pines and variation in the amount of amino acids in pea and oat root exudates. Diverse carbohydrates are released by young maples compared to mature trees, which exude more and diverse amino acids.

Even the organic acids released in root exudates vary. Study conducted by Schilling et al. (1998) revealed that root exudates of *Zea mays* found to contain citric acid, where as in *Triticum turgidum* var. *durum* L. it was oxalic acid and acetic acid and acetate is a dominant acid released by roots of *Linum usitatissimum* L. (Cieslinski et al. 1997). This shows composition of root exudates varies with several factors and it is specific to plant species.

Ultimately it is the quantity and quality and type of carbon sources released in root exudates that decide the composition of microbial communities in the rhizosphere (Merbach et al. 1999). It is not only beneficial organisms; even pathogenic fungi such as *Rhizoctonia*, *Fusarium*, *Sclerotium*, *Aphanomyces*, *Pythium*, *Colletotrichum*, *Verticillium* and *Phytophthora* are allowed to germinate in response to specific metabolites released by the roots (Vancura 1964). Plants can maintain high number of antagonists by providing specific nutrients required for the growth of these organisms to develop resistance against specific pathogens.

Raja et al. (2006) reported another interesting observation that even rhizosphere microflora influence composition of root exudates. They observed that the composition varies after application of bioinoculants, viz. *Azospirillum lipoferum*-A2 204, *Bacillus megaterium* var. *phosphaticum* and *Pseudomonas fluorescens* pf-1, into the soil. It was also supported by rRNA gene profiling and community-level physiological profiles conducted by Miethling et al. (2000). Gomes et al. (2001) reported the alterations in rhizosphere microflora even during senescence.

These studies indicate that the interaction between rhizosphere microflora and plant is not simple and it is the interface which is gaining importance nowadays as a hot spot of plant-microbe interactions, whether it is beneficial or pathogenic. As discussed earlier, this interaction is very specific and influenced by several abiotic and biotic factors including light and temperature, which directly alters the composition of the root exudates and through which metabolic exchange between rhizosphere community and roots is also altered (Berg and Smalla 2009; Harmer 2009). Hence it is the right time to study the alterations in the composition of the root exudates in general and rhizosphere microbial population in particular. If it is not done, the beneficial interaction of specific microbes with specific plant root through metabolites is not going to be established, and it may remain as a major setback in developing microbial formulations for generalized field applications. Sustainable agriculture hence may be achievable only through overall information on plant and its response to various environmental signals in the era of drastic climate change.

14.3 Climate Change and Plant Response

Significant statistical change in distribution of weather patterns over an extension period of time, ranging from decades to millions of years, refers to climate change. It is caused by oceanic circulation, variation in solar radiation, plate tectonics, volcanic eruptions and even human interferences. These changes lead to loss of sea ice, increased in sea level, intense heat waves, extended drought periods and increase in tropical storms. Another important drastic change is the increase in global surface temperature in range of 1.8–3.6 °C by 2100 as a result of increased CO_2 levels derived from both anthropogenic and natural sources (IPCC 2007).

The world is witnessing drastic environmental fluctuations such as local cooling, increased global temperature, shifting of vegetation and extreme weather due to climate change. Is it not influencing the CR, plant physiology and root exudation?

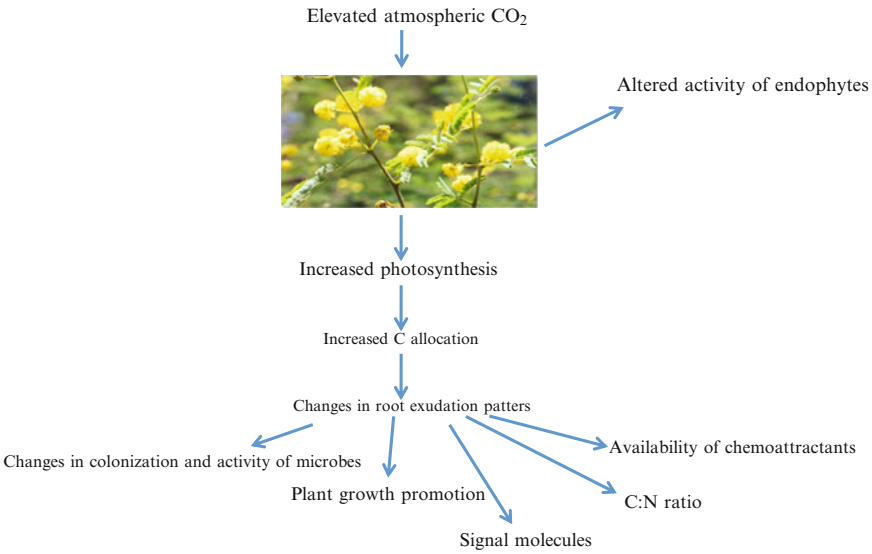

Fig. 14.1 Influence of elevated CO_2 on plant physiology

Scientific reports support the influence of altered environmental conditions on all these plant processes. Especially elevated CO_2 increases carbon allocation to root zone and also alters the composition of the root exudates (Fig. 14.1). It is also influenced by C/N ratio, nutrient availability, elevated temperature and drought (Kandeler et al. 2006; Haase et al. 2008). Hence Drigo et al. (2008) opine that the climate change substantially impacts the diversity and activities of microorganisms leading to impaired beneficial effects of these organisms on plant growth and health. It is the right time to develop a strategy to develop holistic approach involving all the factors influencing the composition of root exudates to favour the growth of the beneficial rhizosphere microflora and antagonists to confer resistance to plant pathogens.

Also, these alterations indirectly alter the nature of soil and hence are known to influence the rhizosphere microbiome. Increase in CO_2 levels, one of the causes of global warming, is known to alter the root exudation patterns which in turn decide the soil food web structure and functioning by increasing the rate of photosynthesis (Haase et al. 2008; Stevnback et al. 2012; Drigo et al. 2013). The world is also witnessing changing weather pattern, for example, change in precipitation level with time is also reported to have significant influence on soil microbial population (Sheik et al. 2011; Castro et al. 2010). Singh et al. (2010) also observed that climate change induced alterations in natural ecosystems and microbial population will have similar changes in the biogeochemical cycles mediated by these microbes. They also reported that there could be addition of new processes to ecosystem due to altered microbial activities which is beneficial or detrimental to plants.

Forchetti et al. (2007) reported the altered plant-associated communities as a result of drought stress. They observed the different subpopulations of endophytes colonizing sunflower grown under drought conditions. Interestingly they could isolate endophytic bacteria with more plant growth-promoting ability in sunflower cultivated under drought than the cultivar grown with sufficient irrigation. Different PGPRs, ecto- or endomycorrhizal taxa, however, are also reported to respond differently to droughts in terms of their patterns of abundance. Examples are from Mediterranean shrubs such as *Pinus muricata*, *Pinus oaxacana*, etc. where drought significantly decreased the microbial colonization process (Compant et al. 2010a, b).

In view of these, proper exploitation of agricultural land and associated beneficial microbes remains as a best choice for climate change resilience farming systems as it supports the proper management of soil, water, biodiversity and local resource usage (Sharma et al. 2014).

14.4 Rhythm in Plants and Its Influence on Plant Processes

Intestines of the animals resemble rhizosphere of the plants in many aspects. Several host functions are regulated by microbes inhabiting these zones. Recently, in animals, feeding and diet of the host were reported to alter intestinal microbiota of humans (Leone et al. 2015) and mice (Liang et al. 2015; Zarrinpar et al. 2014) due to diurnal oscillations. It is also proven to silence the host molecular clock genes leading to gut dysbiosis (Thaiss et al. 2014). Harmer (2009) reported the plant innate ability to estimate time within 24 h period to synchronize biological events via circadian clock. Photosynthetic pattern and other physiological activities of the plant may also alter the rhizosphere microbiome similar to animals.

CR in plants regulates central metabolic pathways of carbon (Kolling et al. 2015), expression of genes, stomatal function and photoperiodism associated with seasonal reproduction (Michael et al. 2003; Yanovsky and Kay 2001). This clock shows variation in response to natural variation both between and within species leading to individual plant performance and fitness (Sulpice et al. 2014; Konmonth-Schultz et al. 2013; Yerushalmi et al. 2011) (Fig. 14.2). It also enhances the adaptations of plant to different environments by regulating physiological and developmental states periodically (Graf et al. 2010; Harmer 2009). Even plant pathogens regulate life cycle in response to diurnally regulated host plant metabolism. On the other hand, plant innate immune response for its fitness is regulated by CR through cellular metabolism (Seo and Mas 2015; Roden and Ingle 2009). Hence it serves as a fascinating adaptive force of life on earth. Obviously, it is endogenous helping in keeping the time of day and night for all living organisms. Photosynthetic organisms record such activity in response to different wavelengths of light as they use light as a source of energy. It is compulsory for them to adapt to daily and seasonal fluctuations of light which serves as a selective force to determine time in a circadian manner (Jarillo et al. 2003).

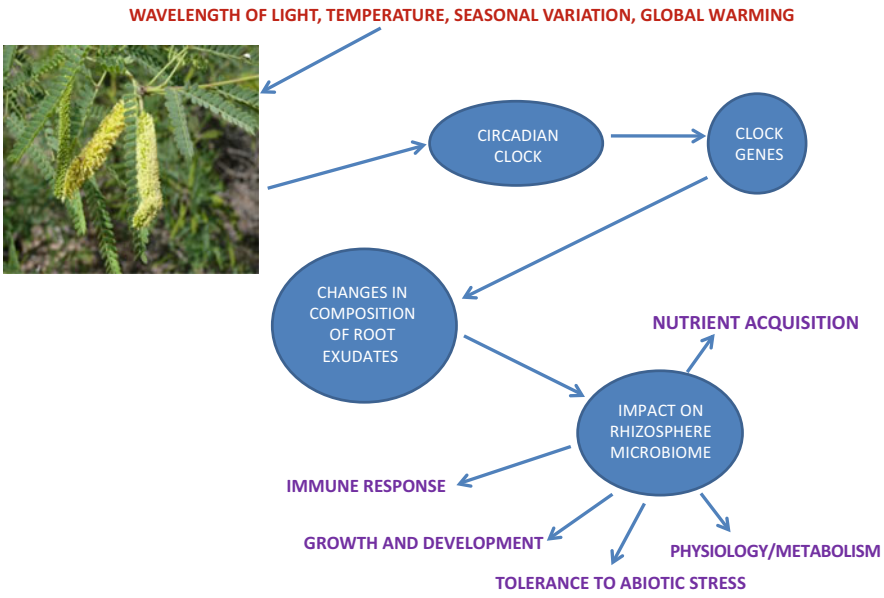

Fig. 14.2 Influence of climatic change on CR and rhizobiome

The list of plant processes regulated by CR is increasing; it is playing a vital role in expression of genes, cytosolic ion concentration, phosphorylation of proteins, movement of chloroplast, stomatal regulation, elongation of hypocotyl, movement of leaf and cotyledon, production of hormones, fitness and responsiveness. Its role in synchronizing developmental processes such as flowering time is well documented. Any change in the clock-associated genes was also reported to alter the photoperiodic control of flowering. Over the years even stem elongation, root pressure, cell membrane potential and CO_2 exchange are also included in the list (Hubbard et al. 2017). Activity of the plants regulated by CR is tabulated in Table 14.1, and it highlights the need of understanding the phenomenon in other plants too.

Johnsson (2007) observed that the rhythmic transpiration reflects rhythmic cellular control by guard and subsidiary cells which regulates assimilation of CO_2 and transpires water vapour by stomatal openings. In 1979, Raschke expressed the need of a model system to understand the regulation of water system in plant in association with photosynthesis and CO_2 transport through stomata. Even before this in 1729, French astronomer De Mairan reported his observation of persistent leaf movements of *Mimosa pudica* for several days even after the plants were placed in darkness. This laid a foundation for plants' accurate timing mechanism to synchronize their physiology with daily environmental fluctuations. It was Bunning (1931) who first identified the plant clock which monitor the duration of day and night. He proved its importance by inducing a mutation in a bean gene involved in clock regulation. Recent studies proved beyond doubt that CR increases ability of plants to anticipate and prepare for changes in the environment that occur during day and night.

Table 14.1 CR in plants and its associated activities

Plant	Activity	References
Mimosa pudica	Daily leaf movements	De Mairan (1729)
Phaseolus coccineus	Periodical movement of leaf	Bunning (1931)
Pea	Influence of light-harvesting chlorophyll a/b binding protein (CAB), small subunit of ribulose-1,5-bisphosphate carboxylase/ oxygenase and an early light-induced protein	Kloppstech (1985)
Wheat	Transcription rate for the Cab-1 gene	Nagy et al. (1988)
Tamarindus indica and *Mimosa pudica*	Rhythmic movement of leaf in legumes driven by turgor-induced expansion and contraction of the pulvinus	Kim et al. (1993)
Arabidopsis thaliana	Elongation rate of the abaxial and adaxial cells of the petiole	Engelmann and Johnsson (1998)
	Rate of hypocotyl elongation	Dowson-Day and Millar (1999)
	Elongation rate of inflorescence stem	Jouve et al. (1998)
	Transcription rate and transcript accumulation of *Arabidopsis* LHCB	Millar and Kay (1991)
	Other genes	McClung and Kay (1994)
	A short fragment of the *Arabidopsis* LHCB13(CAB2) promoter	Millar et al. (1992)
	Multiple metabolic pathways	Schaffer et al. (2001), Harmer et al. (2000)
	35% of the transcriptome	Michael and McClung (2003)
	Sugar metabolism	Blasing et al. (2005)
	Ability to respond to abiotic stresses such as cold	Fowler et al. (2005)
	Rates of chlorophyll production and carbon fixation	Dodd et al. (2005), Green et al. (2002)
	mRNA abundance of the CAT2 and CAT3 catalase genes	Zhong and McClung (1996)
	Glycine-rich RNA-binding protein (ATGRP7/CCR2) and a germin-like protein (AtGER3)	Strayer et al. (2000), Staiger and Apel (1999), Staiger et al. (1999)
	mRNA abundance of nitrate reductase	Pilgrim et al. (1993)
	RCA gene	Liu et al. (1996)
	Genes encoding phytochrome B (PHYB), cryptochrome 1 (CRY1), cryptochrome 2 (CRY2) and phototropin (NPH1)	Harmer et al. (2000)
	Genes CRY1 and CRY2 coding for homologs of the blue light photoreceptor	Dunlap (1999)
	SPA1 and RPT2 genes involved in downstream mediators of phototransduction pathways	Harmer et al. (2000)
	Desaturases involved in lipid modifications	Harmer et al. (2000)

(continued)

Table 14.1 (continued)

Plant	Activity	References
	Auxin efflux carriers PIN3 and PIN7	Taiz and Zeiger (1998)
	Flowering induction by photoperiodism	Samach and Coupland (2000)
	Twenty-three genes encoding enzymes in the phenylpropanoid biosynthetic pathway were coordinately regulated to peak before dawn at CT20	Landry et al. (1995), Li et al. (1993)
	Community structure of the rhizosphere during drought	Zolla et al. (2013)
	Increase the growth and fitness through stress signalling	Muller et al. (2014)
Tomato	Growth improvement	Hillman (1956)
	Sucrose phosphate synthase activity	Jones and Ort (1997)
	LHCA genes	Kellmann et al. (1999)
Beans	Regulation of stomatal opening and gas exchange along with Calvin cycle reactions	Hennessey and Field (1991)
Sorghum	Levels of gibberellic acid	Foster and Morgan (1995)
	ACC oxidase activity and increasing the availability of mRNA coding for 1-aminocyclopropane-1-carboxylic acid (ACC) transcribed by $SbACO_2$ gene	Finlayson et al. (1999)
Robinia pseudoacacia	Leaflet movement	Gomez and Simon (1995)
Angiosperms	LHCB mRNA abundance	Piechulla (1999), Fejes and Nagy (1998)
CAM plants	Phosphorylation and dephosphorylation of PEPc	Nimmo (2000)
Many plants	Regulates the composition of the root exudates	Hubbard et al. (2017), Greenham and McClung (2015)
	Plant stress response	Guadagno et al. (2018)

14.5 Mechanism of CR in Plants in Brief

Mechanism of CR regulated by circadian clock is well established in *Arabidopsis*; the clock was reported to consist of a series of transcriptionally and post-transcriptionally regulated intertwined feedback loops (Harmer 2009). Even though it is proved in this plant, its existence in other plant species needs to be evaluated (Song et al. 2010). The circadian clock has been found to influence a variety of metabolic functions in the plant including chlorophyll biosynthesis, transport photosystems, starch synthesis and degradation and nitrogen and sulphur assimilation. The clock timing was found to be altered to different concentrations of several metabolites such as glutamate, nitrate, glutamine and sucrose (Gutierrez et al. 2008; Knight et al. 2008). However, due to differences in methodology, these results

are sometimes inconsistent across studies, highlighting a need to consider photoperiod duration and the time of sample collection when describing results.

Advances in the identification and characterization of components of the plant circadian system have been made largely through genetic studies in *Arabidopsis*. The number of genes regulating *Arabidopsis* circadian clock is approximately 20, in contrast to smaller number of genes regulating the circadian clock of insects, mammals and fungi. As in the mammalian circadian clock, several clock-associated genes from *Arabidopsis* have overlapping functions. The complexity of phototransduction pathways in plants may contribute to the large number of genes implicated in clock function (Jarillo et al. 2003).

As in other organisms, the circadian system in plants consists of input pathways that provide temporal information from the environment to the clock, the central oscillator mechanism itself and a set of pathways through which the temporal information provided by the clock is used to generate overt rhythms in several processes. During the course of evolution, photoreceptors of plant have developed capability to detect light over a large range of wavelengths and transduce the signal-specific genes regulating the clock. There are three main classes: the phytochromes, having the ability to absorb the red and far-red region of electromagnetic spectrum, and the cryptochromes and phototropins which absorb blue and UV A region of spectrum (Jarillo et al. 2003).

14.6 Plant Rhythm and Its Influence on Rhizosphere Microflora

Waldon et al. proved that the rhizobacteria respond and adapt to increased temperature which in turn regulates the CR. They could isolate rhizobia from nodules of desert woody legume *Prosopis glandulosa* which is better adapted to 36 °C compared to other strains grown in normal conditions. This proves that the bacteria colonizing distinct soil sites respond differently to certain environmental conditions. Increase in temperature from 10 to 30 °C will decrease the ability of an endophyte *Burkholderia phytofirmans* to colonize tomato rhizosphere (Pillay and Nowak 1997). It is also reported that bacterial endophytic populations, which colonize plant internal tissues such as stems, roots, leaves, shoots as well as flowers, fruits and seeds, may be affected in a similar manner (Compant et al. 2005, 2008, 2010a, b; Hallmann 2001). Even mycorrhizal hypha reduces its growth in response to elevated CO_2 concentrations (Madhu and Hatfield 2013).

Composition and abundance of rhizosphere populations associated with strawberry, potato and oil seed was reported to change over the field season, and this alteration could be because of alternation in time.

Daniel et al. (2004) assessed cycling dynamics in *A. thaliana* diel cycle associated with exposure to dark and light periods, and they involved study associated with acyclic *Arabidopsis* line having cca1 gene ectopically overexpressed and also

another plant *Brachypodium distachyon* to prove any alterations in the rhizosphere community among species, wild types and mutants. The data obtained by them completely disproved the observations of Bulgarelli et al. (2012) and suggested that rhizosphere microflora is highly dynamic and are influenced by biotic and abiotic factors along with circadian clocks. This served as clear-cut evidence that CR plays a vital role in deciding both composition of root exudates and also the diversity of rhizosphere microbial community. Even recent reports involving next-generation sequencing of the 16S rRNA gene, soil organic matter composition in the rhizosphere characterized by high-resolution mass spectrometry and 21T Fourier transform ion cyclotron resonance mass spectrometry support this observation (Staley et al. 2017).

These reports suggest the possible role of circadian clock on the rhizosphere community. The timing of bacterial cycling in relation to that of *Arabidopsis* further suggests that diurnal dynamics influence microbial association with plant carbon metabolism and exchange. In view of this, Grayston et al. (2001), Staley et al. (2017) and Dunfield and Germida (2003) suggest that previous studies done without relevance to time of day may need to be reevaluated with regard to the impact of diurnal cycles on the rhizosphere microbial community. Along with this, they also suggest that caution should be taken when conclusions are drawn about root-associated microbial community structure based on the results of a single time point.

14.7 Conclusions and Outlook

Plant-rhizosphere microbiome interactions are highly relevant because rhizosphere microflora is reported to strongly influence plant fitness and biomass which in turn inform evolutionary studies of adaptation, agronomic practices and conservation much needed for sustainable agriculture. Climate change and global warming are the major threats to living organisms, resulting in alterations of normal process of evolution. It is a forced artificial evolution; inevitably all the organisms have to respond and adopt. Especially elevated CO_2 and pattern of light radiation are affecting several natural phenomena including plant-microbe interactions in rhizosphere. If this harmony is not understood and integrated with the bioinoculant performance in the field, the desired effect of bioinoculants on plant growth is naturally affected. It is the right time to evaluate the efficacy of all bioinoculants with special reference to individual plant CR responses.

Genes regulating CR are highly sensitive and regulated by several environmental parameters. Alterations in CR are reported to alter the rhizosphere community structure due to changing pattern of diurnal fluxes of carbon, water or nutrients from plant roots. Clock misfunction would bring in differences in this structure in general and alterations in rare taxa in particular leading to differences in community function required for plant performances. It is the right time to understand the clock genes associated with plants, after which rhizosphere engineering or suitable microbial consortia or bioinoculants can be developed to increase the plant processes associated with plant health, growth and yield.

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Chapter 15
Actinobacteria and Their Role as Plant Probiotics

Esther Menendez and Lorena Carro

Abstract *Actinobacteria* is one of the largest phyla within the domain *Bacteria.* This phylum comprises more than 400 genera heterogeneously distributed in up to 50 families, 20 orders and 6 classes, being composed with very diverse groups of microorganisms. Members included within this phylum were recovered from a wide range of aquatic and terrestrial environments and also from a huge number of higher organisms, including plants. *Actinobacteria* inhabiting soils and plants are well known as producers of bioactive molecules and as biocontrol agents, possessing antimicrobial activities mostly against pathogenic fungi and/or bacteria. Moreover, some of them have the capacity to exert beneficial effects on plant growth and development via different plant growth-promoting mechanisms, i.e., phytohormones biosynthesis, siderophore production, and phosphate solubilization, among others. The available genomic data revealed that members belonging to this phylum have a huge potential as Plant Probiotic *Actinobacteria*. A plethora of studies reported the isolation and identification of plant endophytic actinobacteria possessing those features and also their performance under controlled conditions. However, few studies show the effects of the inoculation of these actinobacteria on real field conditions. In this chapter, we will provide an overview of the available data on the *Actinobacteria* displaying plant growth-promoting features, particularly in the ones that already had applications in agriculture. Together with a correct taxonomic classification, we will present evidence that the Plant Probiotic *Actinobacteria* should be considered as a source of bacterial candidates that will be important for a future sustainable agriculture.

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15.1 Introduction

The *Actinobacteria* is a phylum of Gram-positive bacteria and one of the largest taxonomic units within the domain *Bacteria* (Barka et al. 2015). The majority of the *Actinobacteria* are free-living organisms, being well known for their ubiquitous presence in soil and aquatic habitats and their contribution to organic material recycling. Between these bacteria, we found indeed some of the most well-known producers of antibiotics, exemplified by the genera *Streptomyces, Micromonospora* and *Actinomadura* (Raja and Prabakarana 2011). The *Actinobacteria* establishes close relationships with their environment and the organisms of their surroundings, with key molecular exchanges that allow their coexistence. Within the phylum, we found pathogenic bacteria for humans (*Mycobacteria*, *Nocardia*, or *Tropheryma*), for plants (*Streptomyces scabiei*, which cause scab in potatoes), and for animals (*Corynebacterium, Mycobacteria*). However, beneficial actinobacteria are also found for all these organisms, *Bifidobacterium* being well known for their implication in human and animal health, *Pseudonocardia* for the protection of ant's gardens, or *Frankia* for their symbiotic relationship with actinorhizal plants. New studies on bacterial communities have shown that *Actinobacteria* composition is related to plant health (Wang et al. 2017), inducing a new interest in the study of *Actinobacteria*'s role as plant endophytes. This is clearly remarkable in the number of new species described from plant tissues in the last 10 years (Table 15.1), with more than ten new species published on the *International Journal of Systematic and Evolutionary Microbiology* only in the last 3 months (August–October 2018). High numbers of actinobacterial taxa found in healthy plant tissues have compelled us to think that these microorganisms have the capacity to improve plant health and could act as plant probiotics.

The FAO/WHO Expert Consultation Report defines Probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill et al. 2014). Consequently, plant probiotics should be defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the plant". Between the actinobacteria, *Frankia* genus could be considered the first and most studied plant probiotic actinobacteria. This genus has been studied for more than a century due to its ability to fix atmospheric nitrogen, which is exchanged with the plants with which it establishes symbioses (Beijerinck 1901; Carro et al. 2015). Presence of *Frankia* strains has been also related to improvements in stress tolerance, as high salinity concentration (Ngom et al. 2016) or soil degradation (Diagne et al. 2013). Nevertheless, many other genera have been included in the list of Plant Probiotic *Actinobacteria* in the last years, exemplified by *Streptomyces*, which have been shown to improve plant vegetative growth and to induce and contribute to plant defense from pathogen attacks (Conn et al. 2008); by *Micromonospora*, which are able to improve plant growth and the tripartite symbioses with rhizobia in legumes (Carro 2010; Martínez-Hidalgo et al. 2014); or by *Arthrobacter*, which are able to increase iron-stress resistance (Sharma et al. 2016). Most of the actinobacteria tested as plant probiotic bacteria have been directly

Table 15.1 A selection of new *Actinobacteria* species described from plant tissues in the last 10 years

Genus	Species	Plant	References
Actinocorallia	*A. populi*	*Populus adenopoda*	Li et al. (2018c)
Actinomadura	*A. barringtoniae*	*Barringtonia acutangula*	Rachniyom et al. (2018)
Actinomycetospora	*A. callitridis*	*Pinus sp.*	Kaewkla and Franco (2018)
	A. endophytica	*Podochilus microphyllus*	Sakdapetsiri et al. (2018)
Amnibacterium	*A. endophyticum*	*Aegiceras corniculatum*	Li et al. (2018d)
Arthrobacter	*A. endophyticus*	*Salsola affinis*	Wang et al. (2015)
Brachybacterium	*B. endophyticum*	*Scutellaria baicalensis*	Tuo et al. (2018)
Frankia	*F. canadensis*	*Alnus incana*	Normand et al. (2018)
	F. torreyi	*Comptonia peregrina*	Nouioui et al. (2018a)
	F. irregularis	*Casuarina equisetifolia*	Nouioui et al. (2018b)
Glycomyces	*G. anabasis*	*Anabasis aphylla*	Zhang et al. (2018)
Jiangella	*J. alba*	*Maytenus austroyunnanensis*	Qin et al. (2009)
Kocuria	*K. arsenatis*	*Prosopis laegivata*	Roman-Ponce et al. (2016)
Kribella	*K. podocarpi*	*Podocarpus latifolius*	Curtis et al. (2018)
Marmoricola	*M. endophyticus*	*Thespesia populnea*	Jiang et al. (2017)
Micromonospora	*M. luetiviridens* *M. luteifusca* *M. noduli* *M. phytophila* *M. pisi* *M. ureilytica* *M. vinacea*	*Pisum sativum*	Garcia et al. (2010), Carro et al. (2016a, b, 2018b), Carro and Nouiuoi (2017)
	M. zeae	*Zea mays*	Shen et al. (2014)
	M. costi	*Costus speciosus*	Thawai (2015)
	M. globae	*Globba winitii*	Kuncharoen et al. (2018)
	M. oryzae	*Oryza sativa*	Kittiwongwattana et al. (2015)
	M. parathelypteridis	*Parathelypteris beddomei*	Zhao et al. (2017)
	M. sonneratiae	*Sonneratia apetala*	Li et al. (2013)
	M. taraxaci	*Taraxacum mongolicum*	Zhao et al. (2014)
	M. terminaliae	*Terminalia mucronata*	Kaewkla et al. (2017)
	M. tulbaghiae	*Tulbaghia violacea*	Kirby and Meyers (2010)
	M. violae	*Viola philippica*	Zhang et al. (2014)

(continued)

Table 15.1 (continued)

Genus	Species	Plant	References
Naumannella	*N. huperziae*	*Huperzia serrata*	Sun et al. (2017)
Nesterenkonia	*N. endophytica*	*Glycyrrhiza uralensis*	Li et al. (2018a)
Nocardioides	*Z. zeicaulis*	*Zea mays*	Kämpfer et al. (2016)
Phytoactinopolyspora	*P. endophytica*	*Glycyrrhiza uralensis*	Li et al. (2015)
Solirubrobacter	*S. phytolaccae*	*Phytolacca acinosa*	Wei et al. (2014)
Streptomyces	*S. dioscori*	*Dioscorea bulbifera*	Wang et al. (2018a)
	S. alni	*Alnus nepalensis*	Liu et al. (2009)
	S. populi	*Populus adenopoda*	Wang et al. (2018b)
	S. geranii	*Geranium carolinianum*	Li et al. (2018b)
	S. ginkgonis	*Ginkgo biloba*	Yan et al. (2018)

inoculated on plants, in most of the cases to evaluate the protection against some pathogenic microorganisms. However, contrary to other bacteria, many of them have not been tested for the general characteristic evaluated to determine a plant growth-promoting bacteria (PGPB): nitrogen fixation capacity, phosphate solubilization, production of plant hormones (IAA, ACC desaminase), etc. In this chapter, an overview of *Actinobacteria* known as plant growth promoters will be given, with emphasis on their taxonomic position and their use in agriculture.

15.2 Current Taxonomy of *Actinobacteria*: Classic and NGS-Based Classification

The taxonomic status of a strain, according to the polyphasic taxonomy, is determined by both phenotypic and genotypic characterization. A combination of chemotaxonomic analysis and other phenotypic features (tolerance tests, enzyme production, ability to metabolize carbon and nitrogen sources) together with other genetic traits of the taxon (16S rRNA phylogeny, GC content, DNA–DNA hybridization) was classically used for new actinobacteria species descriptions (Carro and Nouiuoi 2017). The use of multilocus sequences analyses (MLSA) greatly improved the relationships between these new isolates (Carro et al. 2012) and the upstream taxa, as exemplified by the analysis done by Adekambi et al. (2011). Lately, the new sequencing technologies developed and its availability for the vast majority of researchers have introduced new methods for phylogenomic reconstructions, allowing a better classification regarding higher taxa never seen before. Specifically,

the works developed by Sen et al. (2014) for the class *Actinobacteria* and Nouioui et al. (2018c) for the whole phylum have greatly rearranged their respective status.

The phylum *Actinobacteria* was first described by Cavalier-Smith (2002) and include six classes: *Acidimicrobiia* (Norris 2012), *Actinobacteria* (Stackebrandt et al. 1997), *Coriobacteriia* (König 2012), *Nitriliruptoria* (Ludwig et al. 2012), *Rubrobacteria* (Suzuki 2012) and *Thermoleophilia* (Suzuki and Whitman 2012). From these classes, 450 genera are unequally distributed, the majority of them (418) being within the class *Actinobacteria*. Endophytic bacteria have been described only in the class *Actinobacteria* and in the class *Rubrobacteria*. This latter class just comprises one plant-associated species, *Solirubrobacter phytolaccae* (Wei et al. 2014).

After last reclassification based of whole-genome sequences (Nouioui et al. 2018c), the class *Actinobacteria* comprises 20 orders: *Acidothermales*, *Actinomycetales, Bifidobacteriales, Catenulisporales, Corynebacteriales, Cryptosporangiales, Frankiales, Geodermatophilales, Glycomycetales, Jiangellales, Kineosporiales, Micrococcales, Micromonosporales, Nakamurellales, Nitriliruptorales, Propionibacteriales, Pseudonocardiales, Sporichthyales, Streptomycetales* and *Streptosporangiales*. Most of these orders contain endophytic strains; only in six of them, no plant related strains have been isolated (*Acidothermales*, *Actinomycetales, Bifidobacteriales, Catenulisporales, Nitriliruptorales* and *Sporichthyales*). Strains belonging to those orders are related to human samples or extreme habitats. All the other orders contain genera in which some or most of their species have been described as plant endophytes (isolated from within the plant tissues). Among them, the most important genera of plant pathogens are mainly found in the order *Corynebacteriales*, including *Corynebacterium*, *Nocardia*, and *Rhodococcus*; in the order *Micrococcales*, including *Clavibacter*, *Curtobacterium*, *Leifsonia*, and *Rathayibacter*; and in the order *Streptomycetales*, including some species of the genus *Streptomyces*, such as the phytotoxin-producer *S. scabies* (Lozi 1994). Although several species of these genera have been found to be pathogenic, in most cases other species within the same genus have been described as nonpathogenic endophytes or even plant growth-promoting bacteria, i.e., the strain BMG51109 of *Nocardia* (Ghodhbane-Gtari et al. 2018) or the strain SK68 of *Streptomyces* (Damodharan et al. 2018). Although not an exact distribution between pathogen and PGPB could be established between the genera of *Actinobacteria*, some relationships could be observed mainly due to these double functions of some genera (Fig. 15.1). Most of the pathogens appear in genera from the family *Microbacteriaceae* of the order *Micrococcales*, while *Frankiales*, *Jiangellales* or *Micromonosporales* include mainly PGPB or asymptomatic endophytic strains.

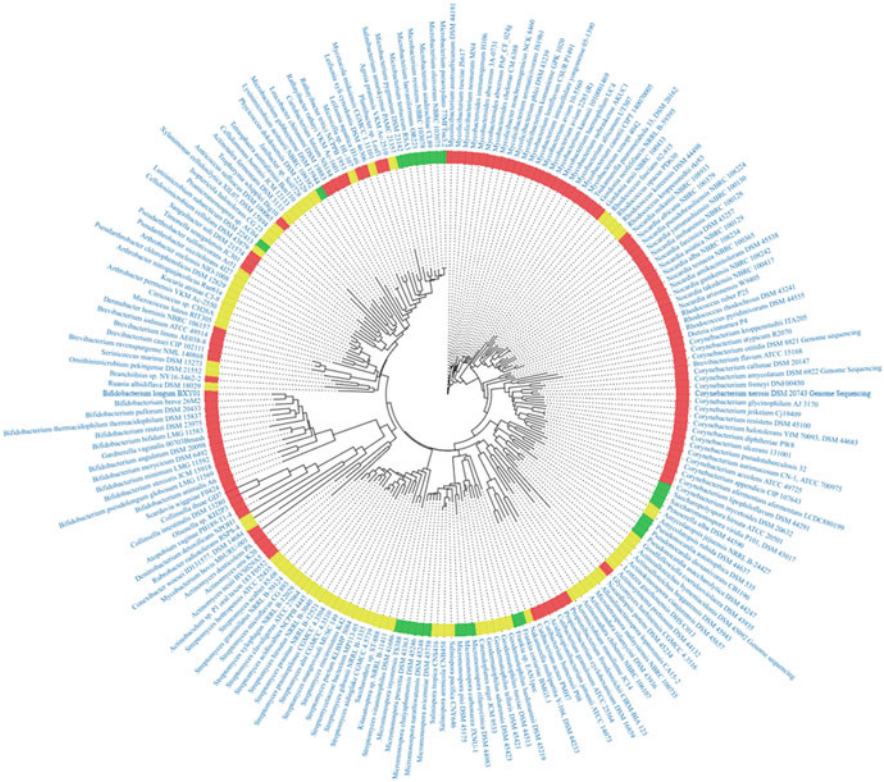

Fig. 15.1 Phylogenetic distance tree of selected *Actinobacteria* genus generated by distance tree tool of IMG 3.2. Groups are based on most abundant species found for a genus as beneficial endophytes (green), clinical samples, plant pathogens (red) and other sources, including soil and rhizosphere (yellow)

15.3 Genomes Data Mining of PGP Traits on *Actinobacteria*

New technologies have encouraged the research of genes from plant endophytes related to their abilities for plant growth promotion, generating full sets of candidate genes to be further analyzed due to their potential. Trujillo et al. (2014) and Carro et al. (2018a) identified some of these genes in several species of the genus *Micromonospora*, including genes related to plant hormones production, phosphate solubilization, or siderophores production, among the most common ones, and also genes related to the biosynthesis of trehalases or other degrading enzymes (amylases, cellulases, chitinases, pectinases, and xylanases), among the most interesting ones for biotechnological applications.

Most of the plant endophyte genomes have been shown to harbor a whole set of genes for central carbohydrate metabolism that could be related to the utilization of root exudates as energy source (Kang et al. 2016). Other frequently found genes

include the ones related to nutrient deficiencies, oxidative stress, drought tolerance, as well as secretion mechanisms and signaling (Trujillo et al. 2015). Genes related to biosynthesis pathways of plant growth modulators, such as auxins and cytokinins, are generally found in most plant probiotic bacteria, which combined with others related to degradation of ethylene through 1-amino-cyclopropane-1-carboxylic acid deaminase generate further improvements of plant status under stress conditions. Some genes that contribute to efficient colonization and competitiveness are also important in first steps of interactions (Francis et al. 2016).

Genome analysis has also put in evidence the importance of actinobacteria as secondary metabolites producers and its possible use in agriculture for biological control. The production of several peptides and antibiotics observed in actinobacteria probiotics could be used to defend the host plant against pathogens (Paterson et al. 2017; Remali et al. 2017). The mechanism of biocontrol also involved induction of plant defense response by, for example, the upregulation of PR10a, NPR1, PAL, and LOX2 genes in colonized plants by *Streptomyces* (Patel et al. 2018).

15.4 Applications of Plant Probiotic *Actinobacteria* in Agriculture

The members of the phylum *Actinobacteria* have a huge and well-appreciated range of biotechnological applications. As we have seen before, the metabolic potential and the biological significance of several groups of actinobacteria are well known, which are of paramount importance in the biotech industries, mostly related to biomedicine (Golinska et al. 2015; Barka et al. 2015; Passari et al. 2017). Actinobacteria associated with plants, namely endophytic actinobacteria, have been studied for its application in agriculture (Palaniyandi et al. 2013), mainly in biocontrol and suppression of plant diseases and, in some cases, in plant growth promotion (Ganapathy and Natesan 2018; Singh and Dubey 2018) (Table 15.2). However, studies showing the effects of Plant Probiotic *Actinobacteria* on crop yields are still scarce (Viaene et al. 2016; Araujo et al. 2017). Some of those works are enumerated in Table 15.3.

Among *Actinobacteria*, the streptomycetes are one of the most abundant bacterial groups in soils, accounting for up to 10% of the total microbiome (Janssen 2006). The genus *Streptomyces* is the most studied genus within the phylum *Actinobacteria*, not only due to its uncountable properties but also because of the versatility of the species within this genus (Viaene et al. 2016).

The vast majority of the studies about the potential of *Streptomyces* strains as plant growth promoters and biocontrollers present effects under in vitro controlled conditions due to its innate ability to produce secondary metabolites (including antibiotic and antimicrobial compounds). Strains belonging to different species of the genus *Streptomyces* isolated from wheat rhizosphere and root endosphere

Table 15.2 List of genera from the phylum *Actinobacteria* with confirmed plant growth promotion potential

Actinobacteria	Plant host	References
Actinoplanes	*Cucumis sativus*	El-Tarabily et al. (2009)
Agromyces	*Oryza sativa*	Bal et al. (2013)
Arthrobacter	*Triticum aestivum*	Upadhyay et al. (2012)
	Brassica *Hordeum vulgare* Weed	Kim et al. (2011)
Curtobacterium	Weeds	Kim et al. (2011)
	Hordeum vulgare	Cardinale et al. (2015)
Frankia	*Atriplex cordobensis* *Colletia hystrix* *Trevoa trinervis* *Talguenea quinquenervia* *Retanilla ephedra*	Fabri et al. (1996)
Kocuria	*Vitis vinifera*	Salomon et al. (2016)
	Prosopis laegivata	Roman-Ponce et al. (2016)
Microbacterium	*Hordeum vulgare*	Cardinale et al. (2015)
	Oryza sativa	Bal et al. (2013), Banik et al. (2016)
	Saccharum officinarum	Lin et al. (2012)
	Arabidopsis thaliana	Schwachtje et al. (2012)
	Vitis vinifera	Salomon et al. (2016)
	Brassica Weeds	Kim et al. (2011)
Microbispora	*Pisum sativum*	Misk and Franco (2011)
Micromonospora	*Medicago sativa*	Martínez-Hidalgo et al. (2014)
	Lupinus angustifolia	Trujillo et al. (2010, 2015)
	Discaria trinervis	Solans (2007)
Nocardia	*Casuarina glauca*	Ghodhbane-Gtari et al. (2018)
Streptomyces	*Aristida pungens* *Cleome arabica* *Solanum nigrum* *Panicum turgidum* *Astragallus armatus* *Peganum harmala* *Hammada scoparia* *Euphorbia helioscopia*	Goudjal et al. (2014)
	Triticum aestivum *Solanum lycopersicum*	Anwar et al. (2016)
	Triticum aestivum	Jog et al. (2014)
	Discaria trinervis	Solans (2007)
Rhodococcus	*Oryza sativa*	Bertani et al. (2016)
	Hordeum vulgare Weeds	Kim et al. (2011)

Table 15.3 Plant Probiotic *Actinobacteria* with known effects on plant performance and development in field and greenhouse conditions

Actinobacterial taxa	Origin	PGP traits	Plant host and cultivation conditions	Effects caused on crops	References
Streptomyces	Chickpea rhizosphere	Antifungal activity Hydrolytic enzymes IAA HCN Siderophores	Chickpea/ Greenhouse and field	Increase of plant biomass and grain yield	Alekhya and Gopalakrishnan (2017)
Micromonospora	Nodules of naturally-occurring *Medicago sativa* plants	Hydrolytic enzymes IAA	Alfalfa/ Greenhouse	Increases in plant biomass and essential microelements	Martínez-Hidalgo et al. (2014)
Arthrobacter sp	Rapeseed roots	P solubilization AHL-like molecules	Rapeseed/ Field	Higher yields and weight per 1000 seeds	Valetti et al. (2018)
Streptomyces *Microbispora*	Whole roots of *Lens esculentus*, *Cicer arietinum*, *Pisum sativum*, *Vicia faba* and *Triticum vulgare*	Antimicrobial activity Siderophores P solubilization HCN	Chickpea/ Greenhouse	Bioprotection against *Phytophtora* and improved plant development	Misk and Franco (2011)
Actinoplanes campanulatus, *Micromonospora chalcea*, *Streptomyces spiralis*	Cucumber roots	Antagonistic activities Plant growth regulators (PGRs)	Cucumber/ Greenhouse	Reduced damping-off disease of cucumber seedlings (Table 3) and root and crown rots of mature cucumber, reduced damping-off disease of cucumber seedlings (Table 3) and root and crown rots of mature cucumber Reduced disease incidence Increased plant development and production	El-Tarabily et al. (2009)

(continued)

Table 15.3 (continued)

Actinobacterial taxa	Origin	PGP traits	Plant host and cultivation conditions	Effects caused on crops	References
Streptomyces griseus/ Micromonospora aurantiaca related strains	Mine soil	Antimicrobial activity, Siderophores P solubilization IAA	Wheat/ Greenhouse	Increase dry weight of wheat plants infected with *Pythium*	Hamdali et al. (2008a, b)
Arthrobacter woluwensis	Rhizospheric soil	IAA ABA Siderophores Halotolerance Organic acids	Soybean/ Greenhouse	Increase on plant length and biomass and higher levels of chlorophyll (SPAD) under saline stress	Khan et al. (2018)
Streptomyces spp.	*Vitis vinifera* rhizosphere/ endosphere	Antifungal activity	Grapevine/ Field	Reduced disease in grafted *Vitis* plants	Alvarez-Pérez et al. (2017)
Streptomyces	Wheat anthers	Antifungal activity	Wheat/ Greenhouse and field	Reduction of disease incidence	Palazzini et al. (2007, 2017)
Arthrobacter spp.	Burned holm oak rhizosphere	IAA Hydrolytic enzymes Siderophores	Alfalfa and pepper/ Greenhouse	Increase of plant biomass	Fernández-González et al. (2017)
Streptomyces spp.	Roots of native plants from India	IAA Siderophore Ammonia production	Wheat/Field	Increase of grain yield and plant biomass	Yandigeri et al. (2012)

showed several activities, such as chitinase and phytase activities, as well as phosphorous solubilization. These strains are also able to produce different compounds, such as IAA, siderophores, organic acids and antifungal metabolites (Jog et al. 2014). Wheat plants in growth chamber (lab-controlled conditions) inoculated with *Streptomyces* strains showed higher plant biomass, number of lateral roots and branches, and nutritional content (essential elements) in comparison with uninoculated control plants (Jog et al. 2014).

In tomato plants, Palaniyandi et al. (2014) isolated a *Streptomyces* strain, called PGPA39, from an agricultural soil, which possess ACC deaminase, biosynthesize IAA and solubilize phosphate. This strain was also halotolerant. Spores of this strain were mixed in sterilized soil and sown with tomato plants, alleviating stress in those plants and showing higher plant biomass and root development than that of noninoculated salt-stressed tomato control plants.

As shown, there are several species and strains belonging to this genus that have plant growth potential, but there are few reports regarding studies showing improvements in crop yields under real field conditions (Viaene et al. 2016; Araujo et al. 2017) (Table 15.3).

Alekhya and Gopalakrishnan (2017) performed a screening of actinobacteria isolated from chickpea rhizosphere to find strains with antagonistic potential. Seven strains belonging to different species of the genus *Streptomyces* and displaying several PGP traits (broad spectrum antifungal activity, hydrolytic enzymes, IAA and HCN biosynthesis and siderophore production) were selected and tested under greenhouse conditions and also, in a field assay. Under greenhouse conditions, inoculated chickpea plants exhibit an increase in shoot weight (up to 84%), root weight (up to 57%), pod number (up to 102%) and pod weight (up to 84%). At harvest time, field assays also showed better performance of chickpea plants inoculated with the selected *Streptomyces* strains: seed number (up to 22%), stover yield (up to 86%), grain yield (up to 17%) and total dry matter (up to 51%).

Studies on grafted *Vitis vinifera* plants showed also the beneficial effects of *Streptomyces* strains under field real conditions (Alvarez-Pérez et al. 2017). In this work, several actinobacterial strains were isolated from young grapevine plants rhizosphere and endosphere. The isolates displayed in vitro antifungal activity, which was confirmed in field assays conducted in three experimental open-root field nurseries of grafted plants. The presence of phytopathogenic fungi affecting grafted *Vitis* plants was dramatically reduced (Alvarez-Pérez et al. 2017).

In cereals, there are also some examples of studies confirming the PGP potential of *Streptomyces* strains under field conditions. Yandigeri et al. (2012) isolated several *Streptomyces* strains from roots of 5 different native plants from India. Those isolates produce IAA, ammonia and siderophores. Three of these strains were tested in wheat plants in a field assay under drought conditions. Their findings revealed that the strains were drought-tolerant and improved seedling vigor after inoculation. At harvest time, wheat plants had higher biomass and there was a significative increase in grain yields.

With the aim of identifying good biocontrol agents, Palazzini and colleagues isolated several strains from wheat anthers and later identified one of them as a good

biocontrol strain, *Streptomyces* sp RC87B (Palazzini et al. 2007). This strain presented antifungal activities, particularly against *Fusarium graminearum sensu stricto* under in vitro and in a greenhouse assay using a wheat cultivar that is susceptible to *Fusarium* infections. Ten years later, a study using the same strains confirmed that this potential can also be translated to field conditions. Wheat susceptible to *Fusarium* infection experienced a reduction of disease incidence (Palazzini et al. 2017).

Not only *Streptomyces* but also other actinobacterial genera, i.e., *Micromonospora, Microbispora, Microbacterium, Actinoplanes*, or *Arthrobacter*, were also tested alone or in combination with other bacterial members such as rhizobia or other actinobacteria, mostly under lab-controlled conditions or greenhouse assays, even that there are some of these studies that involved field trials.

Co-inoculation of leguminous plants with actinobacteria and rhizobial strains produced beneficial effects in those plants, increasing the nodule number, symbiotic efficiency and the plant biomass in most of the cases. *Micromonospora* strains, able to produce hydrolytic enzymes and IAA, alone and in combination with *Ensifer (Sinorhizobium)* strains produced significative increases in shoot and root dry weights and shoot C, N, P and K elements in *Medicago sativa* plants under in vitro and greenhouse conditions (Martínez-Hidalgo et al. 2014).

A study involving a set of field trials with soybean plants showed that the co-inoculation of *Bradyrhizobium japonicum* USDA110 with a strain of *Streptomyces* leads to an enhancement of nitrogen fixation and the production of a higher plant biomass and grain yield (Soe et al. 2012).

Misk and Franco (2011) co-inoculated two strains of *Mesorhizobium ciceri* and different biocontrol-tested *Streptomyces* spp. on chickpea plants under greenhouse conditions. Some of those *Streptomyces* strains suppressed the incidence of *Phytophthora* root rot disease and, in combination with both mesorhizobial strains, also enhanced vegetative growth. Interestingly, these authors also identified a non-streptomycete strain belonging to the genus *Microbispora*, which showed biocontrol and PGP traits; sadly, this strain was not tested in the greenhouse assays.

Interestingly, there is a study reporting the beneficial effects of a triple inoculation of three actinobacterial strains, closely related to the species *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis*, on cucumber plants affected with damping-off disease produced by the phytopathogenic oomycete *Pythium*. The three isolates produced the highest level of growth promotion when together (El-Tarabily et al. 2009). Moreover, all three actinomycete strains, alone and in combination, significantly increased root and shoot production in the presence or absence of *Pythium aphanidermatum* in comparison with the untreated control.

Arthrobacter is another genus that is cited frequently as potential plant growth promoter and as bioremediation agent in agriculture. Khan et al. (2018) identified a rhizospheric strain of *Arthrobacter woluwensis*, strain AK1, which showed ABA and IAA production under saline conditions. This halotolerant strain mitigated salt stress and promoted rice growth under in vitro conditions and also promoted soybean growth under greenhouse conditions.

In a search for phosphate solubilizers, Valetti et al. (2018) isolated an *Arthobacter* strain that significatively increased the yield of rapeseed crops when compared with the yield produced by the negative control plots (no fertilized and non-inoculated). Interestingly, the harvest index derived from the *Arthrobacter* sp. LRCP-11 is superior to the one derived from the negative control and fertilized uninoculated treatment.

Furthermore, there is a recent study discussing the potential role of the genus *Arthrobacter* in burned forests (Fernández-González et al. 2017). These authors performed a metagenomic analysis of the holm oak rhizosphere of undisturbed and burned oak forests. *Actinobacteria* was the most abundant phyla in both cases but is more abundant in the burned one. The genus *Arthrobacter* was one of the genera in burned rhizospheres, showing a significant increase in abundance with respect to other genera of *Actinobacteria*. Isolates from this genus displayed hydrolytic enzyme activities and IAA production and some of them lead to the significant increase of alfalfa and pepper vegetative growth under greenhouse conditions.

15.5 Conclusions and Futures Perspectives

The use of *Actinobacteria* as plant probiotics is still in a very early stage compared with the use and application of other PGP bacteria. However, the high number of new species described having a close relationship with plants, including endophytic and rhizosphere actinobacteria, as well as the importance of these microorganisms revealed by plant microbiomes, make them a very interesting alternative to solve agricultural problems. These microorganisms have an excellent potential for plant protection due to its ability to produce inhibitory compounds that will not allow the development of plant pathogens, as well as inducing the natural defense systems of the plants, even from an early stage of development. The sequencing and further analysis of complete or nearly complete genomes have also evidenced the potential of the *Actinobacteria*. Future studies will help in the discovery of new molecules implicated in plant–endophyte symbiotic interactions. The actinobacteria are also soil microorganisms, a feature that will help in their permanence for a long period of time in this unpleasant environment. Until now, the application of these microorganisms in real agricultural conditions has been limited; however, the limitations of the use of pesticides and chemical fertilizers in several worldwide countries and the global acceptance of the use of Plant Probiotic Bacteria as a “Green” alternative will encourage the use of these Plant Probiotic *Actinobacteria* in real crop production.

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Chapter 16
Organic Fertilizer from Algae: A Novel Approach Towards Sustainable Agriculture

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Abstract To meet the global demand for food requirement, today's farmer is using synthetic fertilizers and pesticides enormously. Although such supplements have helped many developing countries to increase the crop yield, simultaneously it has also raised many issues. The use of synthetic fertilizers has not only increased the cost of food production, but also there is decrease of soil fertility and degradation of local ecosystem due to increase in pollutants in soil, water and air. Therefore, there is a need to look for such alternatives which not only can help in combating the pollution problem but can also be used to increase the crop production. The organic fertilizers or the biofertilizers are one of the alternatives, which are eco-friendly, cost-effective and enhance the soil quality without degrading the ecosystem. Amongst various available fertilizers, the organic fertilizers from algae are considered as a potential alternative to mainstream synthetic fertilizers, as these are rich in macronutrients, micronutrients, some growth regulators, etc. which directly help in improvement of growth and yield of crop plants. In the present chapter, various aspects and potentiality of both microalgae and macroalgae as organic fertilizer have been discussed.

16.1 Introduction

Algae are a diverse group of organisms that include unicellular to multi-cellular complex organisms, which are traditionally being used as agar, alginate, carrageenan, food, feed, fodder, and other phytochemicals (Sahoo 2000). Since many years, microalgae is used as biofertilizer or organic fertilizer in rice fields and now

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macro-algae is also being looked upon as potential resource of organic fertilizer or soil conditioner (Zodape 2001; Kumar 2008; Kumar and Sahoo 2011). Presently, the use of natural algal-based fertilizers is proposed as an innovative solution to address the challenges to sustainable agriculture to ensure optimal nutrient uptake, crop yield and tolerance to various biotic and abiotic stresses (Khan et al. 2009; Kumar et al. 2012). Several reports show a large range of such useful effects from algal-based organic fertilizers and seaweed extracts on plants (Hankins and Hockey 1990; Blunden 1991; Norrie and Keathley 2006; Khan et al. 2009). Applications of algae in agriculture as fertilizers, soil conditioners and green manure are just because of the presence of high amount of macronutrients, micronutrient, growth regulators, vitamins and amino acids for plant's better growth, yield and development (Fletcher et al. 1982; Tay et al. 1985; Khan et al. 2006; Craigie 2011).

16.2 Advantages of Algae as Fertilizer in Sustainable Agriculture

Algal-based organic fertilizers are found to be better in comparison to farmyard manure and chemical fertilizers due to the presence of good amount of organic content, which maintains the moisture-retaining capacity and the availability of minerals in the soil (Aitken and Senn 1965; Kumar and Baweja 2018). Algal-based fertilizers are easy to handle and convenient for use, are cost-effective, have longer shelf life, and increase aeration, humus formation and soil's moisture-retaining capacity along with increased nutrient uptake; there is also increased rate of seed germination and overall growth and yield of treated plants. It is also reported that algal-based fertilizers improve plant resistance against several diseases, pests, insects, nematodes and various stresses like drought, frost, salinity, etc. (Kumar et al. 2012).

16.3 Types of Algae Used as Fertilizer

Algae are of various types ranging from microalgae to macroalgae and unicellular to multicellular forms. Amongst microalgae, blue-green algae (BGA) occupy a unique position and share some common features of bacteria and plants. Microalgae are widely distributed throughout the tropical, subtropical and temperate regions and have been found in almost all the conceivable habitats from the Arctic to the Antarctic, being more common in tropical and subtropical regions. They form microbial mats, biofilms and benthic communities in relationship with other entities (Zutshi and Fatima 2015). Blue-green algae find a highly favourable abode in the waterlogged conditions of rice fields where they are popularly known as 'Algalization' (Pabbi 2015). Their genome sequence (16S and 5S RNA) and

metabolic system resemble bacteria, whereas the presence of phycobiliprotein and chlorophyll 'a' ensure an autotrophic mode of nutrition like eukaryotic plant cells (Li and Ley 1992). They are generally blue-green in colour, the chief pigments being chlorophyll a, carotenes, xanthophylls, c-phycocyanin and c-phycoerythrin. The photosynthetic product is glycogen. These algae are characterized by the absence of flagellated reproductive bodies and sexual reproduction has not been recorded so far. Blue-green algae (BGA) have great ecological and agricultural importance and have the ability to carry out both photosynthesis and nitrogen fixation (nitrogen-fixing BGA includes both free-living and symbiotic forms). Furthermore, with more advantages, such as high biomass yield, capability of growing on non-arable lands in a wide variety of water resources (including fresh water, contaminated and polluted waters) and reducing greenhouse gas emissions, along with their water-holding capacity, BGA have become a precious bioresource for sustainable development (Singh et al. 2016). Traditionally, BGA are grouped under Cyanophyta (Myxophyta, Cyanophyceae), but in the recent decade, they have been grouped as *Cyanobacteria*. They exhibit a great diversity of morphology, with their broader spectrum of physiological properties showing their wide distribution and tolerance of environmental stress. Some of the prominent blue-green algae are *Anabaena*, *Nostoc*, *Cylindrospermum*, *Calothrix*, *Plectonema*, *Anabaenopsis*, *Tolypothrix*, *Oscillatoria*, *Aphanothece*, *Tolipothrix, Aulosira, Calothrix, Cylindrospermum* and *Phormidium*. Amongst these, only very few such as *Anabaena variabilis*, *Nostoc muscorum*, *Aulosira fertissima* and *Tolypothrix tenuis* have been found to be effective as biofertilizers (Table 16.1).

Table 16.1 Some common algae being used as biofertilizers

S. no.	Name of algae	Class	References
1.	*Caulerpa* sp.	Chlorophyceae	Uthirapandi et al. (2018)
2.	*Enteromorpha* sp.	Chlorophyceae	Mathur et al. (2015)
3.	*Ulva* sp.	Chlorophyceae	Sridhar and Rengasamy (2002)
4.	*Ascophyllum nodosum.*	Phaeophyceae	Ali et al. (2016)
5.	*Palisada perforate*	Rhodophyceae	Duarte et al. (2018)
6.	*Sargassum* sp.	Phaeophyceae	Kumar and Sahoo (2011)
7.	*Ecklonia maxima*	Phaeophyceae	Temple and Bomke (1989)
8.	*Macrocystis pyrifera*	Phaeophyceae	Temple and Bomke (1989)
9.	*Laminaria japonica*	Phaeophyceae	Kuwada et al. (2006)
10.	*Undaria pinnatifida*	Phaeophyceae	Kuwada et al. (2006)
11.	*Turbinaria decurrens*	Phaeophyceae	Sivasankari et al. (2006), Uthirapandi et al. (2018)
12.	*Gracillaria* sp.	Rhodophyceae	Pise and Sabale (2010)
13.	*Rosenvingea intricata*	Phaeophyceae	Thirumaran et al. (2009)
14.	*Kappaphycus alvarezii*	Rhodophyceae	Rathore et al. (2009)
15.	*Dictyota dichotoma*	Phaeophyceae	Sasikumar et al. (2011)

Marine macroalgae, commonly known as seaweeds, are being used as organic fertilizers in many countries. They are distributed globally in oceans and are broadly divided into three categories: green, brown and red algae. Marine algae is being commercially cultivated in many countries such as China, Japan, Korea, the Philippines, etc. Seaweeds affect the biological, chemical and physical properties of soil which influence plant growth, crop yield and development (Temple and Bomke 1988, 1990). The use of seaweeds and seaweed extracts boosts soil structure and soil flora by increasing moisture retention capacity. The seaweed liquid extracts of many seaweeds contain several bioactive compounds, which are used in many agricultural and horticultural fields. The seaweeds extracts are gaining popularity for many important crops such as vegetables, cereals, flowers, etc. Some of the common marine algae which are being used as soil conditioner or biostimulant are *Ulva*, *Enteromorpha*, *Caulerpa*, *Laminaria*, *Undaria*, *Sargassum*, *Turbinaria*, *Gracilaria*, etc. (Fig. 16.1; Table 16.1).

16.4 Microalgae as Biofertilizer

16.4.1 Nitrogen Fixation by Blue-Green Algae

Blue-green algae are diazotrophs (able to fix atmospheric nitrogen). The annual turnover of nitrogen in biosphere varies from estimated 100–200 million metric tonnes, of which 2/3 comes from biological sources where blue-green algae play an important role (Pabbi 2015). Watanabe et al. (1977) demonstrated at IRRI, Manila, that 23 successive crops can be grown successfully continuously for 12 years by using only blue-green algae without any added nitrogen fertilizer. However, total nitrogen fixation by blue-green algae depends upon the physicochemical properties of soil and many other climatic and biotic factors, for example, alkalinity of the soil favour *N* fixation by blue-green algae (Roger and Kulasooriya 1980). Crop plant can utilize fixed nitrogen only when it is available extracellularly either as extracellular products or by mineralization of their intracellular contents through microbial decomposition after death (Pabbi 2015). Crop plants are able to utilize more nutrients from the soil in the presence of algal inoculation because of the slow release of the fixed and metabolized nitrogen. The nitrogen fixation by blue-green algae has a switch 'on' mechanism which is activated when the level of combined nitrogen falls below a threshold level (~40 ppm) due to progressive utilization and loss from soil atmosphere (Pabbi 2015). It has been established by the ^{15}N tracer technique that about 90% of the *N* accumulated by cyanobacteria is derived from the air (Inubushi and Watanabe 1986). It has been also reported that excessive nitrogenous fertilizers (except urea) have negative effect, whereas optimum doses of these fertilizers have a positive effect on growth and development of blue-green algae (Watanabe 1973). Till date, all known nitrogen-fixing organisms are prokaryotes. Atmospheric nitrogen can be easily fixed by these nitrogen-fixing organisms because of the enzyme nitrogenase which fixes atmospheric nitrogen into ammonium using 16 ATP for

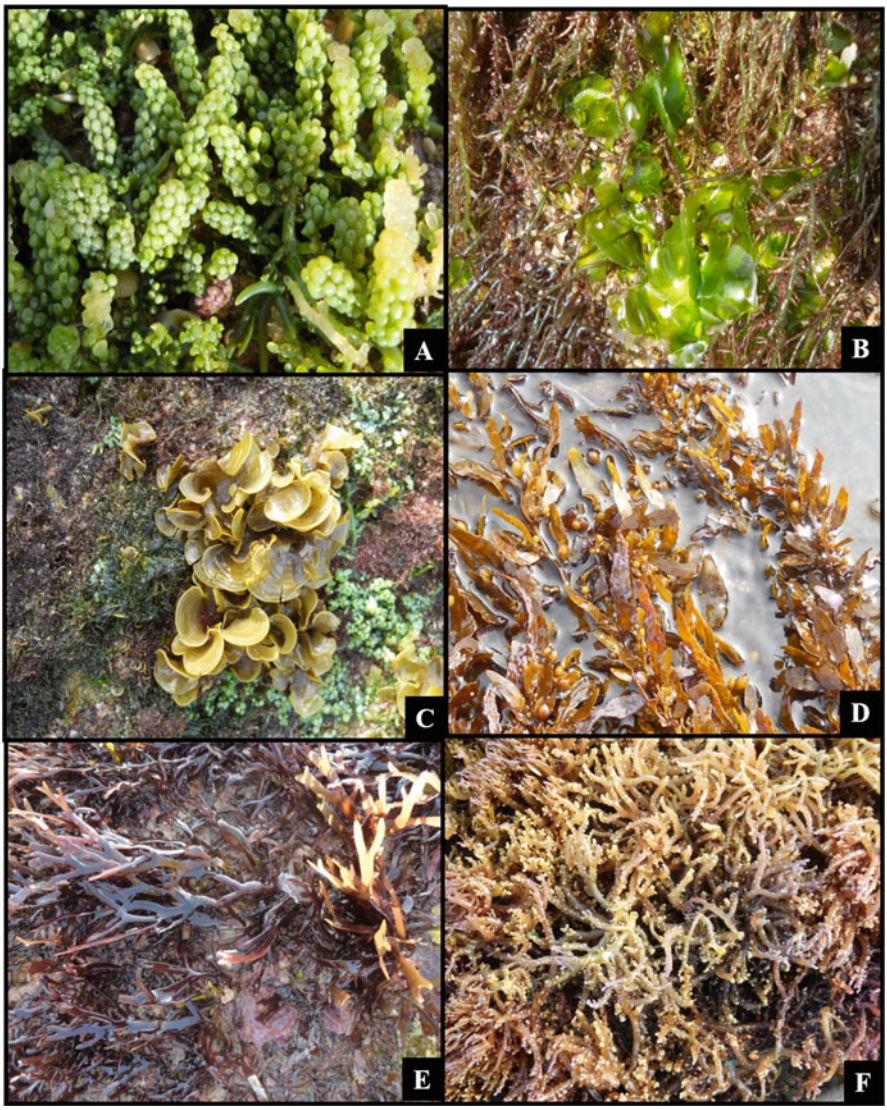

Fig. 16.1 Some common marine algae being used as a source of organic fertilizers. (**a**) *Caulerpa* sp.; (**b**) *Ulva* sp.; (**c**) *Padina* sp.; (**d**) *Sargassum* sp.; (**e**) *Gracilaria* sp.; (**f**) *Kappaphycus* sp.

each molecule of N_2 as a source of energy (Lee 2008). 2-Oxoglutarate from the citric acid cycle fixes this ammonium by the enzyme glutamate dehydrogenase to form initial glutamate (glutamic acid) and after the addition of a second ammonium produces glutamine. Glutamine can be transferred from one cyanobacterial cell to another (Fig. 16.2c). Like bacteria, nitrogenase of blue-green algae is also composed of two components, dinitrogenase reductase (iron protein) and dinitrogenase

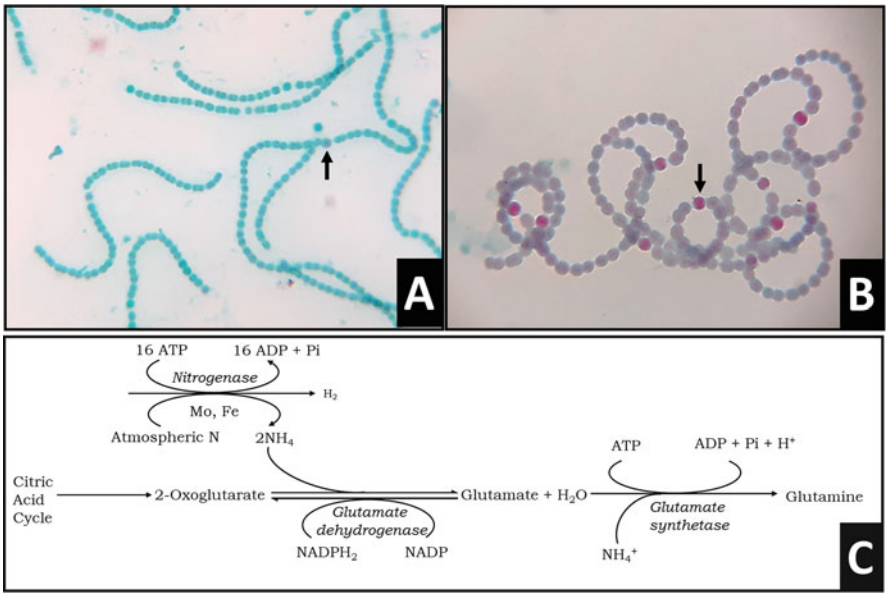

Fig. 16.2 Microalgae used for N_2 fixation in crop fields. (**a**) *Nostoc*, (**b**) *Anabaena*, (**c**) process of nitrogen fixation by blue-green algae; arrow heterocyst, site for nitrogen fixation

(molybdenum-iron protein) encoded by the *nif* HDK operon (Lee 2008; Henson et al. 2004) (Fig. 16.2).

16.4.2 Symbiotic Blue-Green Algae

Apart from acting directly as a soil conditioner, there are few symbiotically competent blue-green algae which have some excellent features that make them particularly significant in an attempt to extend the list of N_2-fixing symbiosis to include plants of commercial interest. These symbiotic blue-green algae occur in basically in two types of associations: extracellular and intracellular. Symbiotic blue-green algae are not only restricted to roots, but also they have a wide range of host plant tissue. The major plant hosts are bryophytes, cycads, the angiosperm *Gunnera*, the water fern *Azolla* and fungi (all extracellular). Unlike rhizobia, most of the symbiotic blue-green algae carry their own mechanism for nitrogenase protection. In addition to supplying fixed nitrogen to their host, blue-green algae also provide fixed carbon to non-photosynthetic parts of the host.

Amongst various other symbiotic association of blue-green algae, the BGA's association with a fern known as '*Azolla-Anabaena azollae*' makes a unique mutually beneficial 'relationship'. This relationship has many ecological and economic significance. It is commercially used as fertilizer in various fields and especially for

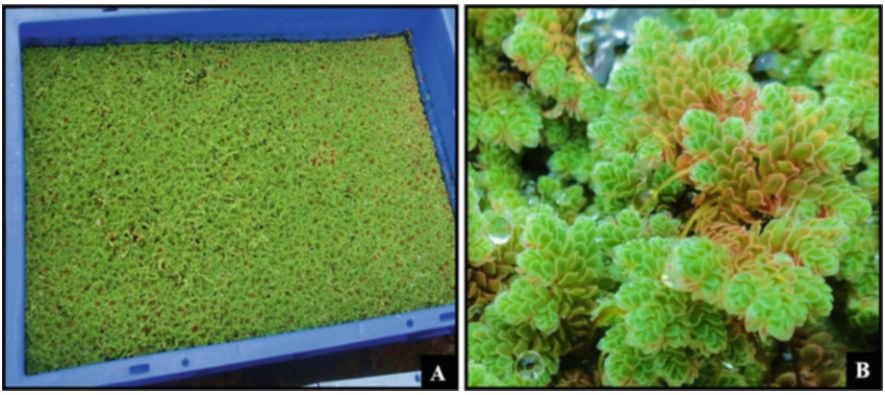

Fig. 16.3 Commercial cultivation of *Azolla* sp. (**a**) *Azolla* sp. growing in a cultivation tank and (**b**) *Azolla* plants

the cultivation of rice, where it plays a very important role in rice production. *Azolla* provides an enclosed environment for *Anabaena* within the fern's aerial dorsal leaf lobes. In return, *Anabaena* sequesters nitrogen directly from the atmosphere, which is required by *Azolla* for its growth. *Azolla* and *Anabaena* have never been apart for 70 million years, and for centuries, *Azolla-Anabaena azollae* symbionts have been used as 'green manure' in China and other Asian countries to increase rice production without any crop rotation (Deepali 2017). In general, free-living *Anabaena* can have maximum 8% heterocyst compared to vegetative cells, whereas in *A. azollae* is about 30%, which is very high, the highest for free-living *Anabaena* being about 8% (Hill 1975). *Azolla* is able to form green mat over water and is readily decomposed to ammonia and produces large quantities of biofertilizers (Fig. 16.3a, b). *Azolla* leaf consists of a thick, greenish (or reddish) dorsal (upper) lobe and a thinner, translucent ventral (lower) lobe immersed in water. Filaments of *Anabaena* generally remain in the upper lobes.

16.4.3 Beyond Nitrogen Fixation

Cyanobacteria have been shown to be the most important in nitrogen fixation for maintaining and improving the productivity of crop plants. In addition to this, they also play an important role in increasing soil fertility in many ways. Blue-green algae generally have a polysaccharide sheath and extracellular polymeric secretions that exert a mechanical effect on soil particles as they form a gluing mesh and bind soil particles on their surface (MalamIssa et al. 1999, 2001; Nobles et al. 2001). The extracellular polymeric secretions from blue-green algae material not only play a significant role in water storage by increased water retention capacity, maintaining

pH and temperature of the soil, but it also protects soil from erosion (Hu et al. 2002, 2003; Pandey et al. 2005). Interwoven filaments of blue-green algae growing on soil surface increases the soil aggregate size which in turn reduces soil compaction. Thus, blue-green algae have been used as inoculants to improve soil structure, increase soil fertility or recover damaged soil crusts.

Another important aspect where blue-green algae can benefit crop plants is by producing plant growth regulators (PGRs) such as gibberellins, auxin, cytokinin, ethylene and abscisic acid like substances which improve growth and production of crop plants (Venkataraman and Neelakantan 1967; Mishra and Kaushik 1989a, b; Zaccaro et al. 2006). Furthermore, blue-green algae as a result of algal inoculations also increase significant amount (5–32%) of organic matter to the soil (Singh and Bisoyi 1989). Blue-green algae play a significant role in the reduction of the oxidizable matter content of the soil by the oxygen liberated during photosynthesis which is a phenomenon of great importance for areas where more than one crop of rice is sown in a year (Pabbi 2015). In organic matter rich soils, the availability of phosphorus (another major nutrient required for crop plants) is greatly enhanced through microbial activity, and some blue-green algae play an important role in solubilizing phosphorus (Yandigeri et al. 2011).

16.5 Macroalgae as Fertilizers

Seaweeds or marine macroalgae also make an effective organic fertilizer. They are generally used as biostimulants and soil conditioner in agricultural fields. The seaweeds are in abundance around the world and can be collected from the beaches around the globe as the drifted ones. They themselves do not fix atmospheric nitrogen like microalgae and are a good source of growth regulators and various micro- and macronutrients. Seaweeds are used in the form of liquid extracts (SLE) or pulp residue left after the extraction. There are also reports of the direct application of seaweed in dried powder form in agricultural fields as fertilizers. Several studies have been conducted on various crops using organic fertilizers prepared from different seaweeds and the significant increase has been observed in the crop yield. Application of seaweed extract accelerates the seed germination percentage even if the SLEs are applied at lower concentrations. The commercially available SLEs are majorly prepared from brown seaweeds, and they vary in viscosity, colour, odour and pH. Brown seaweeds are chief source of fucoidans and alginates, thus the chelating and gelling properties of these polysaccharides make these compounds very important in agriculture (Cardozo et al. 2007; Khan et al. 2009; Craigie 2011; Kumar et al. 2012). Alginate found as a mixed salt with the major cations like Na, Ca, Mg and K along with minor metal ions in the cell wall of brown seaweeds (Khan et al. 2009). Alginate combines with these ions and form chelates in the soil that absorb moisture and improve soil structure and porosity. It results in improved plant root system as well as accelerated soil microbial activity (Eyras et al. 1998; Khan et al. 2009; Moore 2004). Alginates improve soil properties and influence growth of

beneficial Arbuscular Mycorrhizal (AM) fungi (Ishii et al. 2000). Kuwada et al. (2006) observed that seaweed liquid extract from *Undaria pinnatifida* and *Laminaria japonica* could be used as an AM fungus growth promoter. The growth-promoting activity in roots has been reported when the seaweed extracts are used in crop plants (Biddington and Dearman 1983). Seaweed liquid extract accelerate proper root development by increasing lateral root formation (Atzmon and van Staden 1994; Vernieri et al. 2005; Kumar 2008) and enhanced total volume of the root system too (Slàvik 2005; Mancuso et al. 2006). A superior root system developed in crop plants may be due to the presence of endogenous auxins and other active compounds in the seaweed liquid extracts (Crouch et al. 1992). Nutrient uptake capacity has also been found to be increased by the use of SLEs, thus promoting growth and yield of crop plants (Crouch et al. 1990), Also, seaweed liquid extracts and seaweed manure accelerates early flowering and fruit set in several crop plants (Abetz and Young 1983; Featonby-Smith and van Staden 1987; Arthur et al. 2003).

16.5.1 Growth Regulators in Macroalgae

Several plant growth bioassays undoubtedly point towards the presence of some plant growth regulators in algal-based biofertilizers (Mooney and van Staden 1986; Tay et al. 1985; Williams et al. 1981; Kumar et al. 2012). Moreover, seaweed liquid extracts and seaweed manure showed a large range of growth responses which indicates the presence of several plant growth regulators such as auxins, betains, cytokinins, gibberellins, abscisic acid, jasmonic acid, brassinosteroids, polyamines, etc. (Tay et al. 1985; Crouch and van Staden 1993; Khan et al. 2009; Kumar et al. 2012). Auxins which are important for rooting in plants are present in all groups of algae and have been reported from *Undaria pinnatifida* (Abe et al. 1972), *Porphyra perforata* (Zhang et al. 1993), *Caulerpa paspaloides*, etc. Auxins such as indole acetic acid (IAA), indole-3-carboxylic acid (ICA), *N*,*N*-dimethyltryptamine (NNPT), indole-3-aldehyde (IAld) and *N*-hydroxyethylphthalimide have been identified in commercial seaweed-based fertilizers (Stirk et al. 2014). Similarly, cytokinins which regulate shooting, bud formation, protein synthesis, delays leaf senescence, etc. have also been identified in various species of all red (*Euchema maxima*), brown (*Fucus serratus*) and green algae (Sharma et al. 2014) (Table 16.2).

16.5.2 Macro- and Micronutrients

Several studies have also been conducted on the nutrient content of macroalgae. The report shows that algae are rich in both micro- and macronutrients (Rioux et al. 2007; Khan et al. 2009; Kumar et al. 2012). Algae have ability to accumulate

Table 16.2 Plant growth regulators (PGRs) in some common macroalgae

S. no.	PGRs	Algae	References
1.	Auxins	*Ascophyllum nodosum*	Stirk and Van Staden (1997)
2.	Betaines	*Ascophyllum nodosum*	Blunden et al. (1986)
3.	Cytokinins	*Ascophyllum nodosum, Ecklonia maxima, Macrocystis pyrifera, Sargassum* sp.	Khan et al. (2009)
4.	Gibberellins	*Ascophyllum nodosum*	Crouch and Van Staden (1993)

Table 16.3 Macro- and micronutrient contents of some common macroalgae

S. no.	Seaweeds	Major nutrients	References
1.	*Ascophyllum nodosum*	Ca, K, P	Craigie (2011)
2.	*Durvillaea potatorum*	Fe, Zn, B, Mn	Khan et al. (2009)
3.	*Sargassum* sp.	Ca, Fe, K, P, Mg	Pise and Sabale (2010), Hernández-Herrera et al. (2014), Kumar and Sahoo (2017)
4.	*Ecklonia maxima*	Ca, K, Zn, P	Temple and Bomke (1989)
5.	*Macrocystis pyrifera*	K, P, Fe, Ca	Temple and Bomke (1989)
6.	*Gracillaria* sp.	K, P, Fe, Mg, Ca, Zn	Pise and Sabale (2010)
7.	*Caulerpa* sp.	Na, Mg, Ca, K	Hernández-Herrera et al. (2014)
8.	*Ulva* sp.	Na, P, Mg, Ca, K	Pise and Sabale (2010), Hernández-Herrera et al. (2014)
9.	*Padina* sp.	Na, P, Ca, K	Hernández-Herrera et al. (2014)
10.	*Turbinaria conoides*	Mg, Fe, B, Zn	Murugaiyan et al. (2012)

certain micro- and macronutrients (Sharma et al. 2014). In addition, some reports are also there which show that leftover pulp of seaweeds after extraction of phycocolloids can also be a good source of these nutrients (Kumar and Sahoo 2017) (Table 16.3).

16.6 Algal Cultivation for the Production of Organic Fertilizers

The algae can be cultivated conveniently, as it is simple and easy to handle. It is more convenient to culture microalgae, technically and economically in comparison to marine macroalgae, as the availability of seawater is a limitation for its growth and development. In general, the various algal production methods include pond/tank/pit method for mass cultivation of *Azolla* for symbiotic *Anabaena* sp. and for the cultivation of free-living microalgae, field cultivation method for seaweeds.

16.6.1 *Pond/Tank Method: Mass Culture of* Azolla *sp. for the Commercial Production of Anabaena (Modified After Pillai et al. 2002; Pabbi and Dhar 2008; Datta 2011)*

Azolla can be maintained easily in open ponds, tanks or even in trays on soil-based continuous cultures. For mass cultivation, garden soil and cow dung in a ratio of 8:1 and tap water are mixed and added in the culture vessels for making 1 cm layer of soil and cow dung mixture with 5 cm water layer. The depth of this is maintained at 5 cm in height throughout the culture. To settle suspended matter, the culture vessel is left undisturbed overnight, and next day 5 g of healthy and fresh *Azolla* fronds are suspended in it. A pinch of Single Super Phosphate (SSP) is added if required or if fronds show P deficiency symptoms. To maintain the cultures, regular trimming is also done. *Azolla* plants grow within 2 weeks. The optimum environmental factors for *Azolla* production are temperature, 25–30 °C; light, partial shade; pH, 4.5–8.0; for optimum moisture, the minimum level of water should be maintained, phosphorus, >25 ppm. Fresh *Azolla* at 0.5–1.0 ton/ha after 7–10 days of transplantation of rice is inoculated, and SSP is applied at 20 kg/ha in split doses to maintain the *Azolla* plants (Fig. 16.3).

16.6.2 *Pond/Tank/Pit Method: Cultivation of Free-Living Microalgae*

For the cultivation of free-living microalgae, small ponds/pits are made in the field either by digging the field, using shallow tanks or using galvanized iron sheets. The size of the tank can be adjusted according to the requirement. In 5 kg of river soil, superphosphates and sodium molybdate are added in the ratio of 20:0.4 g. After 10 hours, 250 g of mother culture of BGA is added to the tank and is left undisturbed. Periodic evaporation and pH (neutral) are checked, and measures are also taken to control the contamination and mosquito growth. After 10–15 days, the culture is ready and can be seen as growing as flakes on the soil. These cultures can be collected and stored in plastic bags (500 g each) and can also be used as mother cultures for future (Sahu et al. 2012).

16.6.3 *Field Cultivation Method for Seaweeds*

Seaweeds are being cultivated and exploited extensively in many countries for the extraction of various commercial products. The seaweeds can also be harvested from the natural fields and also the drifted plants can be collected from seashores.

Generally, two methods of seaweed farming are in use, which is pond cultivation and open sea cultivation. The seaweed cultivation is only feasible for the farmers in coastal areas. In both methods, seaweeds germ-lings are tied to a nylon/jute/coir rope and are kept in seawater to make the harvest double in amount. The number of days to be decided depends upon the species of seaweed being cultivated, and it can vary from 30 to 120 days. The seaweed cultivation is also technically and economically viable for the farmers.

16.6.4 Application of Azolla *in Field*

In the rice field, *Azolla* is applied at 1 ton/ha and a water depth of 2 in. is maintained. After 2–3 weeks, a thick mat of *Azolla* is found and then rice can be transplanted. This accounts for 10–20 ton *Azolla* contributing 20–40 kg nitrogen/ha. In case of pest or insect attack, Furadon at 2–3 kg/ha can be applied. The incorporated *Azolla* dies within 8–10 days and releases nitrogen. Each crop of *Azolla* during dual cropping contributes 30 kg nitrogen/ha on an average.

16.6.5 Application of BGA in Field

The stored BGA packets can be used for the field and 500 g is recommended for 1 acre of rice field. About 500 g BGA is mixed with 4 kg of farm soil and is sprinkled on standing water. For the proper growth and development of BGA, the field must always be waterlogged, and additional algal material can be used for the fast multiplication and growth of algae. Phosphate fertilizers also enhance the growth of BGA, so they can also be used along with the BGA inoculum (Sahu et al. 2012).

16.6.6 Preparation of Seaweed Liquid Extract (Modified After Bhosle et al. 1975)

The sun-dried seaweeds are washed thoroughly using tap water to remove sand, salt and other debris from plants. The water is drained off and the plant material is spread on blotting paper to remove excess water and kept for shade drying. After complete drying, 500 g of seaweed is finely chopped and boiled with 500 ml distilled water for an hour in the water bath, and the extract is filtered through muslin cloth; this filtrate is taken as 100% concentration of the seaweed extract. Different concentrations of seaweed extracts (5%, 10%, 20%, 30%, 40% , 50%, etc.) are prepared by diluting this extract with distilled water (Kumar 2008). The pulp leftover after extraction can also be used as a fertilizer and can be directly applied to the field (Kumar and Sahoo 2017) (Fig. 16.4).

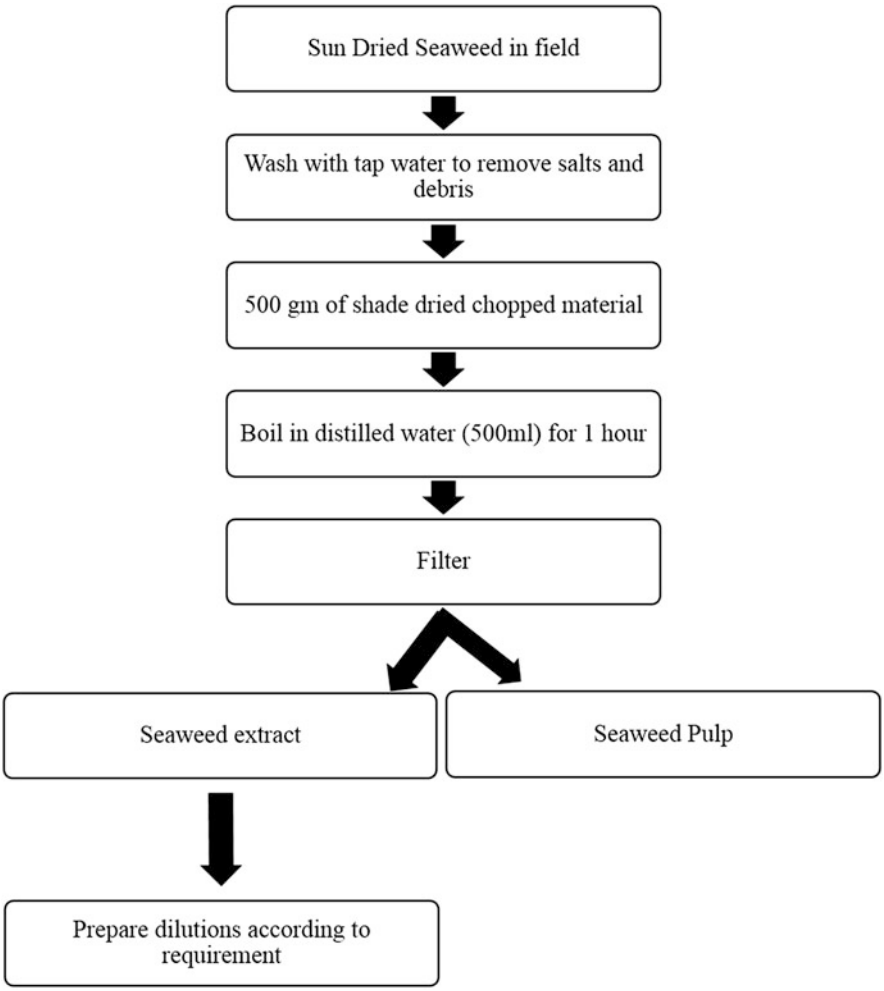

Fig. 16.4 Commercial preparation of seaweed liquid extract

16.6.7 Application of Seaweed Liquid Extract in Field

16.6.7.1 Seed Soaking

The seeds are soaked overnight in the desired concentration of SLEs and then they are sown in the field. The soaking enhances the percentage of seed germination, seedling vigour by increased levels of plant defence enzymes (Burchett et al. 1998; Sivasankri et al. 2006; Kumar and Sahoo 2011).

16.6.7.2 Foliar Application

The SLEs are applied as foliar sprays in low concentrations and found to be effective in increasing fruit or tuber formation, chlorophyll content, also there is a reduction in fungal disease incidences. The increased growth and yield have been observed in various food crops such as wheat, maize, rice, spinach, tomato, potato, carrots, etc. (Sharma et al. 2014).

16.6.7.3 Direct Application in Soil

The direct soil application of SLEs is the most common practice around the world. There is also a practice to add the dried powdered seaweeds directly into the filed since ancient times. It has been a common practice in coastal areas. The seaweeds either in powdered form or SLEs, act as soil conditioners, and the nutrients present are directly available to plants.

16.7 Conclusion

The enormous potential and properties of algae as organic fertilizer, makes them suitable to be used in agricultural fields, in diversified crops, to improve yield and quality. It is now a well-known fact that algae contain various bioactive compounds including nutrients and growth hormones and therefore, algal interaction with soil community benefits the crop production. Algae and its products have a complex interface with the plants and their environment, helping them to combat various abiotic and biotic stresses. Algal fertilizer also increases beneficial microorganisms and can also convert CO_2 to O_2. The organic fertilizers prepared from algae provide considerable benefits to farmers including both economic and environmental aspects and even farmers can cultivate them at their own convenience. Thus, the fertilizers from algae are the finest alternative for synthetic fertilizers, which not only boost crop yield but also promote sustainable agriculture.

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Chapter 17
Phosphate-Solubilising Fungi and Their Potential Role in Sustainable Agriculture

Sanjana Kaul, Supriya Sharma, Apra, and Manoj K. Dhar

Abstract Phosphate-solubilising fungi harness the phosphate available in the soil-plant systems and make it available to the plants. They solubilise or mineralise phosphate that is present in the sparingly soluble organic and inorganic form in the soil, thereby improving growth and yield of a wide variety of crops. Various mechanisms governing the plant growth promotion by phosphate solubilisation are being investigated. Development of an efficient management system to improve agricultural productivity is of current interest in agricultural biotechnology. Use of phosphate-solubilising fungi (PSF) as conventional phosphate fertilisers is a promising strategy to improve global demands of improved agricultural productivity, depletion of soil fertility, water pollution and accumulation of toxic elements. It provides an environmentally acceptable agro-technique for enhanced agricultural sustainability. Despite the significance of PSF in plant growth promotion, they are still to be replaced with conventional chemical fertilisers. This review mainly focuses on the fungi that can solubilise phosphorus and thus have the potential to be used as biofertilisers. The mechanism of phosphate solubilisation is being highlighted with its significance, thereby depicting the success of this technology. Finally, the agronomic effectiveness of PSF has been discussed, which concludes that this technology is ready for commercial exploitation in various regions worldwide.

Keywords Agro-techniques · Biofertilisers · Mineralise · Phosphate-solubilising fungi

17.1 Introduction

Phosphorous plays an important role in the growth and development of plants. It is the second essential mineral nutrient after nitrogen, limiting the growth of crops (Tak et al. 2012) and constitutes only 0.2% of plant's dry weight. Although it is available

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in the soil in both organic and inorganic forms, its availability is still restricted to the plants. The dynamics of the phosphorous present in the soil is characterised by various physicochemical and biological processes. Phosphorus is an important structural component of biomolecules like coenzymes, phosphoproteins, phospholipids and nucleic acids that are involved in various physiological processes of plants and animals especially in photosynthesis, carbon metabolism and membrane formation of living organisms (Wu 2005; Anand et al. 2016). In living system, it is involved in the transfer and storage of energy which is used for growth and reproduction. Deficiency of this macronutrient may affect the architecture of roots and development of seeds that adversely affects the crop maturity (Borch et al. 1999; Williamson et al. 2001). In plants, phosphorus is readily translocated from older to younger tissues as the plant forms cells and develops roots, stems and leaves. A major amount of phosphorus absorbed by the plant is accumulated inside the grains as phytase, and its deficiency negatively affects grain yield.

The soil constitutes 0.05 (w/w) of phosphorous; however, only 0.1% of the phosphorous is utilised by plants (Alori et al. 2017). The major reason for its unavailability is the presence of phosphorous in insoluble form. Organic matter accounts for 20–80% of phosphorous in the soil (Richardson 1994). Although the elemental phosphorus occupies an integral importance in the life of plants, it is not easily available to plants due to some reasons. Firstly, the microorganisms present in the soil convert the available phosphate into organic forms. Secondly, the available phosphorus (inorganic form) is adsorbed by the soil particles. Thirdly, the pH of the soil should be 4–8; otherwise, phosphorus begins to form bonds with other compounds (Khan et al. 2018). It is present in the soil abundantly both in its organic and inorganic forms. In order to solubilise these fixed and insoluble forms, different management strategies are being employed so that the growth of the plants can be improved (Satyaprakash et al. 2017). One of the strategies that can be addressed to combat this challenge is the application of fertilisers (containing phosphorous). However, only a small amount of the applied fertiliser is available to the plants; repeated and injudicious addition of the fertilisers forms precipitates on reacting with Al^{3+} and Fe^{2+} in acidic and Ca^{2+} in calcareous or normal soil that cause adverse effects on the environment like contamination of the waterbodies, eutrophication, etc. This demands sound, eco-friendly and economically feasible strategies that can replace or produce highly efficient phosphorous fertilisers so that crop production is increased and no harm is caused to the environment. Among other approaches used to change the current scenario is the use of microflora. This group of microorganisms are referred to as phosphorus-solubilising microorganisms (PSM). They may circumvent the deficiency of phosphorous in the Indian soil and can supply phosphate to the plants in eco-friendly and sustainable manner. Although microorganisms have the potential to solubilise phosphate, however, rarely sufficient data has been published on their phosphate-solubilising potential.

Microbial population in the soil comprises eubacteria, cyanobacteria, arbuscular mycorrhizal (AM) fungi, algae and actinomycetes (Thakur et al. 2014). Besides soil-microbial population, endophytes, the microbes dwelling inside the plants, also empower plant growth by phosphate solubilisation (Oteino et al. 2015). Although

host-microbe interactions are the determinants of soil fertility and plant growth, however, a complete understanding of the complex interactions taking place among various components of host-soil-microbes is required for the successful application of such microbes (Satyaprakash et al. 2017). These microbes enhance the plant nutrient acquisition and are involved in various biological activities such as a phosphate solubilisation by using different mechanisms.

Among microbes, the phosphate-solubilising fungi constitute 0.1–0.5% of the total fungal population found in the soil. Unlike bacteria, they have been reported not to lose their phosphate-solubilisation activity on subsequent subculturing (Kucey 1983). The salt-tolerant or halophilic fungi that also exhibit the ability to solubilise insoluble phosphorus facilitate the development of saline-alkali soil-based agriculture (Alori et al. 2017). Zhou et al. (2018) demonstrated the role of *Trichosporon asperellum* in alleviating the suppression effect of salt stress involving the change of phytohormone levels in cucumber plant and its ability of phosphate solubilisation. Fungi assorted qualities are viewed as critical for sustaining/upholding the manageability of agriculture and horticulture systems (Walia et al. 2017). Various fungi have been reported to mobilise the poorly available phosphorous via solubilisation and mineralisation. *Aspergillus niger* and *Penicillium* sp. have been reported to be the most common fungi possessing phosphate solubilisation (Nelofer et al. 2016; Chadha et al. 2015).

Endophytic fungal isolate *Byssochlamys nivea* obtained from *Pistacia vera* possesses phosphate-solubilising potential (Dolatabad et al. 2017). These organisms have the ability to produce more acids than (such as gluconic, citric, lactic, 2-ketogluconic, oxalic, tartaric and acetic acid) bacteria and can traverse long distances within soil more easily as compared to bacteria (Sharma et al. 2013a, b). A nematofungus, *Arthrobotrys oligospora*, has been reported to solubilise the phosphate rocks. The diversity and dominance of phosphate solubilisers depends on biotic and abiotic factors prevailing in a particular ecological niche (Bhattacharyya et al. 2016). The success of fungi to reach and colonise a patch of soil can be attributed to their competitive saprophytic ability and tolerance to heavy metals (Khan et al. 2009). Therefore, fungal inoculants (biofertilisers) can be considered as an environment-friendly alternative to further applications of mineral phosphate fertilisers possessing phosphate-solubilising activity in crop productivity. Besides, phosphate-solubilising fungi augment plant growth by enhancing the availability of other trace elements, efficiency of nitrogen fixation, phytoremediation of heavy metals or bioleaching of rare earth elements for mined ores (Ahemad 2015; Shin et al. 2015). Conflicting results for the effect of temperature on phosphate-solubilisation activity of fungi have been observed. Some workers have reported 28 °C as the ambient temperature for phosphate solubilisation, whereas other workers have reported high phosphate solubilisation at either high or low temperatures (Abdel-Ghany and Alawlaqi 2018).

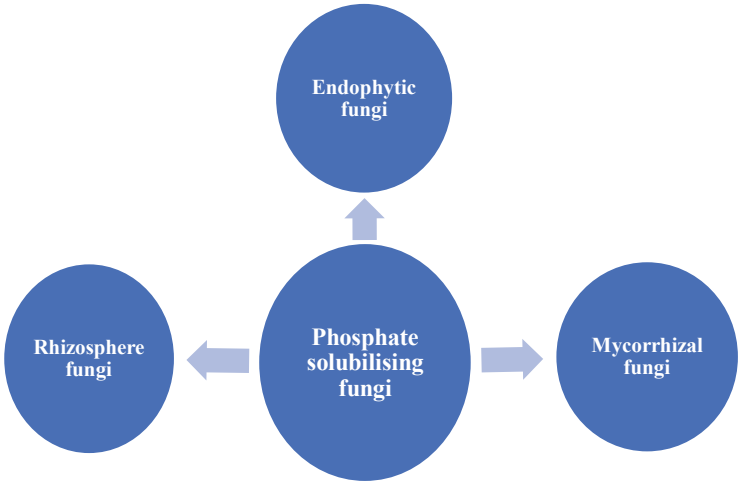

Fig. 17.1 An overview of phosphate-solubilising fungi

17.2 Phosphate-Solubilising Fungi

The unavailability of phosphorus makes soil deficient in phosphate element that can be overcome by certain microorganisms referred to as phosphate-solubilising microorganisms (PSM). They have the capacity to dissolve insoluble phosphorus for plants by the process of mineralisation and solubilisation. These microorganisms may include bacteria, fungi and actinomycetes. Of these microbes, phosphate-solubilising fungi (PSF) are generally isolated by using serial plate dilution method or by enrichment culture method on suitable media (Khan et al. 2007). A clear halo zone is formed around the culture which confirms the culture to have phosphate-solubilising potential. These PSF are then selected on the basis of their P-solubilising potential. Further, these potential fungi are cultured on large scale, and their inoculant is developed which is tested at field level against economically important plants (Khan et al. 2007). There are various regions from where PSF can be isolated and play role in promoting phosphate-solubilising activity for plants (Fig. 17.1). These regions are:

(a) Rhizospheric region
(b) Endophytic region
(c) Mycorrhizal region

17.2.1 Phosphate-Solubilising Fungi Associated with Rhizospheric Region

Rhizospheric microorganisms contribute to chemical and physical modifications that directly affect plants and their health. In a recent study, rhizospheric thermo-halotolerant fungi *Aspergillus terreus* isolated from the rhizosphere soils of *Suaeda monoica*, a wild halophilic plant in Jizan, Saudi Arabia, had been checked for its P-solubilising as well as zinc-phosphate-solubilising capacity (Abdel-Ghany and Alawlaqi 2018). Khan et al. (2018) isolated 19 P-solubilising fungi from the rhizosphere soil of wheat plant, and out of 19 isolates, 12 isolates showed positive results for P solubilisation. The best isolate that had excellent potential to solubilise phosphate was *Aspergillus* spp. Similarly, Elias et al. (2016) isolated rhizospheric microorganisms from soil region of different plants. Among them *Penicillium*, *Aspergillus* spp. and *Fusarium* spp. were isolated as the dominant phosphate-solubilising fungi. Dominance of *Aspergillus* spp. as major phosphate solubilisers among microorganisms isolated from the rhizospheric soil region of betel vine plant has been reported (Jain et al. (2012)). A study confronts the potential and the effect of phosphate-solubilising fungi, *Aspergillus awamori*, on the growth of mung bean plant (Tallapragada and Seshachala (2012)). Phosphate-solubilising potential of about eight *Trichoderma* spp. from the rhizospheric region of *Calophyllum brasiliense* has been reported (Resende et al. 2014). Yin et al. (2017) conducted a report in which *Aspergillus aculeatus* isolated from the rhizosphere of wheat plant was evaluated for its P solubilisation potential. For the development of *Pongamia pinnata* (medicinal plant), two fungi, *Aspergillus ustus* and *Aspergillus tamarii*, isolated from its rhizosphere were investigated for excellent P solubilisation potential (Pany et al. 2018).

17.2.2 Phosphate-Solubilising Fungi Associated with Endophytic Region

Endophytes are the microorganisms living inside the plant where they appear to enhance and improve growth of the plant by using various mechanisms. One such mechanism is the uptake of element P from the soil. There are several endophytes that have been discovered that exhibit phosphate-solubilising activity. In one such report, fungal isolates were isolated from the roots of *Cardiospermum halicacabum*, and it was found that *Aspergillus oryzae* had potential P-solubilising activity (Devi and Packialakshmi 2018). Sarbadhikary and Mandal (2018) reported that an endophytic fungal strain of *Aspergillus* isolated from the leaf of *Schima wallichii* had potent plant growth promotion potential in terms of various plant growth promoting activities including P-solubilising activity. An endophytic fungi *Xylaria regalis* isolated from the cones of *Thuja plicata* was evaluated for its various plant growth

promotion activities including improvement in crop plants by the mechanism of P solubilisation (Adnan et al. 2018).

Similarly, *Trichoderma gamsii* isolated from the lateral roots of *Lens esculenta* had significant phosphate-solubilising potential (Rinu et al. 2014). *Penicillium funiculosum* was investigated for its P-solubilising potential and also for its role on the physiology of host plant, *Glycine max*, growing under salinity stress (Khan et al. 2011). The plant growth-promoting traits of some epiphytic and endophytic yeast isolates obtained from the leaves of rice and sugar cane were evaluated in which calcium phosphate-solubilising capabilities of selected yeast were investigated (Nutaratat et al. 2014). In a study conducted by Nath et al. (2015), *Penicillium sclerotiorum*, an endophytic fungi isolated from the tea plants (*Camellia sinensis*) of Assam tea gardens, was suggested to be the most efficient P solubiliser. Chadha et al. (2015) isolated endophytic fungi from the roots of tomato and demonstrated the P-solubilising potential to be significant in the isolates like *Aspergillus versicolor, Aspergillus niger*, *Fusarium fusarioides* and *Chaetomium globosum.* An endophytic fungal isolate *Byssochlamys nivea* obtained from the plant *Pistacia vera* was suggested as a P-solubilising fungus (Dolatabad et al. 2017). According to a study, a root endophytic fungi, *Piriformospora indica*, was assessed for its P-solubilising capacity (Ngwene et al. 2016). Two endophytic fungi, *Aspergillus fumigatus* and *Fusarium proliferatum*, were isolated from the roots of *Oxalis corniculata* and then screened positive for P solubilisation (Bilal et al. 2018). Lubna et al. (2018) conducted a report in which the screening of *Aspergillus flavus* for P solubilisation besides other growth-promoting activities was performed. Other reports on isolation of endophytic PSF have been tabulated (Table 17.1). Endophytic fungal isolates forming clear halos on Pikovskaya agar have been shown in Fig. 17.2.

17.2.3 Phosphate-Solubilising Fungi Associated with Mycorrhizal Region

Mycorrhizae are the symbiotic association of fungus with the roots of vascular plants. They are called arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi when they colonise root tissues intracellularly and extracellularly, respectively. The mycorrhizal fungi have been investigated for their role in the increased plant uptake of phosphate and other micronutrients. It has also been demonstrated that the inoculation of these fungi in the plant help plant to use more soluble phosphate from the fertiliser. This is so because mycorrhizae have increased root phosphate-absorbing sites due to the presence of extraradical mycelium (Khan et al. 2007). It has also been well established that mycorrhiza engage other microorganisms on their surface, as they are known to produce significant hyphae biomass in soil and also help them to release inorganic P in the soil for plants (Scheublin et al. 2010; Agnolucci et al. 2015; Zhang et al. 2018). In one such report, Zhang et al. (2018) studied that the fructose exuded by AMF, *Rhizophagus irregularis*, helped the (PSB)

Table 17.1 Phosphate-solubilising fungi

S. no.	Habitat	Organism	Reference
1.	Rhizosphere	*Aspergillus terre*us	Abdel-Ghany and Alawlaqi (2018)
		Aspergillus spp.	Khan et al. (2018)
		Aspergillus ustus and *Aspergillus tamarii*	Pany et al. (2018)
		Aspergillus aculeatus	Yin et al. (2017)
		Aspergillus awamori	Jain et al. (2012)
		Aspergillus niger and *Penicillium notatum*	Malviya et al. (2011)
		Aspergillus niger	Tallapragada and Seshachala (2012)
		Penicillium oxalicum	Singh and Reddy (2011)
		Penicillium spp. and *Talaromyces* spp.	Scervino et al. (2010)
		Absidia spp.	Nenwani et al. (2010)
2.	Endophytic region	*Piriformospora indica*	Wu et al. (2018)
		Aspergillus oryzae	Devi and Packialakshmi (2018)
		Aspergillus spp.	Sarbadhikary and Mandal (2018)
		Aspergillus fumigatus and *Fusarium proliferatum*	Bilal et al. (2018)
		Aspergillus flavus	Asaf et al. (2018)
		Byssochlamys nivea	Dolatabad et al. (2017)
		Xylaria regalis	Adnan et al. (2018)
		Piriformospora indica	Ngwene et al. (2016)
		Aspergillus versicolor, Aspergillus niger, Fusarium fusarioides, Chaetomium globosum	Chadha et al. (2015)
		Penicillium sclerotiorum	Nath et al. (2015)
		Trichoderma gamsii	Rinu et al. (2014)
3.	Mycorrhizal	*Rhizophagus irregularis*	Zhang et al. (2018)
		Rhizophagus irregularis and *Penicillium aculeatum*	Efthymiou et al. (2018)
		Glomus fistulosum	Osorio and Habte (2013)
		Glomus aggregatum and *Glomus mosseae*	Zhang et al. (2011)
		Glomus intraradices, Glomus mosseae	Suri et al. (2011)

bacterium, *Rahnella aquatilis*, in increasing the expression of phosphate genes and the rate of phosphatase release in the growth medium. Similarly, a report conducted by Yousefi et al. (2011) suggested the interaction of PSB and AMF in increasing inorganic P uptake by the wheat plant. The dual action of fungus *Mortierella* spp.

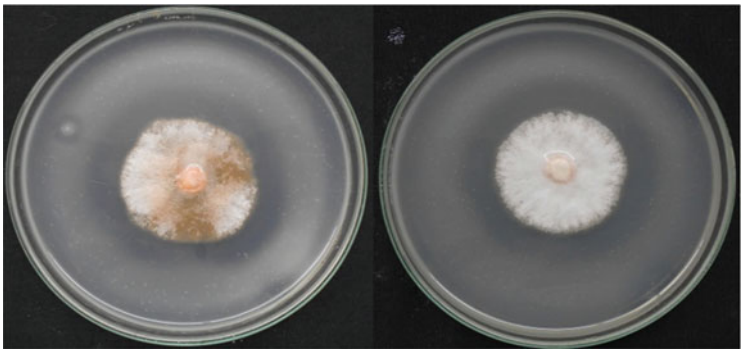

Fig. 17.2 Solubilisation of inorganic phosphate by phosphate-solubilising fungi on Pikovskaya agar plates

with two other mycorrhizal fungi, *Glomus aggregatum* and *Glomus mosseae*, was investigated for the increased P-solubilising action (Zhang et al. 2011).

17.3 Mechanism and Significance of Phosphate Solubilisation

Phosphate solubilisation is accomplished through various biological processes or mechanisms. Since phosphorous is present in the soil in both organic and inorganic forms, therefore, phosphate-solubilising activity is determined by the ability of fungi to release various metabolites like organic and inorganic acids, proton extrusion, enzymes, etc. Sims and Pierzynski (2005) reported major processes affecting the soil phosphorous concentrations. This involves dissolution-precipitation (mineral equilibria), sorption-desorption (interaction of phosphorous in solution with soil solid surfaces) and mineralisation-immobilisation (biologically mediated conversions of phosphorous between organic and inorganic forms). Majority of the global cycling of phosphorous in the soil is attributed to bacteria and fungi (Sharma et al. 2011). An overview of the plant growth promotion by phosphate-solubilising fungi is shown in Fig. 17.3.

Phosphate-solubilising fungi (PSF) employ three main mechanisms for the phosphate solubilisation which includes (a) release of metabolites, (b) biochemical mineralisation and (c) biological mineralisation. PSF augment the solubilisation of inorganic phosphorous by the release of metabolites such as complexing or mineral-dissolving compounds like low-molecular-weight acids (both organic and inorganic acids), siderophores, protons, hydroxyl ions, CO_2, etc. On the other hand, biochemical and biological mineralisation of organic phosphorous is mediated as a consequence of synthesis of a variety of different extracellular enzymes like phosphatases catalysing the hydrolysis of phosphoric esters and the release of phosphorous during

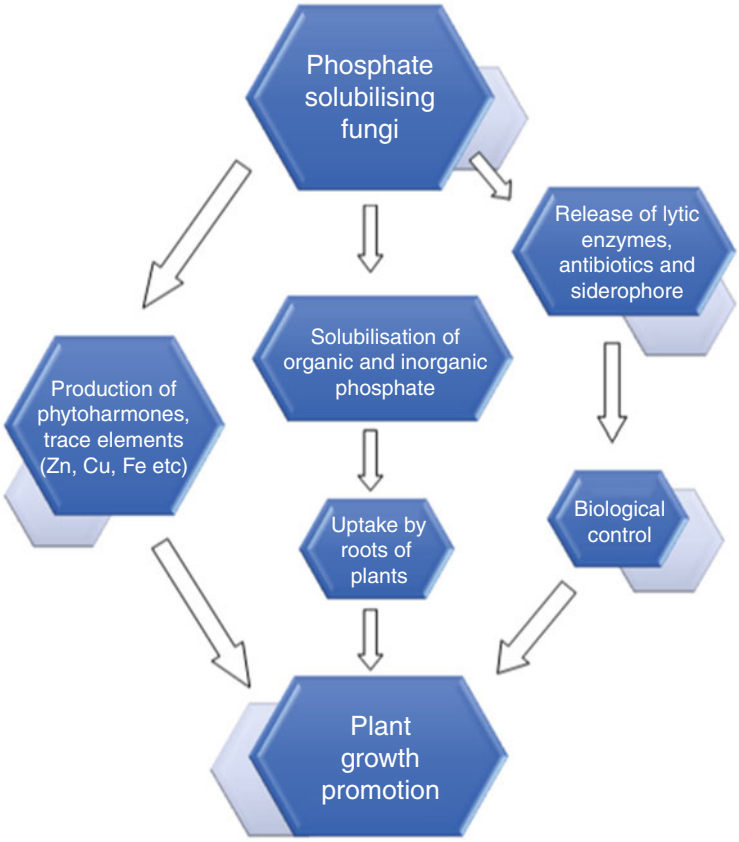

Fig. 17.3 Schematic representation of plant growth promotion by phosphate-solubilising fungi

substrate degradation (McGill and Cole 1981; Glick 2012; Sharma et al. 2013a, b) respectively.

17.3.1 Solubilisation of Inorganic Phosphorous

The solubilisation of inorganic phosphorous by PSF occurs mainly by the release of organic acids. Organic acid production by PSF results in lowering of pH of medium (Maliha et al. 2004). They are synthesised on the outer face of cytoplasmic membrane by direct oxidation pathway such as oxidative respiration and fermentation of organic carbon sources (Zaidi et al. 2009). The direct dissolution of phosphate ions is augmented by exchange of phosphate anions with acid anions (Omar 1997). In addition, phosphate-solubilising fungi (PSF) follow chelation-mediated mechanism to act on sparingly soluble phosphorous present in the soil. This is mediated by direct

Table 17.2 Organic acids produced by phosphate-solubilising fungi isolated from different ecological niches

Phosphate-solubilising fungi	Ecological niche	Organic acid	Reference
Aspergillus and *Penicillium* sp.	–	Formic, propionic, lactic, acetic, glycolic, fumaric acid	Abdel-Ghany and Alawlaqi (2018)
Aspergillus niger	–	Carboxylic acid	Sahoo and Gupta (2017)
Aspergillus niger	Soybean rhizosphere	Citric and oxalic acid	Li et al. (2016)
Penicillium oxalicum	Maize rhizosphere	Oxalic, formic and tartaric acid	Li et al. (2016)
Aspergillus niger	Tropical soil	Gluconic and oxalic acid	Thakur et al. (2014)
Aspergillus flavus, A. niger, Penicillium canescens	Stored wheat grains	Oxalic, citric and gluconic acid	Thakur et al. (2014)
Aspergillus sp., *Penicillium* sp., *Chaetomium nigricolor*	Lateritic soil	Oxalic, succinic, citric and 2-ketogluconic acid	Thakur et al. (2014)
Aspergillus niger, Penicillium sp.	Soil	Citric, glycolic and succinic acid	Thakur et al. (2014)

dissolution of phosphate ions by chelation of cations associated with phosphate ions. The hydroxyl and carboxyl groups of organic acids produced by PSF act on the cations (Al, Fe, Ca), thereby chelating these cations and releasing the phosphate ions for utilisation by plants (Sharma et al. 2011; Vassilev et al. 2015). Previous studies have reported secretion of organic acids by the phosphate-solubilising fungi. Various species of *Aspergillus* and *Penicillium*, viz. *A. niger, A. flavus, A. candidus, A. awamori, A. foetidus, A. terricola, A. japonicum, A. tamari, A. amstelodami* and *A. fumigatus* and *P. oxalicum, P. canescens, P. rugulosum, P. variabile, P. radiacum* and *P. bilaji*, are known to produce organic acids. The predominant acids produced by them are succinic, gluconic, citric, malic, maleic, acetic, tartaric, oxalic, ketogluconic and fumaric acids (Khan et al. 2010). Some other phosphate-solubilising fungi, their ecological niches and organic acids produced by them are tabulated in Table 17.2.

Plants assimilate phosphorous as mono ($H_2PO_4^-$) or dibasic ions (HPO_4^{2-}); however, monovalent phosphate ions are the only soluble form of inorganic phosphate. Since this form is solubilised at low pH, an increase in pH leads to an increase in dibasic and tribasic forms of Pi that makes it inaccessible to the plants. PSF convert it into soluble form by lowering the pH. As already mentioned, this is achieved through the production of various organic acids that result in the acidification of their surrounding environment, ultimately releasing phosphate ion from the mineral by substituting H^+ bound to it (Goldstein 1995). In addition, solubilisation of inorganic phosphate has also been reported to be carried out by PSF by releasing some inorganic acids like HCl. Various studies support the solubilisation of phosphorous by different inorganic acids. Some of the genera like *Penicillium* and

Aspergillus have been reported to produce inorganic acids for solubilisation of phosphate (Whitelaw 1999). However, there are other processes (nitrate formation or CO_2 release) that might be contributing toward the solubilisation of the phosphate, but they are considered as less effective as compared to the solubilisation by organic acids (Rudolph 1922). A model proposed by Krishnaraj (1998) depicted that there could be an alternative mechanism of phosphate solubilisation. According to this model, protons (H^+) released during NH_4^+ assimilation are pumped out of the cells and solubilise phosphate. This model ruled out the direct involvement of organic and inorganic acids in phosphate solubilisation. Asea et al. (1988) reported that in some of the fungi, NH_4^+-driven proton release is the sole mechanism supporting phosphate solubilisation. Besides, H_2S released by PSF reacts with ferric phosphate and forms ferrous sulphate, thereby releasing phosphate (Swaby and Sperber 1958) and playing a role in the solubilisation process.

17.3.2 Mineralisation of Organic Phosphates

Solubilisation of organic phosphate is regarded as mineralisation of organic phosphorous. It is accomplished by the release of various enzymes such as phosphatases, phytases and phosphonatases. Phosphonatases act on C-P bonds of organophosphonates and lyse them (Sharma et al. 2013a, b). Although phytate is the major component of organic phosphorous in the soil and constitutes the major stored form of organic phosphorous in pollens and seeds, its availability is limited to plants. In order to make it soluble, microorganisms like fungi come into play by releasing the enzyme phytase which acts on the substrate phytate, degrade it and release phosphorous (Richardson 1994).

In an experiment to improve the growth of *Arabidopsis* plants, Richardson et al. (2001) transformed the plant with phytase gene (phyA) derived from *Aspergillus niger*. Observation of the results revealed that the growth and phosphorous content of the transformed plants was much higher than the control plant supplied with inorganic phosphate. Other enzymes like phosphatases which dephosphorylate phosphor-ester or phosphor-anhydride bonds of organic matter are also known to solubilise organic phosphate. These are non-specific acid phosphatases and among them phosphor-mono-esterase have been reported to be produced by PSF (Sharma et al. 2013a, b). They can be further categorised as acidic and alkaline phosphor-mono-esterases (Nannipieri et al. 2011). Further studies are required to be done for understanding the mechanism of phosphatases in the mineralisation of organic phosphorous (Chen et al. 2003).

It is quite evident that various mechanisms are active in various organisms for the solubilisation of phosphate. Each organism can, therefore, act on insoluble phosphorous either by one or more ways. Although detailed studies are required to be done to find out the single mechanism involved in phosphate solubilisation by a particular PSF, however, mechanism involving production of organic acids seems to be of great significance.

17.3.3 Other Molecules in Phosphate Solubilisation

A few workers have reported the significance of other molecules such as siderophores and exopolysaccharides in phosphate solubilisation. Siderophores are low-molecular-weight compounds chelating free iron present in soil (Mukherjee et al. 2018). Different kinds of siderophores, viz. siderophorin, ferricrocin and glomuferrinare, are produced by fungi (Winkelmann 2017; Karmakar et al. 2018). Exopolysaccharides are carbohydrate polymers that are released by the microorganisms outside their cell wall. They may be further categorised as homo- or heterosaccharides depending upon their structure and composition. Their potential role in phosphate solubilisation has been reported. Although very few reports are available, certain secondary metabolites like hydrogen cyanide (HCN) have been reported to play an important role in geochemical processes in the substrates. They can chelate metal ions and thus indirectly increase the availability of phosphate (Rijavec and Lapanje 2016) (Fig. 17.4).

17.4 Agronomic Effectiveness of Phosphate-Solubilising Fungi

The use of phosphate-solubilising fungi as live microbial biofertilisers provides a promising alternative to chemical fertilisers and pesticides. They can promote nutrient exchange and show biocontrol against various pathogens, thereby increasing the plant growth. Besides antagonism, the utilisation of the conventional phosphorous fertilisers in agriculture would also meet some global issues like increased global food demands, pollution of surface and groundwater, waterway eutrophication, depleted soil fertility and accumulation of toxic metals (Se, As) in soil. Quite a number of soil microorganisms are capable of improving the growth and yield of a wide variety of crops by solubilising/mineralising insoluble soil phosphate to release soluble P and making it available to plants. Thus, inoculating seeds/crops/soil with PSF is a promising strategy to improve world food production without causing any environmental hazard. An overview of significant contribution of PSF in agriculture has been represented schematically in Fig. 17.5.

17.4.1 Plant Growth Promotion

The excessive use of chemical fertilisers to improve soil fertility and plant health is not a long-lasting approach, as it has limitations. Alternatively, the use of phosphate-solubilising fungi opens up innovative research mechanisms for better plant productivity as well as protecting the environment from hazards of agrochemicals (Gomez-Munoz et al. 2017). There are a number of reports of a wide range of microorganisms

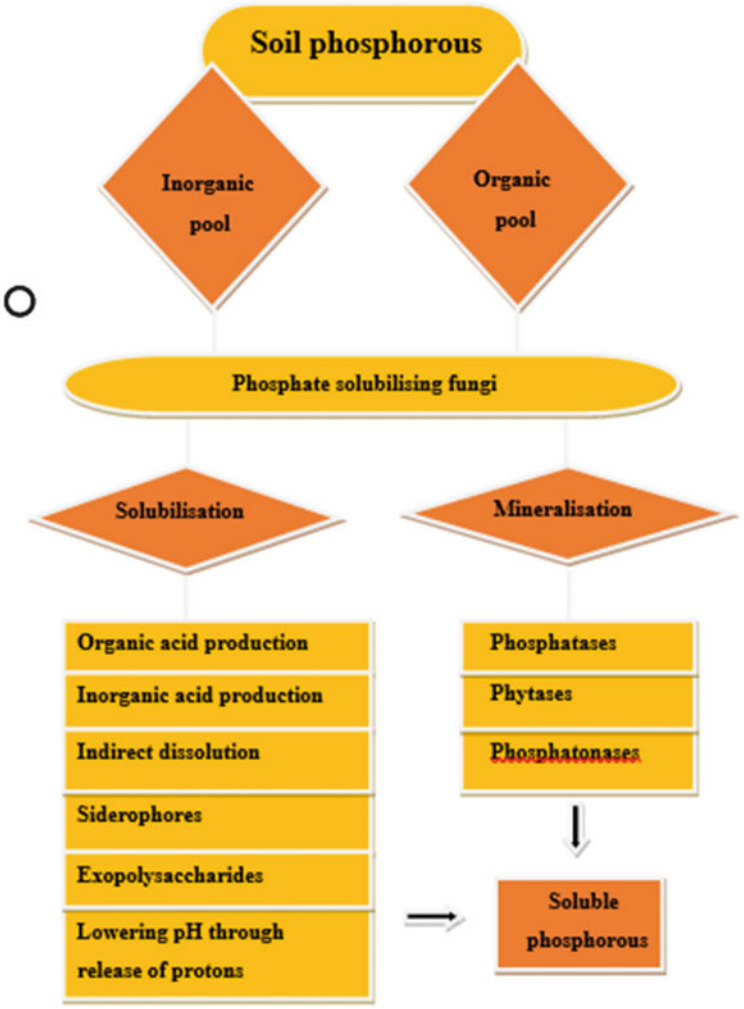

Fig. 17.4 An overview of mechanism involved in phosphate solubilisation

with potential role in plant growth promotion by increasing P uptake by plants. In one such report, the effect of *Penicillium bilaiae* on the growth of wheat plant was studied, and it was found that the increase in the growth of root of the wheat plant was due to the increased availability of P (Gomez-Munoz et al. 2017). Another study reported the inoculation of *Aspergillus* spp., isolated from different rhizospheric soils of Indian regions, in the plants like wheat and chickpea promoted their growth significantly, thus suggesting its potential in plant growth promotion (Pandya et al. 2018). Also, in some other report, *Aspergillus niger* was evaluated for its plant growth promotion potential in improving the growth of wheat plant. Rojas et al. (2018) suggested the effectiveness of AMF and two strains of P-solubilising fungi

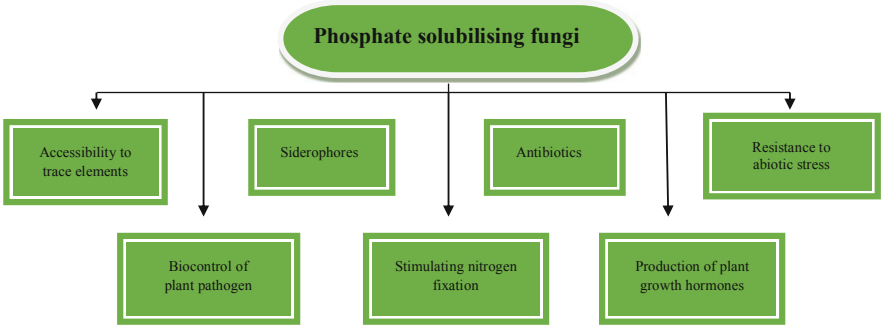

Fig. 17.5 An overview of significant contribution of PSF in agriculture

(PSF), *Aspergillus niger* and *Penicillium brevicompactum*, in increasing available soil phosphorus for the growth and development of coffee plants. The solubilisation of P by a biocontrol fungal strain *Trichoderma harzianum* has been investigated in tomato plant, and it was concluded that this biocontrol agent has the potential to enhance the P uptake, thereby increasing growth and nutrient uptake by the plant (Li et al. 2015).

A comparative study of P solubilisation and the host plant growth promotion ability of *Fusarium verticillioides* and *Humicola* spp. under salt stress was conducted, and the result of this study was that the endophytic fungus *F. verticillioides* was more efficient to protect soybean plants from oxidative damage than *Humicola* spp. (Radhakrishnan et al. 2015). An endophyte *Piriformospora indica* promotes growth of *Brassica napus* by the combined effect of P-solubilising activity and higher gene expression (Wu et al. 2018). Similarly, endophytic *Aspergillus* spp. were subjected to field trail, and it was found that its application enhanced various growth and yield parameters significantly in tomato and brinjal (Sarbadhikary and Mandal 2018).

17.4.2 Biocontrol Activity

Fungi-mediated solubilisation of insoluble phosphates is also associated with biochemical mechanisms and production of metabolites which take part in biological control against soilborne phytopathogens. In vitro studies have also reported the potential of P-solubilising microorganisms, for the simultaneous synthesis and release of metabolites like siderophores, pathogen-suppressing metabolites, phytohormones and lytic enzymes for suppressing the growth of pathogen. Various studies have shown that indole acetic acid and siderophores are among most frequently studied metabolites secreted by phosphate-solubilising fungi (Vassilev et al. 2006). Siderophores are low-molecular-weight compounds that can chelate free iron present in soil (Winkelmann, 1991). Different kinds of siderophores produced

by fungi includes Ferricrocin from *Trichoderma virens* (Mukherjee et al. 2018), mixed ligand siderophores from *Arthroderma cuniculi* (Karmakar et al. 2018) and glomuferrin from arbuscular mycorrhizal fungi *Glomus* (Winkelmann 2017). Chelation of freely available iron in the soil results in competition among the microbes for available iron (Lemanceau et al. 1986). Iron is an important molecule for metabolism of the microorganisms, as it can act as cofactor for various enzymes. Therefore, siderophore production is beneficial to the plants, as it enhances the growth of siderophore-producing fungi and limits the growth of other fungi (Prabhu et al. 1996). The plant growth promoters especially auxins play a significant role in host– parasite interactions. Among auxins, IAA is involved in the interaction between a plant pathogen and its host (Hamill 1993). Two hypotheses have been proposed for the mechanisms of biocontrol action of IAA by some workers. One hypothesis proposed that IAA together with glutathione S-transferases is potentially involved in defence-related plant reactions (Hahn and Strittmatter 1994; Droog 1997), and the second hypothesis supported the fact that spore germination and mycelium growth of different pathogenic fungi are inhibited (Brown and Hamilton 1993). Association of AM fungi with plant roots reduces the chances of pathogen attack (Morandi, 1996). This may be because of the accumulation of certain metabolites like flavonoids, isoflavonoids, phytoalexins, etc. or the production of hydrolytic enzymes (Pozo et al. 1998).

Besides, PSF can suppress the growth of various pathogens by limiting the supply of essential nutrients required by the plants competing for space, etc. (Bhattacharyya et al. 2016). Since fungal microflora have been reported to produce diverse array of bioactive metabolites, the soil sustaining such microflora is rich in antifungal antibiotics and suppresses various disease. Various PSF such as *Trichoderma harzianum*, *Aspergillus* and *Penicillium* sp. (Altomare et al. 1999) are considered as potential hub for diverse antibiotics. However, the mechanism of biocontrol activity is known but that responsible for plant growth promotion still needs to be studied in detail. Antibiotics produced by antagonistic fungi have either biostatic or biocidal effects on soil-borne plant pathogens. Certain metabolites like siderophores, HCN, organic acids or lytic enzymes produced by PSF *Trichoderma harzianum, Aspergillus niger, A. awamori, P. digitatum, P. variavile* and arbuscular mycorrhizal fungi show significant role in their antagonistic potential (Vassilev et al. 2006). Field trials of *A. awamori* and *P. digitatum* using root dip application on *Fusarium* wilt in tomato caused by *F. oxysporum* resulted an increase in tomato yield from 28 to 53% (Khan and Khan 2001, 2002).

17.4.3 Phosphate-Solubilising Fungi as Biofertilisers

The phosphate-solubilising fungi have gained the interest of scientific community especially the agronomists (Khan et al. 2010). Their role in plant growth promotion has revealed their potential as biofertilisers. The term ‘microphos’ has been proposed for viable microbial preparations possessing solubilisation of insoluble phosphorous

under in vitro conditions. The production of microphos firstly involves screening, selection and evaluation of phosphate-solubilising potential of the fungal isolates. This is followed by the development of fungal inoculants. This process also involves selection of carriers with which the inoculum could be mixed. The carrier materials for using fungal inoculants as microphos are soil, peat, manure, cow dung-cake powder, etc. Instead of using a single culture (monoculture), mixed cultures (co-culture) either of same or different groups can be used for the development of microphos. Mixed cultures may also involve mixing of same or different groups like using one or more fungi as co-inoculants or using one or more fungi with bacteria as co-inoculants. The compatibility of the two cultures plays a vital role in the development of mixed inoculants. Had there been no compatibility or some sort of antagonistic activity, it would have not been possible to use them under laboratory conditions. Once the fungal inoculants are developed, they undergo a quality check and hence released for distribution to the farmers. There are a few reports where some fungal inoculants have been released commercially, viz. *Penicillium bilaiae* (JumpStart; Philom Bios, Saskatoon, Canada) and *Penicillium radicum* (PR-70 RELEASE; BioCare Technology, Somersby, Australia).

Li et al. (2015) investigated the capability of *Trichoderma harzianum* to solubilise sparingly soluble phosphate and other minerals. The results of this study suggest that the induction of increased or suppressed plant growth occurs through the direct effect of *T. harzianum* on root development. Presence of organic acids including lactic acid, citric acid, tartaric acid and succinic acid was also detected by HPLC and LC/MS in these isolates. Chagas et al. (2016) reported efficiency of *Trichoderma* spp. as a growth promoter of cowpea (*Vigna unguiculata*). They were found to have a greater ability to synthesise IAA and solubilise phosphate than the controls. Steiner et al. (2016) reported increased dry matter yield of sorghum upon inoculation with *P. pinophilum* and *A. terreus* and application of phosphorous rock. In an experimental trial, three endophytic species of *Penicillium*, viz *P. oxalicum, P. glabrum* and *Penicillium* spp., isolated from *Piper longum* were applied as biofertilisers, and outstanding results were observed. It was observed that spike development in the control plants occurs after 180 days, whereas maturation of the fruits and spike development in the plants treated with *Penicillium glabrum* and *Penicillium* spp. occurred after 150 days (Sahoo and Gupta 2017). In a greenhouse experiment conducted on cherry tomato, the potential of three *Trichoderma* isolates and two homeopathic preparations (Phosphorus 6CH and Carbo vegetabilis 6CH) was observed. *Trichoderma asperellum* was found to enhance the leaf area and dry mass of leaves and roots, while the homeopathic preparations applied did not show any effect (Franca et al. 2017). A current study conducted on thermohalotolerant *Aspergillus terreus* isolated from rhizospheric soil depicted that it increases the biomass and phosphorous content of *Hordeum vulgare* plants; it can improve crop production by maintaining the levels of available phosphorous in the saline soil. Therefore, *A. terreus* can be used as a substitute for chemical fertilisers (Ghany et al. 2018). Synergistic effects of phosphate-solubilising fungi on the growth and development of plants have also been reported (Abdel-Ghany and Alawlaqi 2018).

17.4.3.1 Mode of Application of Phosphate-Solubilising Fungal Inoculants

Traditionally, two methods have been used commonly for the application of biofertilisers. One of the most common and widely used methods is the application of the inoculant on the surface of the seeds prior to sowing. The process of application of microphos involves soaking of the selected seeds into the liquid culture medium and mixing of the seeds with fungal inoculants adhered to their carriers. The proper mixing allows fungal inoculants to attach onto the surface of the seeds. Although it is the most widely used method, it has some drawbacks, viz. less population of PSF may be attached to the seed surface and survivability of the inoculated fungi is adversely affected by the chemicals and fertilisers applied to the seeds and soil after planting respectively. As discussed earlier, two approaches can applied for application of microphos: monoculture approach (MCA) and co-culture approach (CCA).

An alternative method involves application of inoculants directly to the soil. Soil application method results in increased population of PSF per unit area. Unlike the first method, this method reduces direct contact of the fungal inoculants with chemically treated seeds. This method is quicker, since it does not involve mixing of seeds with inoculants. In contrast to the carrier-based inoculants, these inoculants can withstand low-moisture conditions in a better way.

17.4.3.2 Factors Affecting the Survival of Phosphate-Solubilising Fungal Inoculants

Addition of PSF as inoculants to the soil results in certain changes in the community composition, its structure and function as well. These changes in the environments exert a selection pressure on the inoculants for adaptation to a new condition (Khan et al. 2010). An exhaustive study to understand various factors influencing fungal community composition, how they are affecting it, what type of response is generated by the PSF and how these responses improve the phosphate-solubilising potential of the fungal inoculants is required to be done. Various factors affecting the survival of the PSF in the soil have been reported like physicochemical properties of soils (Bashan et al. 1995), moisture content (Van Elsas et al. 1991), genotype, age of the plants, composition of the phytochemicals and root exudates, presence of environmental pollutants (heavy metals, fertilisers, pesticides) in soils (Taiwo and Oso 1997) and the presence of recombinant plasmids (Van Veen et al. 1997). Since the composition, type and amount of exudates produced by the plants vary from species to species, the community composition in the rhizosphere varies accordingly. The exudates produced by the plant species include high- and low-molecular-weight compounds. High-molecular-weight compounds produced by the plant species are proteins, mucilage, etc., whereas low-molecular-weight compounds are phenolics, sugars, amino acids, organic acids and various secondary metabolites.

Although it has been reported that the density of the fungal inoculants decreases rapidly upon inoculation into the soil, therefore, a better understanding of various interactions existing in the fungal inoculants with their surrounding for establishment in the competitive environment under different agro-ecological regions of the world would help in development of potential PSF as biofertilisers.

17.5 Conclusion and Future Prospects

Phosphate-solubilising fungi have significant potential in plant growth promotion; therefore, they can be used as biofertilisers. They enhance sustainable agriculture by mobilising soil inorganic or organic phosphate and making it available to the plants. Nowadays, it is a great responsibility of agronomists to find out different ways to improve soil phosphorous availability without applying the chemical P fertilisers. Besides improving the fertility and productivity of the soil, PSF also protect the environment from agrochemicals. PSF as microbial inoculants is a new horizon for better plant productivity. Extensive and consistent efforts are required by the scientific community so as to screen, identify and characterise more PSF. This would help in the development of phosphate-solubilising fungal inoculants that could be applied by the farming communities under field conditions.

Additional insights on PSF as biofertilisers such as better management, development of more effective microbial inoculants through the genetic manipulation of specific organisms or with a combination of these approaches would likely improve their use and help in establishment of sustainable agriculture, and our movement from a green revolution to an evergreen revolution can be accomplished. Although the practice of microbial application to enhance the fertility of soil is extensively used in developed countries like the UK and USA, in the developing countries like India, these practices have now been initiated and subsequently need to be developed. Since the focus of consumers of agricultural products is on the health, quality and nutritional value, employment of PSF as biofertilisers is an option to increase food production without imposing hazardous effects on the environment.

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Chapter 18
Fungi as Biological Control Agents

Savita and Anuradha Sharma

Abstract Nowadays, use of a fungal biocontrol agent (BCA) is considered to be a rapidly developing natural phenomenon in research area with implications for plant yield and food production. Fungal biocontrol agents (BCAs) do not cause any harm to the environment, and they generally do not develop resistance in various types of insects, pests, weeds, and pathogens due to their complex mode of action. They have been proved to be an alternative against the undesirable use of chemical pesticides. The advantage of fungi to be used as biological control agents is that they need not be ingested by the insect hosts, but they can invade directly through the insect's cuticle and control all insect pests including sucking insects, but in the case of viruses and bacteria, this is not possible. The present literature includes mechanisms of fungal biological control agents, advantages and limitations of BCAs, and list of commercially available BCAs against the insects, pests, weeds, nematodes, and plant pathogens.

18.1 Introduction

According to the most recent estimate by the UN, the population of the world is 7.3 billion, which may reach up to 9.7 billion by the end of 2050. This increase in population may result in food demand to increase anywhere between 59% and 98% by 2050 (Ray et al. 2013). Farmers worldwide will need to increase crop production. To fulfill the growing demand for food quality and quantity, we need to increase the crop production either by increasing the amount of agricultural land to grow crops or by enhancing productivity by controlling the crop losses caused by plant pathogens, pests, animals, and weeds (Strange and Scott 2005). Roughly 20–40% direct yield losses are caused by weeds, pathogens, and animals (Oerke et al. 1994; Teng and Krupa 1980; Teng 1987; Oerke 2006).

In the 1960s–1980s, synthetic insecticides, herbicides, and fungicides were introduced for the successful control of agricultural pests to increase the agricultural

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output. Ideally, the pesticides must be specific to their target, but actually, this is not the case. There is no doubt that the use of pesticides has increased the production of food and fibre, but they also have resulted in serious health implications to man and his environment because they are not specific to their target. Nowadays, enough evidences are available which prove that some of these chemicals are responsible for environmental damage and they have also adversely affected the human health (Forget 1993; Igbedioh 1991; Jeyaratnam 1985; Zeise et al. 2013; Eduati et al. 2015). Almost each and every segment of population has been exposed to pesticides, and the estimated number of worldwide deaths due to chronic diseases caused by pesticide poisoning is about 1 million per year (WHO 1990; Environews Forum 1999). Organochlorine (OC) compounds have polluted all life forms on the earth including air and water bodies such as lakes, rivers, and oceans (Hurley et al. 1998; Yusof et al. 2016). According to US National Academy of Sciences, the DDT metabolite DDE caused the decline in the population of bald eagle due to eggshell thinning (Liroff 2000). The pesticides, also known as endocrine disruptors, adversely affect the human health by antagonizing natural hormones in the body. The long-term and low-dose exposure of these chemical pesticides can cause immune suppression, reproductive abnormalities, hormone disruption, and cancer (Crisp et al. 1998; Hurley et al. 1998; Brouwer et al. 1999; Roghelia and Patel 2017).

Nowadays, strict regulations have been formulated against the use of chemical pesticide. Therefore, the alternative approaches are being developed by the pest management researchers to replace the use of synthetic chemicals for controlling the plant pathogens and the pests. Among few potent alternatives, the biological control agents are preferred eco-friendly approaches. It is considered to be a natural method for controlling the pests by using the living organisms. Those living organisms which are used to control the invasive species, and which are generally the natural enemies of the same are called as the "biological control agents." Biocontrol means the use of living organisms to suppress the growth of the population of a pest. It is also called as "biological suppression". Nowadays, fungi are considered as a new means of biological control against weeds and pathogens to improve the plant yield and food production. The present literature includes the past and current progress of fungal biocontrol agents and understanding about the mode of mechanism.

18.2 Fungi as Biocontrol Agents

Nowadays, various biocontrol products are being produced commercially by using fungi to control the insect pests and plant diseases. The successful use of fungi as biocontrol agents is reported by Hasan (1972), Cullen et al. (1973), Hasan and Wapshere (1973), Emge et al. (1981), Shah and Pell (2003), Faria and Wraight (2007), and Lacey et al. (2015). Natural methods alone are not efficient to control the plant diseases, insect pests, and weeds because they are more labour-intensive than chemical pesticides. However, fungal biological control agents (BCAs) do offer several benefits which are as follows:

- Fungi are ubiquitous in distribution.
- They have high degree of host specificity.
- They are persistent, and they have dispersal efficiency, and they can cause destruction of the host.
- It is easy to culture and maintain the fungi in the laboratory.
- Fungi do not adversely affect the environment, and they are specific to their target, while the chemicals are not target specific.

18.3 Mechanism of Fungi-Mediated Biocontrol

Fungi use several mechanisms to prevent infection or to suppress the growth of insect pests and weeds, which include the following methods for effective biocontrol.

18.3.1 Direct Antagonism (Hyperparasitism)

Direct antagonism is a process in which a pathogen is killed by other microorganisms. It is also called as hyperparasitism (Baker and Cook 1974). If a fungus is parasitic on other fungi, then it is called as a mycoparasite. *Ampelomyces quisqualis* (deuteromycete hyper-parasite) reduces the growth of mildew colony through hyperparasitism and eventually kills them by producing pycnidia (fruiting bodies) within powdery mildew (Erysiphales) hyphae, conidiophore, and cleistothecia. *Trichoderma lignorum* (*T. viride*) control the damping off of citrus seedlings by parasitizing the hyphae of *Rhizoctonia solani* (Weindling 1932; Lo 1997; Harman et al. 2004; Asad et al. 2014; Abbas et al. 2017). *Trichoderma* species shows hyperparasitism against many economically important plant pathogens that makes *T. species* more suitable for the development of biocontrol strategies (Harman et al. 2004; Motlagh and Samimi 2013).

18.3.2 Antibiosis

When two or more organisms interact with each other and that interaction is harmful to at least one of them, this type of association is known as antibiosis. It can also be an association between an organism and the metabolic substances produced by another. Antagonistic fungi secrete antimicrobial compounds to suppress the growth of pathogenic fungi in the close proximity of its growth area. The loss of activity in nonproducing mutants of the antagonist provides the ultimate proof for the role of these compounds in biocontrol; for example, gliotoxin-minus mutants of *Gliocladium virens* loses its 50% antagonistic effect against the disease-causing

pathogen as compared to the wild type (Wilhite et al. 1994; Vargas et al. 2014; Vinale et al. 2014). Most fungi are capable of secreting one or more compounds and secondary metabolites with the antibiotic activity. The most common species that produce the antibiotics are *Trichoderma* and *Gliocladium* spp.; *Trichoderma virens* (syn. *Gliocladium virens*) produces two major antifungal antibiotics, gliotoxin and gliovirin (Howell et al. 1993, Mendoza et al. 2015). *Trichoderma pseudokoningii* and *T. viride* inhibit *Botrytis cinerea* on strawberry fruits by producing some secondary metabolites (Tronsmo and Dennis 1977). Bae et al. (2001) evaluated the antibiosis of the culture filtrate of *Trichoderma* spp. against *Phytophthora capsici* and their phytotoxic activities against pepper. In this study, the strain DIS 320c (*T. caribbaeum* var. *aequatoriale*) showed 100% antibiosis against *P. capsici*. Nelson and Powelson (1988) reported that *Trichoderma hamatum* reduced the growth of *Botrytis cinerea* which causes grey mould of snap bean pods and blossom by 77–97% by producing inhibitory volatile compounds. Menendez and Godeas (1998) reported the inhibitory effect of *Trichoderma harzianum* in biocontrol of *Sclerotinia sclerotiorum* which is a soilborne plant pathogen which affects the yield of many economically important crops, such as soybean. Calistru et al. (1997) reported that the hyphae of *Trichoderma* spp. and *Fusarium moniliforme/Aspergillus flavus* on co-culturing show antibiosis without hyphal penetration, suggesting that mycoparasitism was not the sole cause for the observed inhibitory effects. Therefore, metabolites such as volatiles, extracellular enzymes, and antibiotics produced by *Trichoderma* spp. were probably responsible for antibiosis. Mendoza et al. (2015) evaluated in vitro antagonistic activity *of* 14 strains of *Trichoderma* spp. against *Macrophomina phaseolina*. Eleven out of 14 isolates showed antagonism by competition and stopped the growth of *M. phaseolina*. Szekeres et al. (2005) reported that *Trichoderma* spp. produce antagonistic secondary metabolites, namely, peptaibols and peptaibiotics. These metabolites are linear, amphipathic polypeptides that have strong antimicrobial activity against gram-positive bacteria and fungi (Wiest et al. 2002; Szekeres et al. 2005).

18.3.3 Competition

Competition is a process in which two organisms compete with each other for nutrients such as macronutrients and micronutrients. Some species of filamentous fungi and yeasts can inhibit fungal pathogens by competition, which reduces the concentration of nutrients that become responsible for the reduced rate of spore germination and in slower growth of germ tube (Blakeman and Fokkema 1982; Blakeman 1993; Elad 1995; Funck Jensen and Lumsden 1999). Competition for limiting nutrients leads to starvation which is the most common cause of death of microorganisms, which results in biological control of fungal phytopathogens (Chet et al. 1993). *Trichoderma* spp. produce a number of secondary metabolites with pharmaceutical and biotechnological importance that include nonribosomal peptides, peptaibols, polyketides, pyrones, volatile and non-volatile terpenes, and

siderophores, (Vinale et al. 2008, 2012; Velázquez-Robledo et al. 2011; Müller et al. 2013). The association of *Trichoderma* with the root system of the plant leads to better nutrient and water uptake and provides protection from pathogenic organisms (Harman 2000; Benítez et al. 2004; Harman 2006; Contreras-Cornejo et al. 2013, 2015). Blakeman (1978) reported that iron, which is extremely limited in the rhizosphere, works as a basic tool for biocontrol based on competition. Iron occurs in ferric form in highly oxidized and aerated soils at very low concentration and at pH 7.4 (Lindsay 1979). Under iron starvation, filamentous fungi secrete iron-binding ligands called siderophores, which facilitate the mobilization of environmental iron (Eisendle et al. 2004). Siderophore biosynthesis is negatively controlled by carbon source in *Aspergillus fumigatus* and *Aspergillus nidulans* (Eisendle et al. 2004). These siderophores increase the rhizosphere competence in *Trichoderma harzianum* which can be used as biocontrol agents against other fungi (Chet and Inbar 1994). For example, *Trichoderma* effectively controls the growth of *Pythium* and *Fusarium oxysporum* in soil depending upon the availability of iron (Tjamos et al. 1992).

18.3.4 Induced Resistance

Induced resistance (IR) is considered as one of the important modes of biocontrol in the plants against soilborne pathogens and foliar pathogens (Sequeira 1983; Kuc 1987; Kloepper et al. 1992). Induced resistance limits the growth and spread of pathogen by secreting defence-related enzymes such as chitinases, proteases, and peroxidases (Hammerschmidt et al. 1982; Metraux and Boller 1986). Induced resistance has been demonstrated in vitro against wilt diseases with avirulent strain of fungi, but under field conditions, induced resistance by nonpathogenic strain of *F. oxysporum* is not so effective in sweet potato against *Fusarium oxysporum* f. sp. *batatas* (Ogawa and Komada 1986).

Salicylic acid produced by T39 of *Trichoderma harzianum* induced resistance against *Botrytis cinerea* in bean (De Meyer et al. 1998). When the leaves and roots of cucumber seedlings were inoculated with *Trichoderma harzianum,* it resulted in increased activity of peroxidase and chitinase (Yedidia et al. 1999). If a biocontrol agent is applied directly on a separated part of the infected plant, it demonstrates induced systemic resistance (ISR), while the use of dead cells of inducer (BCA) to suppress the disease may demonstrate the local induced resistance (IR). For example, the use of dead cells of T39 can inhibit the infection of powdery mildew on cucumber and the infection of *Botrytis cinerea* on tobacco, pepper, and beans.

Redman et al. (1999) reported that mutualistic symbiotic association between the host and the nonpathogenic isolate of fungi confers the disease resistance against other pathogenic fungi; for example, a pathogenic isolate of *Colletotrichum magna* (a common pathogen of cucurbits) was converted to a nonpathogenic isolate by UV radiation and gene disruption. This converted nonpathogenic endophytic mutualist enables the symbiont to confer disease resistance against *Phytophthora, Colletotrichum, and Fusarium.* This phenomenon was defined as "endophyte-

associated resistance" (EAR) (Redman et al. 1999). Mycorrhizal fungi prevent soilborne diseases in plants by inducing EAR. However, mycorrhizal plants may be more susceptible to foliar pathogens because pathogenesis-related (PR) proteins take long time to accumulate in the foliage (St. Arnaud et al. 1994; Shaul et al. 1999).

18.4 Limitations of Biocontrol Agents

- An isolate (BCA) may control the growth of a certain pathogen on one crop, but on another crop, it may not be effective to control the disease. This is because of plant host effect. The host on which BCA is effective certainly provides some soluble and volatile exudates secreted by the root, leaf, flower, and seed, which can support introduced BCAs. While on another host on which BCA is not effective, it does not provide such nutrients. For example, PGPR (BCA) is differently effective on different cultivars of wheat (Chanway et al. 1988).
- Microclimate, abiotic factors largely affect the suppression of diseases by BCAs (Shtienberg and Elad 1997). Various factors such as fluctuating temperature, VPD, surface wetness, gases, and air movement affect the indigenous microflora and BCAs directly (Burrage 1971). For example, *Trichoderma harzianum* T39 is more capable to control grey mould in cucumber (fruit and stem) under dry conditions at temperatures above 20 °C in comparison with wet conditions and temperatures below 20 °C (Elad et al. 1993).
- Plant surface produces some chemical exudates that contain macro- and micro-elements, amino acids, organic acids, sugars, sugar alcohols, and pectic substances. Environmental factors along with the age of plant affect the nature and amount of the exudates released from the plants. These changes may modify the leaf characteristics like morphology, chemistry of the surface, and the metabolic state, which directly or indirectly affect plant surface microflora (Cutter 1976). The community in the rhizosphere changes with colonization by bacteria, yeasts, and filamentous fungi that result in the fluctuation in the concentration of nutrients due to competition among microflora (Blakeman 1985). Similarly, rhizosphere is affected by other abiotic factors like rain events, daytime drought, and weathering processes that result in fluctuation in salt concentration and soil particle structure. These changes in the rhizosphere interfere with the establishment and efficacy of the introduced biocontrol agents (BCAs).

18.5 Fungi-Mediated Biocontrol of Insects

Entomophthorales (*Zygomycota*) is the order consisting of a large number of fungal species which are related to biocontrol of insects. Extensive research has been carried out on the use of *Bauvaria* to control chinch bugs in Kansas (Feng and Poprwaski 1994; Lacey et al. 2001). The common fungi which have been used as the

mycoinsecticides include *Cordyceps* species, *Beauvaria,* and *Paecilomyces* which infect the larvae of beetles, moths, and other insects; *Hirsutella* infects the larva of a citrus mite; *Aschersonia* infects citrus white flies, and *Noumorea* infects soybean looper. *Metarhizium* species has a special character to mention, that is, it infects a number of insects by forming long chains of spores. This feature enables its use in novel roach traps, which is superior to use of chemicals because chemicals will kill only the insects that enter the chamber, whereas insects that become infected with *Metarhizium* will carry the fungus to their hiding places and infect their neighbours. *Coelomomyces* species are able to infect the mosquitoes which are the major concern to people because their bites are painful and they transmit some of the most important diseases like malaria, dengue, and chikungunya. Some commercially available products (BCAs) manufactured by using fungi as control agents against insect pests are listed in Table 18.1. The different modes of treatments which can be used for the biological control of insects are as follows.

18.5.1 Permanent Introduction

This method involves the introduction and establishment of native fungi at the site of host population. This is one of the cheapest methods but labour-intensive, involving the periodic release of fungal spores to maintain a high density of the biocontrol fungus. The resting spores of *Entomophaga maimaiga* were released in 1991 and 1992 at 50 sites, over 4 states, to control the larvae of gypsy moths. After a year of release of the fungal spores, gypsy moth populations were found to be declined not only in the areas of spore release, but cadavers of larvae could be found in areas where release of spores did not occur.

18.5.2 Inoculation Augmentation

This method involves the release of the pathogen in the field for seasonal control of disease, which occurs annually, and the inoculation of the fungus is not expected to carry on over the following years. This method is potentially a dangerous technique of dispersing the fungus; however, there is no report till yet of accidents involving this method. The fungi are applied as a spray or dust with the help of air or ground equipment. The inoculations are applied usually at 3-year intervals. The best suitable example for the inoculation augmentation is the use of *Beauveria bassiana* for the biological control of *Dendrolimus* (the pine moth), in the People's Republic of China.

Table 18.1 Products developed from fungi for the biological control of pests

Fungus	Product	Target	Producer
Verticillium lecanii	Mycotal	Whitefly and thrips	Koppert, the Netherlands
	Vertalac	Aphids	Koppert, the Netherlands
Metarhizium anisopliae	BIO 1020	Vine weevil	Licenced to Taensa, USA
	Biogreen	Scarab larvae on pasture	Bio-Care Technology, Australia
	Metaquino	Spittle bugs	Brazil
	Bio-path	Cockroaches	EcoScience, USA
	Bio-blast	Termites	EcoScience, USA
	Cobican	Sugarcane spittle bug	Probioagro, Venezuela
Metarhizium flavoviride	Green Muscle	Locusts, grasshoppers	CABI—BioScience, UK
Beauveria bassiana	Conidia	Coffee berry borer	Live Systems Technology, Colombia
	Ostrinil	Corn borer	Natural Plant Protection (NPP), France
	Corn guard	European corn borer	Mycotech, USA
	Mycotrol GH	Grasshoppers, locusts	Mycotech, USA
	Mycotrol WP and BotaniGard	Whitefly, aphids, thrips	Mycotech, USA
	Naturalis-L	Cotton pests including bollworms	Troy Biosciences, USA
	Proecol	Army worm	Probioagro, Venezuela
	Boverin	Colorado beetle	Former USSR
	Boverol	Colorado beetle	Czechoslovakia
	Boverosil	Colorado beetle	Czechoslovakia
Beauveria brongniartii	Engerlingspilz	Cockchafer	Andermatt, Switzerland
	Schweizer Beauveria	Cockchafer	Eric Schweizer, Switzerland
	Melocont	Cockchafer	Kwizda, Austria
Paecilomyces fumosoroseus	PFR-97	Whitefly	ECO-tek, USA
	Pae-Sin	Whitefly	Agrobionsa, Mexico
Lagenidium giganteum	Laginex	Mosquito larvae	AgraQuest, USA

18.5.3 Conservation or Environmental Manipulations

In this method, favourable conditions are provided for the growth of the fungus by modifying the environment of the host. For example, the favourable conditions can be provided for the fungal infection by spraying a mild chemical insecticide that would weaken the host, and another means is by maintaining high humidity and wet conditions in order to favour fungal growth. *Medicago sativa*, alfalfa, is infected by a number of common pathogens; among them is the alfalfa weevil, which can be biologically controlled by the introduction of various species of *Erynia*

(*Entomophthorales*). Highly moist and warm microclimatic conditions are maintained along with the light spray of chemical insecticide to encourage the growth and development of *Erynia* sp.

18.6 Fungi to Control the Plant Disease

Some commercially available mycofungicide products (BCAs) to control the plant diseases are listed in Table 18.2. *Trichoderma* is one of the important fungi which have been proved to be the best mycofungicide against many plant diseases such as root rot diseases of many crops, stem blight of peanuts (Ganesan et al. 2007), choanephora wet rot in okra (Siddiqui et al. 2008), and silverleaf of plums (Corke and Hunter 1979), followed by *Verticillium* to control cotton wilt (Hanson 2000), *Sphaerellopsis* to control rust diseases on a number of plants, and several others. Many commercial products as BCAs have been produced by using *Trichoderma* to control various plant pathogens such as *Pythium*, *Rhizoctonia, Fusarium, Sclerotina, Botrytis cinerea*, etc.

Penicillium chrysogenum is responsible for the post-harvest rot of citrus fruits. It can be controlled biologically by applying the yeast *Pichia guilliermondii* to the fruit after harvest but before storage or shipping. *Pythium ultimum* which causes damping off of cotton and *Rhizoctonia solani* can be controlled by the treatment of soil with the fungus *Gliocladium virens*. *Heterobasidion annosum* is a common cause of root rot of conifers. The disease may be controlled by the treatment of the surface of cut pine stumps with a spore suspension of *Phlebia gigantean*, which colonizes the stump surfaces and prevents subsequent colonization by *H. annosum*.

18.7 Biocontrol of Nematodes

Nematodes are small, needle-shaped worms that can infect plants and animals. A large number of crop plants are being infected by plant pathogenic nematodes, and they are costly to control. Thousands of dollars are invested annually to control these diseases. The chemical nematocides are helpful to control nematodes, but they are detrimental to our environment. Nematophagous fungi are the natural enemies of gastrointestinal helminth parasites, and they have been proved to be effective as biocontrol agents against the nematodes (Kerry 2000; Yang et al. 2011; Ward et al. 2012; Araujo et al. 2013). Ovicidal fungi are a group of fungi that colonize and consume the contents of eggs and larvae of nematodes (Frassy et al. 2010; Mello et al. 2013). Important ovicidal fungi which are being used for biocontrol of nematodes include *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium* Goddard), *Paecilomyces lilacinus,* and *Dactyella ovoparasitica* (Lysek and Sterba 1991). *Dactyella* and *Arthrobotrys* have peculiar nets, constricting rings, and knobs that can trap the nematodes, and that is the reason they are known as nematode-

Table 18.2 Fungal products developed for the biological control of plant diseases

Fungus	Product	Target	Producer
Trichoderma harzianum	Trichoderma 2000	*Rhizoctonia solani*, *Sclerotium rolfsii, Pythium*	Mycontrol (EfA1) Ltd, Israel
	Trichopel	Wide range of fungal diseases	Agrimm Technologies Ltd, New Zealand
	T-22 and T-22HB Bio-Trek, RootShield	*Pythium*, *Rhizoctonia, Fusarium, Sclerotina*	BioWorks (=TGT Inc) Geneva, USA
	Trichodex	Fungal diseases, e.g. *Botrytis cinerea*	Makhteshim-Agan, several European companies, e.g. DeCeuster, Belgium
Trichoderma harzianum and T. viride	Trichodowels, Trichoject, Trichoseal, and others	*Chondrostereum purpureum* and other soil and foliar pathogens	Agrimm Technologies Ltd, New Zealand
Trichoderma harzianum and T. polysporum	Binab T	Fungi causing wilt, wood decay	Bio-Innovation, Sweden
Pythium oligandrum	Polygandron, Polyversum	*Pythium ultimum*	Plant Protection Institute, Slovak Republic
Fusarium oxysporum	Fusaclean	*Fusarium oxysporum*	Natural Plant Protection, France
	Biofox C	*Fusarium oxysporum, F. moniliforme*	SIAPA, Italy
Candida oleophila	Aspire	*Botrytis* spp., *Penicillium* spp.	Ecogen Inc., USA
Cryptococcus albidus	YIELDPLUS	*Botrytis* spp., *Penicillium* spp.	Anchor Yeast, S. Africa
Ampelomyces quisqualis	AQ10 Biofungicide	Powdery mildews	Ecogen Inc., USA
Coniothyrium minitans	Cotans WG	*Sclerotinia* species	Prophyta, Germany. KONI, Germany
Gliocladium virens	SoilGard (=GlioGard)	Several plant diseases Damping off and root pathogens	ThermoTrilogy, USA
Gliocladium catenulatum	Primastop	Several plant diseases	Kemira, Agro Oy, Finland
Rotstop	*Phlebiopsis* (=*Peniophora*) *gigantea*	*Heterobasidion annosum*	Kemira Agro Oy, Finland

trapping fungi. As the nematode is trapped by the fungal hyphae, the fungus will invade the body cavity of the nematode, resulting in death. *Lagenidium* (aquatic oomycete) attacks on susceptible aquatic nematodes.

18.8 Biocontrol of Weeds and Noxious Plants

There are about 30,000 species of plants which are considered as weeds, and about 1600 of these can cause serious crop losses. In order to control weeds, agriculturists have started using herbicides or weedicides. The chemical herbicides are detrimental to our environment, and they have contaminated our water bodies including underground aquifers. There are several reports which state that the chemical herbicides can pose serious health implications to human health. Biological control of weeds can solve this problem by using mycoherbicides (bioherbicides) which have advantages over chemical herbicides. Recently, the successful use of a cocktail of three pathogens has been demonstrated in the field to control several weeds (Chandramohan 1999; Chandramohan et al. 2000). Charudattan (2001) reported that broad-spectrum bioherbicides do not have very high levels of host specificity; therefore, they could be used against more than one weed species (e.g. *Dactylaria higginsii* for *Cyperus* spp., *Phomopsis amaranthicola* for *Amaranthus* spp., etc.). Many facultative parasites, such as *Alternaria cassiae, Chondrostereum purpureum, Colletotrichum gloeosporioides, Cylindrobasidium levae, Dactylaria higginsii, Phomopsis amaranthicola, Pseudomonas syringae* pv. *tagetis,* and *Sclerotinia sclerotiorum,* are either registered or being developed as bioherbicides (Charudattan 2001). Table 18.3 shows the list of commercially available mycoherbicides to control the weeds and noxious plants. Mycoherbicides are more host-specific, and their preparation cost is cheaper, and also they are nonhazardous to human health. A number of mycoherbicides have been marketed by Mycogen Co. in San Diego, CA. *Puccinia* species can control the growth of skeleton weed and thistle under greenhouse conditions. Milkweed or strangler vine, a major problem on citrus in south Florida, can be controlled by using the mycoherbicide "Divine" composed of *Phytophthora palmivora.* Jointvetch "Collego" produced a mycoherbicide by using *Colletotrichum gloeosporoides* to control jointvetch, which lowers the market value of rice during harvesting. Sicklepod can be biologically controlled by *Alternaria cassia*. Water hyacinth can be controlled biologically by applying an inoculum of *Cercospora rodmanii*, renamed *C. piaropi*. Some fungi have been discovered to infect *Hydrilla* which causes the most problems to fishermen.

18.9 Conclusion

The use of fungi as biological control agents has achieved a significant progress over the last two decades. Some commercially available BCA products are already being sold in the market. Future use of fungi as biocontrol agents will expand if scientists can successfully develop resting spores and competent mycelia. Biocontrol agents alone are not sufficient to control all kinds of plant diseases under diverse conditions. Nowadays, mechanisms of action of some BCAs are becoming clearer. However, more research and development need to be done in the field of fungal biocontrol

Table 18.3 Fungi developed or commercially available for the biological control of weeds

Fungus	Target	Commercial name	Supplier or country where registered
Alternaria cassiae	Sicklepod (*Cassia obtusifolia*) and coffee senna (*C. occidentalis)* in soybeans and peanuts	Casst	USA
Cercospora rodmanii	Water hyacinth *(Eichhornia crassipes)*	'ABG 5003'	Abbott Labs, USA
Colletotrichum coccodes	Velvetleaf (*Abutilon theophrasti*) in corn and soybeans	Velgo	USA and Canada
Colletotrichum gloeosporioides f. sp. *cuscutae*	*Cuscuta chinensis, C. australis* in soybeans	Luboa 2	PR China
Colletotrichum gloeosporioides f. sp. *malvae*	Mallow (*Malva pusilla*) in wheat and lentils	Biomal	Canada
Colletotrichum gloeosporioides f. sp. *aeschynomene*	Northern Jointvetch (*Aeschynomene virginica*) USA in rice	Collego	Encore Technologies, USA
Chondrostereum purpureum	Black cherry (*Prunus serotina*) in forestry in The Netherlands	BioChon	Koppert, The Netherlands
Phytophthora palmivora	Milkweed vine (*Morrenia odorata*) in Florida citrus	Devine	Sumitomo, Valent, USA

agents for better understanding of their behaviour as BCAs. Genetic transformation of fungi can improve the performance of fungal BCAs under variable environmental conditions. However, the potential risk associated with release of these organisms into the environment should be further studied to enable acceptable guidelines for their implementation.

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Chapter 19
Biocontrol Agents: Potential of Biopesticides for Integrated Pest Management

Archana Singh, Richa Bhardwaj, and Indrakant K. Singh

Abstract Active compounds of biological origin and their synthetic derivatives are in high demand for crop protection over conventional pesticides since synthetic chemicals have reduced availability, adverse toxicological effects, and resistance and pest resurgence issues. Insecticides of biological origin (biopesticides) are less toxic and effective in small quantities and decompose quickly, leaving not much burden on environment. These are mostly target-specific and do not affect nontarget organisms much. Many of the bacteria, fungi, viruses, nematodes, protozoans, plants or plant-derived products (botanicals), pathogen/predator systems, insect pheromones, and plant-incorporated protectants (PIPs) are widely used as biological control agents for insect pest management (IPM). Among all, *Bacillus thuringiensis*-based biological insecticide has been primarily developed and commercialized. Biotechnological approaches such as transgenic technology and nanotechnology have recently come up that have potential to enhance expression and delivery mechanisms of biopesticide. Though the list is huge, only a limited number of living system-derived compounds have been used commercially, which are amenable to mass production and affordable to the growers. This chapter addresses the recent status of microbial control agents as biopesticides, which is used to improve agricultural productivity by restricting pest infestation.

Keywords Microbial pesticides · *Bacillus thuringiensis* · IPM · Bacterial · Fungal · Viral pesticides

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19.1 Introduction

Since ancient time, agriculture has been facing devastating harm caused by weeds, viruses, nematodes, fungi, insect pests, animals, and birds which has led to the decline in crop production. It has been evaluated that there has been a great loss of crop yield due to insects, diseases and weeds. To overcome this problem, various strategies were employed. One of the most commonly used methods to get rid of the pests is to use chemicals/synthetic pesticides (e.g. chlorinated hydrocarbons, carbamates, organophosphates, etc.). In spite of the success gained by the use of chemical pesticides, there are prospective health and environmental hazards/risks related with them. These chemical pesticides have long persistence period. Moreover, undiscerning and continuous application of these chemical products resulted in escalated residual problems, resistance among the pests and loss of some beneficial species. To overcome the hazards related to chemical pesticides, there is a need to adopt a coherent and eco-friendly approach. One such improvement in pest control tactic is to develop biopesticides which are derived from naturally occurring material such as plants, animals, microorganisms or their products. These are effective and biodegradable and pose less impact on the environment. The term 'biopesticide' is misleading in the sense it is not necessary that microbial agent for pest control will completely eradicate the pest, rather it suppresses and allow the crop to adequately develop some deleterious effect on the pest so that crop produce is not affected (Crump et al. 1999; Hynes and Boyetchko 2006).

Now a days, pesticides of biological origins are gaining popularity because of their low environmental impact and as a possible substitute to conventional synthetic pesticides, and a decline in the rate of usage of synthetic insecticides, occurrence of resistance to traditional synthetic pesticide, and increased public awareness about impact of synthetic pesticide on environment and humans have been observed. Some popular IPM strategies employ a combination of chemical and biological crop protection. Use of biological product at an appropriate time can reduce the total need for synthetic pesticides (Sara 2015). New biorational pesticides are also being developed which comprises pest control agents, chemical analogues of biochemicals such as pheromones, insect growth regulators, etc. These are more environment-friendly than synthetic chemical pesticides. The use of microbial control agents offers more realistic approach compared to chemical pesticides since it is an ecologically compatible IPM method (Koul and Cuperus 2007; Koul et al. 2008).

Biopesticides are broadly classified into several classes: microbial pesticides consisting of entomopathogenic bacteria (e.g., *Bacillus thuringiensis*), fungi (e.g., *B. bassiana*), or viruses (e.g., *Baculovirus*) including their metabolites, entomopathogenic nematodes, and protozoa. The member of *Bacillaceae* family, *Bacillus thuringiensis*, is widely used as biopesticide, since it produces a toxin that is active against many classes of insects (Fisher and Garczynski 2012). In addition, herbal/botanical pesticides provide coherent protection from pests and microbial diseases and can be used as plant-incorporated protectant (i.e., genetically modified crops like transgenic *Bt* cotton) though their use as food items is

debatable (Sarwar 2015). Further, in order to improve the delivery methods of pesticide, nanomaterials have been designed as a carrier system that has potential to reduce the concentration of pesticide to be used (De Oliveira et al. 2014).

Improvements have been made in the production and formulation technology of microbial pesticides. But at the same time, the use of biopesticides has been restricted due to various constraints at developmental, registration, and production level. Although there are many developments in terms of novel discoveries of microbial isolates and increase in the ability of genetic manipulation, but concerns related to pest resistance, environmental issues, and human welfare still remain. In the current chapter, we focus on the use of biocontrol agents to control pest attack in order to improve crop production, and we attempt to provide the recent information on it.

19.2 Microbial Pesticides

The largest group of broad-spectrum biopesticides is derived from wide range of microorganisms such as bacteria, viruses, fungi, and nematodes. They are effective against pests and do not have much deleterious effect on nontarget pests and are safe for the environment. Microorganisms growing in the close proximity of plants can be either harmful or beneficial. Plant diseases caused by harmful microorganisms have caused serious loss to crop productivity. On the other hand, beneficial microorganisms increase soil fertility and help in pest control. Therefore, useful microorganisms are encouraged to be utilized in agriculture. Different types of useful microorganisms can be isolated, tested, and commercialized so that they can be used at larger scale (Fig. 19.1). Based on their origin, microbial pesticides have been broadly categorized as bacterial, fungal, viral and nematodal biopesticides.

19.2.1 Bacterial Biopesticides

They are the most widely used and inexpensive means of pest bioregulation (Sarwar 2015). A huge number of bacterial species have been reported with insecticidal properties, but only few could reach the stage of commercialization (Table 19.1).

19.2.1.1 Bt as Microbial Pesticide

The most well-known example of microbial pesticide is the bacterium *Bacillus thuringiensis* or *Bt* which is a Gram-positive, facultative, and spore-forming bacterium. There are nearly 100 well-known subspecies of *Bt* which have been reported to control certain insect pests (Schnepf et al. 1998; Jurat-Fuentes and Jackson 2012). They have wide host range, and they are active against Lepidoptera, Diptera

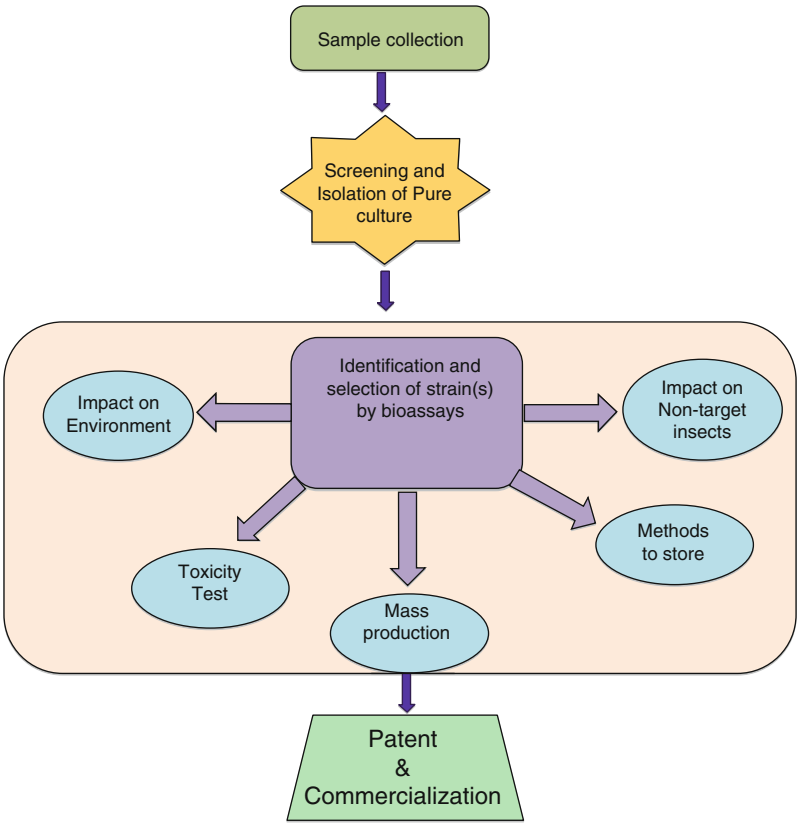

Fig. 19.1 A flowchart to depict the steps that are followed for screening and development of microbial pesticides

Table 19.1 Bacterial biopesticides developed to control pest attack on various crop plants

Name of the bacteria	Target pest
Bacillus popilliae	Members of Coleoptera
Paenibacillus popilliae	Coleoptera: Scarabaeidae: *Popillia japonica*
Bacillus thuringinesis var. kurstaki	Members of Lepidoptera and Coleoptera
B thuringinesis var. aizawal	Lepidoptera
B thuringinesis var. galleriae	*Helicoverpa armigera* and *Plutella xylostella*
B thuringinesis var. israelensis	Diptera: Culicidae, Simuliidae
B. thuringiensis subspecies *japonensis* strain Buibui	Coleoptera: Scarabaeidae
B. thuringiensis subspecies *tenebrionis*	Coleoptera: Chrysomelidae, predominantly *Leptinotarsa*
Lysinibacillus sphaericus	Diptera: Culicidae
Serratia entomophila	*Costelytra zealandica*
Chromobacterium subtsugae	*Leptinotarsa decemlineata*, Hemiptera, Acarina

(Nematocera), and Coleoptera (Chrysomelidae and Scarabaeidae) (Wei et al. 2003; van Frankenhuyzen 2009). Bt possesses the beneficial characteristics of both chemical pesticides and biopesticides, and, therefore, it is the most widely used microbial pesticide. Similar to synthetic pesticide, it is not expensive, can be easily formulated, acts quickly, and has an elongated shelf life; but unlike synthetic pesticides, they do not show much hazardous effect on environment and are specific to target organisms (Birch et al. 2011). The only disadvantage of *Bt* is its sensitivity toward sunlight; therefore, frequent applications are needed. *Bt* pesticides are available as formulated sprayable products of bacterial spores and endotoxin crystals and are used on broad acre crops. High level of selectivity and safety are required, when they are sprayed on fruits and vegetables. *Bt* formulations are not harmful to humans, vertebrates, beneficial organisms, and the environment (Chandler et al. 2011). A continuous monitoring of microbial pesticide is done so that it does not harm any nontarget organism including humans (Gupta and Dikshit 2010). In order to check the attack by lepidopteran insects (leaf rollers and defoliators) in orchards, two subspecies of *Bt*, *B. thuringiensis* subsp. *kurstaki* (Btk, Dipel) and *B. thurinigiensis* subsp. have been used (Glare et al. 2012). The above-mentioned subspecies are also utilized to control lepidopteran pests of crucifers, cucurbits, corn, legumes, cotton, and solanaceous vegetables. Btk is also applied to control the insect pests (*Plodia interpunctella* and *P. operculella*) of stored products such as grain, fruits and potato (Kroschel and Lacey 2009). Among coleopterans, Colorado potato beetle, *Leptinotarsa decemlineata*, is the main target of a subspecies of Bt, *B. thuringiensis* subsp. *tenebrionis* (Btt) (Wraight et al. 2007, 2009).

19.2.1.2 Mode of Action

Bacillus thuringiensis produce pesticidal toxins, namely Cry family of crystalline proteins that are encoded by the cry genes (Mazid et al. 2011). These are responsible for feeding cessation and death of the insect (Khachatourians 2009). Cry proteins possess three specific domains attached together by a single linker (Bravo et al. 2007). They are produced as protoxins of different length of which the longer C-terminal protoxins are involved in crystal formation and causing toxicity (de Maagd et al. 2001). When Cry proteins are ingested by the insects, after solubilization, biologically active endotoxins are released that are resistant to insect proteases (Schnepf et al. 1998; Whalon and Wingerd 2003). The C-terminal domain of this endotoxin binds to the receptors present on the cell membrane of the bush border of midgut after which the hydrophobic region of the toxin also gets linked to the membrane (Rodrigo-Simón et al. 2008). This linkage causes osmotic imbalance and formation of transmembrane pores leading to leakage of gut content and cell lysis in the gut wall (Fig. 19.2).

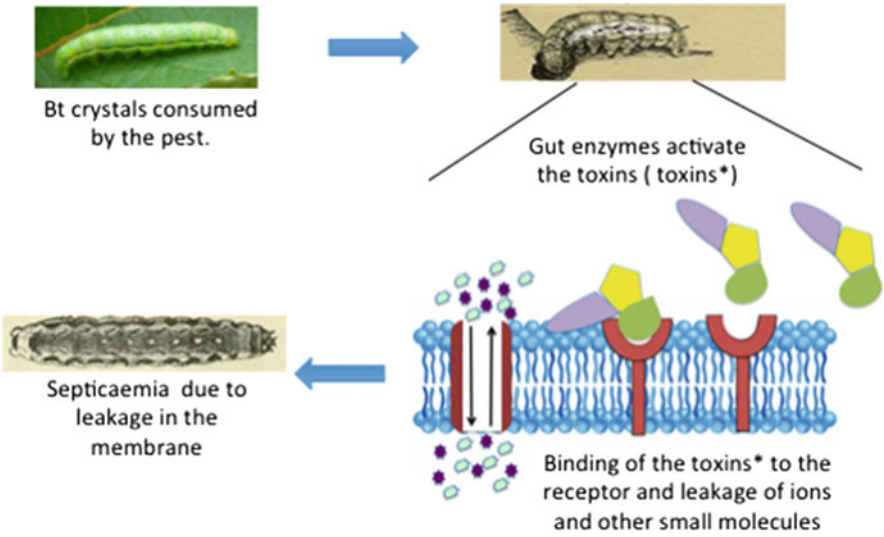

Fig. 19.2 Effects of *Bacillus thuringiensis* (*Bt gene* and *Cry protein*) on insect larvae

19.2.1.3 Bt-Crops

Bt-crops, a Bt product different from microbial pesticides, has been largely used in the last two decades. Genes coding for crystal proteins and vegetative storage proteins (VIPs) have been successfully transferred into different crop plants to form *Bt* transgenic crop varieties. In spite of huge controversy, Bt crops have been widely adopted due to its high efficacy and specificity. Moreover, they are safe for consumers and do not pollute the environment. There is availability of diversity of toxin genes from different strains that can be easily cloned, expressed and transformed to produce *Bt* crops (Kennedy 2008). Currently, approximately 75 classes of Cry toxins and 125 different VIPs are known (Crickmore et al. 2014). Transgenic 'Spunta' potato lines with the *Cry1Ia1* has been a great success providing complete resistance to potato tuberworm in laboratory and field tests (Douches et al. 2002). Another transgenic line of potato expressing Cry3Aa toxin shows significant resistance against *L. decemlineata.* In the last few decades, the area growing *Bt*-crops has increased at high rate. A growing interest in the use of *Bt-Brinjal*, *Bt*-cotton and *Bt*-maize has caused drastic decrease in the usage of chemical insecticides (Brookes and Barfoot 2012) as well as microbial pesticides. Due to high cost for generating GM crops, it is not possible to have transgenic variety for each crop. Therefore, other conventional but eco-friendly methods such as sprayable *Bt* formulations still have a great potential in the coming decades.

Table 19.2 Fungal biopesticides

Name of the fungus	Target pest
Aschersonia aleyrodis	Hemiptera (Aleyrodidae)
B. bassiana sensu lato	Acari, Diptera, Lepidoptera, Hemiptera, Isoptera Coleoptera, Diplopoda, Hymenoptera, Lepidoptera, Orthoptera, Siphonoptera, Thysanoptera
B. bassiana	Coleoptera, Acari, Diptera, Orthoptera, Thysanoptera, Hymenoptera, Hemiptera.
Beauveria brongniartii	Coleoptera (Scarabaeidae)
Conidiobolus thromboides	Acari Hemiptera, Thysanoptera
Hirsutella thompsonii	Acari
Isaria fumosorosea	Acari, Diptera, Coleoptera, Hemiptera, Thysanoptera
Lagenidium giganteum	Diptera (Culicidae)
Lecanicillium longisporum	Hemiptera
Lecanicillium muscarium	Acari, Hemiptera, Thysanoptera
Metarhizium anisopliae sensu lato	Acari, Blattoidea, Coleoptera, Diptera, Hemiptera, Isoptera, Lepidoptera, Orthoptera
Metarhizium acridum	Orthoptera
Nomuraea rileyi	Lepidoptera
Paecliomyces fumosoroseus	Hemiptera

19.2.2 Fungal Biopesticides

Another class of microbial insecticides, mycoinsecticides, are products of entomopathogenic fungi, which are natural pathogens of diverse agricultural pests both insects and acari. There are many suitable characteristic features of fungi, which make them suitable for use as biocontrol agents. They are pathogenic to pests but do not harm nontarget insects such as bees and parasites and predators of pests. They neither cause any risk on growth and development of beneficial organisms such as earthworms and collembola. Therefore, mycopesticides are potential agent for IPM and also useful for long-term agriculture and crop production by safeguarding biodiversity (Goettel et al. 2008; Kim et al. 2010; Koike et al. 2011).

Fungi-based biopesticides were considered for IPM by industrial methods of mass production and formulation for application with the use of few specific mycopathogens (Chandler et al. 2008). IPM using fungi utilizes ecological approaches, and appropriate environmental conditions are maintained to promote infection and spread of the pathogen within the pest (Lacey et al. 2015). Commercially available fungi-based biopesticides (Table 19.2) are mainly derived from *Beauveria* spp., *Metarhizium* spp., *Isaria fumosorosea*, and *Lecanicillium* spp.

Specifically, *Beauveria bassiana* and *Metarhizium anisopliae* are the two ascomycetes that are most commonly used as commercial mycoinsecticide. They are usually applied in the form of conidia or mycelium which sporulates after their

application. Insect-pathogenic fungus *M. anisopliae* has been reported to be used against adult *Aedes aegypti* and *A. albopictus* mosquitoes (Driesche et Al. 2008). Entomopathogenic fungi alone or in combined application of insecticide with fungal entomopathogen could be a useful strategy in IPM (Sarwar 2015). Some mycoinsecticide has been developed for control of locust and grasshopper pests in Africa and Australia (Chandler et al. 2011). It has been observed that when *B. bassiana* have been applied along with sublethal concentration of insecticide, there is high insect mortality in potato beetle (*Leptinotarsa decemlineata*). A combination of *B. bassiana* and *neem* (*Azadirachta indica*) has also been explored, and their compatibility yielded highest mortalities *of B. tabaci* eggs and nymphs.

19.2.2.1 Mode of Action of Mycoinsecticides

The process of infecting pests includes gaining the access to host's hemolymph, producing toxins and growing up by using nutrients present in haemocoel. In some cases, species of pathogenic fungus such as *B. bassiana* and *M. anisopliae* cause muscardine insect disease; in which after killing the host, cadavers become mummified by mycelial growth (Miranpuri and Khachatourians 1995) (Fig. 19.3). Entomopathogenic fungi are the most effective against sucking insect pests such as aphids, thrips, scale insects, mealy bugs, whiteflies, mosquitoes and all kind of

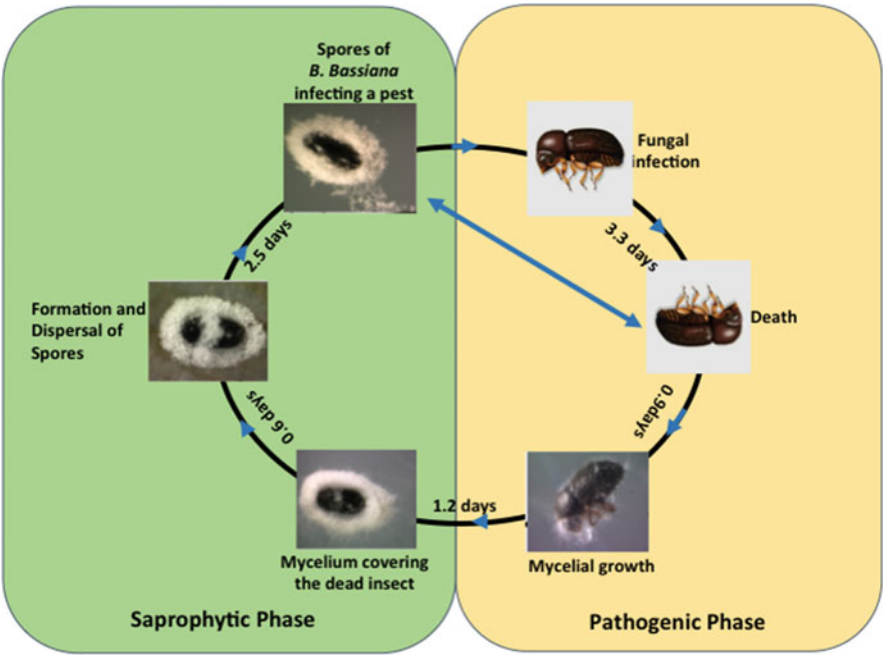

Fig. 19.3 *Beauveria bassiana* targeting coffee berry borer

mites (Barbara and Clewes 2003; Pineda et al. 2007). Certain fungal species, primarily Streptomycetes, are known to produce toxins against insect pest species belonging to Lepidoptera, Homoptera, Coleoptera, Orthoptera, and mites (Cole and Rolinson 1972). Examples of some most active toxins are actinomycin A, cycloheximide and novobiocin.

19.2.3 Mycoinsecticide: A Case Study

Solenopsis invicta Buren, a Hymenopteran, is native to South America and an aggressive ant species (Lowe et al. 2000). They are highly resistant to pathogens due to development of defensive alkaloids (Storey 1990), necrophoric behaviors (Qiu et al. 2014, 2015), trophallactic behavior (De Souza et al. 2008; Qiu et al. 2016), generation of volatiles (Wang et al. 2015), as oral transfer of chemical cues, growth proteins and hormones (Leboeuf et al. 2016). As a result, most of the biological control mediating organisms are not active against this invasive insect. Further, a combination of two species of fungi, *Metarhizium brunneum* and *Beauveria bassiana*, were used to manage *Solenopsis invicta* Buren. Results showed 51.35 and 56.68% of mortality in workers during day 1 and 2 with *M. brunneum* and *B. bassiana* GHA treatments. However, only 9.47 and 35.96% of the mortality could be explained by fungal infection. In *B. bassiana* NI8 treatment 84.48% of mortality was observed within 4–6 days. Mortality occurring in these two treatments can be explained. *M. brunneum* produces a toxin, destruxins (Strasser et al. 2000; Schrank and Vainstein 2010), and releases certain enzymes including lipases, proteases, and chitinases that attack the cuticle of the insects. Field study also showed positive results, and several fire ants were killed by *M. brunneum* and *B. bassiana* (Rojas et al. 2018).

19.2.4 Viral Biopesticides

Virus pesticides act on specific target and are mostly effective against lepidopteran pests of cotton, rice, and vegetables and plant-chewing insects. *Heliothis zea* nucleopolyhedrosis is the first viral insecticide with broad range. There are different groups of entomopathogenic viruses: baculoviruses (BVs), nucleopolyhedrosis viruses (NPVs), granuloviruses (GVs), acoviruses, iridoviruses, parvoviruses, polydnaviruses, poxviruses, reoviruses, cytoplasmic polyhedrosis viruses, nodaviruses, picorna-like viruses, and tetraviruses. Among them, baculovirus (BV) has received maximum focus for biopesticide development at commercial level (Moscardi et al. 2011). Non-BV (Tetraviruses, Cypovirus etc.) viruses have also been used for crop protection but only up to a limited extent (Ramle et al. 2005; Jackson et al. 2005). Baculovirus infects many species belonging to genera *Helicoverpa* or *Heliothis*. *HzSNPV* is efficacious against pests belonging to the

genera soybean, sorghum, maize, tomato and beans (Sarwar 2015). A type of baculovirus namely *HaSNPV* has been reported from India which has been exclusively used in cotton field (Srinivasa et al. 2008).

19.2.4.1 BV as Viral Biopesticides

There are many beneficial aspects of BV because of which it has been picked for commercialization. There is significant information about pathology and ecology of BV, which is helpful in registration and product development. BV has widespread distribution allowing collaborative research and interaction between pesticide companies. It possesses high levels of virulence against pests. Moreover, BV shows great levels of replication, which is of commercial interest. The robust infective stage is the occlusion body (OB), which contains rod-shaped nucleocapsids and circular and double-stranded DNA. The OBs are made up of tough crystalline proteins making it ideal for product formulation, application, and commercialization. There is no requirement of keeping intervals between spray timings, and it is safe for human and nontarget insects. Moreover, OBs are large enough to be visualized and quantified by phase-contrast microscopy. The only limitation in its use is its degradation by sunlight because of which frequent applications are needed (Lacey et al. 2015).

BVs are active against world's most devastating agricultural pests, *Helicoverpa* spp. and *Spodoptera* spp. (Mazid et al. 2011). Two well-known commercial formulations based on *Spodoptera* NPV are available in the United States and Europe. BV-based biopesticides have been widely adopted in many different places including China, India, Thailand, Vietnam, Brazil, Mexico, and Guatemala Southeast Asia, Australia, and South America. Virus-based products are available against cabbage moths, corn earworms, cotton leafworms and bollworms, beet armyworms, celery loopers, tobacco budworms and many other pests (Table 19.3). Recombination technology has also lead to development of potential economical substitutes such as recombinant baculovirus, vEV-Tox34, expressing the gene Tox-34 from a mite *Pyemotes tritici* enhance the rate of killing of the corn earworm, *Helicoverpa zea* (Tomalski and Miller 1991).

19.2.4.2 Mode of Action

Viral infection involves entry of the virus to a target cell via replication in the nuclei or in the cytoplasm. Postinfection, virus exists in three phases: 0–6 h is designated as early phase, 6–24 h is called as second phase, and 24–72 h is labeled as very late phase. OBs/virions are formed during late phase of their life cycle. Infected nuclei per cell can produce hundreds of polyhedra (example in NPVs) or thousands of granules as in GVs. It may cause enzootics leading to the decrease in pest populations. It has been reported in baculovirus, occlusion bodies gets inactivated rapidly when exposed to solar ultraviolet radiations (280–320 nm) (Killick 1990). UV inactivation can be controlled by using plastic greenhouse structures which can

Table 19.3 Viral biopesticides

Name of virus	Target pest
Nudiviruses	
NPV for *Anagrapha falcifera*	*Anagrapha falcifera*
NPV for *A. gemmatalis*	*Mucuna pruriens* and *Diatraea saccharalis*
NPV for *Autographa californica*	*Autographa californica*
NPV for *H. zea* and *H. virescens*	*Helicoverpa zea* and *Helicoverpa virescens*
NPV for *Mamestra brassicae*	*Mamestra brassicae*
NPV for *Orgyia pseudotsugata*	*Orgyia pseudotsugata*
Corn earworm NPV (HezeSNPV)	*Helicoverpa zea*, *Helicoverpa armigera*, and *Heliothis virescens*
Cotton bollworm NPV (HearNPV)	*Helicoverpa armigera*
NPV for *Spodoptera exigua*	*Spodoptera exigua* and *Paradrina clavipalpis*
Unbarred *Spodoptera* moth NPV (SdalNPV)	*Spodoptera albula* (*sunia*)
Beet armyworm NPV (SpexMNPV)	*Spodoptera exigua*
Tobacco armyworm NPV (SpltNPV)	*Spodoptera exigua*
Egyptian cotton leafworm NPV (SpliNPV)	*Spodoptera littoralis*
SeMNPV	*Spodoptera exigua*
Gypsy moth, NPV (LydiMNPV)	*Spodoptera exigua*
Velvetbean caterpillar, NPV (AngeMNPV)	*Anticarsia gemmatalis*
Redheaded pine sawfly NPV (NeleNPV)	*Neodiprion lecontei*
Douglas fir tussock moth NPV (OrpsNPV)	*Orygia pseudotsugata*
Balsam fir sawfly NPV (NeabNPV)	*Neodiprion abietis*
Codling moth GV (CpGV)	*Cydia pomonella*
False codling moth GV	*Cryptophlebia*
CrleGV	*Leucotreta*
AdorGV	*Adoxophyes orana*
Potato tuber moth GV (PhopGV)	*Phthorimaea operculella*
Summer fruit tortrix GV (AdorGV)	*Adoxophyes orana*
Tea tortrix (HomaGV)	*Homona magnanima*
Smaller tea tortrix GV (AdhoGV)	*Adoxophyes honmai*
Alfalfa looper NPV (AucaMNPV)	*Autographa calofornica*
Cabbage looper (TrniSNPV)	*Trichoplusia ni*
Tea moth (BuzuNPV)	*Buzura suppressaria*
Tea tussock moth (Eups NPV)	*Euproctis pseudoconspersa*
Tea geomotrid EcobNPV	*Extropic obliqua*
Teak defoliator (HypeNPV)	*Hyblea peura*
CpGV	*Cydia pomonella*
Imported cabbageworm (PiraGV)	*Artogeia (Pieris) rapae*
Oriental armyworm (LeseNPV)	*Leucania (Mythimna) separata*
Diamond back moth GV (PlxyGV)	*Plutella xylostella*
Reoviridae	
Masson pine moth cypovirus (CPV)	*Dendrolimus punctatus*
Parvoviridae	

(continued)

Table 19.3 (continued)

Name of virus	Target pest
Cockroach densonucleosis virus (DNV)	*Periplaneta fuliginosa*
Others	
Oryctes virus	*Oryctes rhinoceros*
Granulosis virus	Lepidoptera

reduce the intensity of incident UV-B radiations reading by >90% compared with external readings leading to an increase in the prevalence of infection in larvae (Lasa et al. 2007).

The use of formulations such as stilbene can increase susceptibility to NPV infection either by disrupting the peritrophic membrane (Okuno et al. 2003) or by inhibiting shedding or by virus-induced apoptosis of insect midgut cells (Dougherty et al. 2006). Two genetically enhanced isolates of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) from the spider *Diguetia canities* and *Tegenaria agrestis* designated vAcTaITX-1 and vAcDTX9.2 have been commercially evaluated as potential biopesticide against lepidopteran insects (Hughes et al. 1997). Viral pesticides have numerous advantages over chemical pesticides, but their large-scale production, cost-effective methods for producing recombinants, intensive labor, and time-consuming transinfection pose certain difficulties. They are being produced on small scale by various IPM centers and state agricultural departments (Gupta and Dikshit 2010; Lacey et al. 2015).

19.2.4.3 A Case Study on the Use of *Oryctes nudivirus* for the Control of Invasive Coconut Palm Rhinoceros Beetle

Indigenous to Asia/West Pacific areas, *Oryctes rhinoceros* or coconut palm rhinoceros beetle was coincidentally established into Samoa and eventually extended to islands of southwest Pacific regions (Bedford 1980; Jackson 2009). These beetles are key pest of palm and coconut. They minimize the produce by ingesting the vegetation mainly the crown and its destruction, leading to the death of the whole tree (Bedford 1980). Larvae of *Oryctes rhinoceros* has diverse habitat such as inside rotting palm wood, dead tops of living trees, and organic content-rich sites (Bedford 1980). *Oryctes* virus was intensely established in the pest-infested regions of Samoa and other southwest Pacific islands to overcome the devastation produced by the beetles (Bedford 1980; Hüger 2005; Jackson 2009). These viruses were originally collected from Malaysia (Hüger 1966). Remarkable consequences were observed by using this virus as a biological control agent. It regulated and lowered the population of coconut palm rhinoceros beetle and their larvae. Infected adults served as reserves for virus. In beetle populations, virus spread from infected to noninfected larvae through feeding, mating, sites of larval breeding, etc. Larvae with severe infection die within 9–25 days after virus consumption (Hüger 1966; Zelazny 1972). Continuous reviews were conducted in the recent years, which suggest more fatal and

pernicious strains of virus are required to reduce the problem of less efficacy of *Oryctes* virus on some beetle-infected islands (Jackson et al. 2005; Jackson 2009).

19.2.5 Nematode Biopesticides

Entomopathogenic nematodes (EPNs) are one of the most astonishing organisms as they repress insects in their perplexing habitats (such as soil-borne pests and stem borers). They have become an important microbial tool for biotic control.

19.2.5.1 *Steinernema* and *Heterorhabditis*: *EPNs as* Biopesticides

Steinernema and *Heterorhabditis* are the two widely used genera as EPNs in pest management. They are mostly present in all forest and agricultural land. They have an aggregated distribution, which depends upon their behavior, restricted dispersal ability, and changeability in spatial and temporal distribution of their natural enemies (Atwa 2014). EPNs are very often used as biological control agents since they are environment-friendly and do not harm human and nontarget organisms (Akhurst and Smith 2002; Ehlers and Shapiro-Ilan 2005). They are suitable for mass production, and it is easy to register and commercialize EPNs as biopesticide. They have a wide host range including 5–6 orders of insects (Poinar 1979; Klein 1990).

There are more than 10 industries which are involved in the production of EPNs as biocontrol agent, and approximately 15 species have reached up to the level of commercialization (Table 19.4). The efficacy of EPNs as biopesticide depends on environmental factors (biotic and abiotic). Biotic factors include the species of nematode that has been selected and number of times it has been applied. Abiotic factors include desiccation, ultraviolet light, type of habitat, and time of application. EPNs are sensitive to desiccation and ultraviolet light, and it works better if applied early morning or in evening.

Although the basic research on EPN involves figuring out its usage as biopesticide, the recent advanced research focuses on understanding how host attraction and infection can be improved for better efficacy. During this course, it has been concluded that vibration and electromagnetic stimuli can improve attraction toward the host (Torr et al. 2004; Ilan et al. 2013). These discoveries are certainly going to improve the suitability of EPNs as biocontrol agents.

19.2.5.2 Mode of Action

EPNs infect their host via spiracles or cuticle, mouth and anus opening as infective juveniles (IJs) (Kaya and Gaugler 1993; Koppenhöfer et al. 2003). EPNs carry mutualistic symbiotic bacteria such as *Xenorhabdus* spp. and *Photorhabdus* spp. for Steinernematids and Heterorhabditids, respectively (Poinar 1990). They liberate

Table 19.4 Nematode biopesticides

Name of nematode	Target pest
Heterorhabditis bacteriophora	Lepidoptera, cutworms, corn root worms, turf and Japanese beetles, flea beetles, soil insects, white grubs (scarabs), black vine weevils, and citrus root weevils
H. indica	*Galleria mellonella*, root mealybugs, grubs
H. marelata	White grubs (scarabs), cutworms, black vine weevils
H. megidis	Weevils
H. zealandica	Scarab grubs
H. megidis	Coleoptera (Scarabaeidae)
P. hermaphrodita	Slugs
Steinernema glaseri	Root weevils, cutworms, fleas, banana root borers and fungal gnats, white grubs (scarabs, especially Japanese beetle, *Popillia* sp.)
S. kraussei	Black vine weevil, *Otiorhynchus sulcatus*
S. carpocapsae	Lepidoptera, Coleoptera, Diptera, Hymenoptera, and Hemiptera
S. feltiae	Coleoptera, Lepidoptera, and others
S. longicaudum	Lepidopteran and Coleopteran
S. riobrave	*Diaprepes* spp. (citrus root weevils), *Scapteriscus* spp. (mole crickets)
S. scapterisci	*Scapteriscus* spp. (mole crickets)
Deladenus siricidicola	*Sirex noctilio* (Sirex wood wasp)

their bacterial symbionts into the haemocoel of the host, which are mainly responsible for the death of the host within 24–48 h (Dowds and Peters 2002). Entomopathogenic nematodes at most can have three cohorts in IJs and leave the body to infect a new one (Kaya and Gaugler 1993) (Fig. 19.4). EPNs can be produced under in situ or ex situ conditions in solid media or by liquid fermentation (Grewal and Georgis 1999; Shapiro-Ilan et al. 2006). Some successfully produced nematodes in fermenters are *Steinernema carpocapsae*, *S. riobrave*, *Steinernema glaseri*, *Steinernema scapterisci*, and *Heterorhabditis bacteriophora*.

19.2.5.3 A Case Study on *Steinernema scapterisci* for Controlling Invasive Mole Crickets in Florida

Scapteriscus species are key serious pest and known to cause acute destruction to turf especially reported in Florida (Frank 2009). For regulating their growing population several biological control methods were adopted. One such strategy made use of EPNs and parasitoids in Florida. In 1985, nematode species from Uruguay were introduced in Florida to manage and check the population of encroaching mole cricket (*S. scapterisci*). At the beginning, they helped in regulating the pest (Parkman et al. 1993). In Florida, Uruguay's nematode species were released, and they got established into *S. vicinus*, *S. borelli*, and *S. abbreviatus* populations (Hudson et al. 1988; Parkman et al. 1993). Further, two parasitoids (from South America) became established all over Florida. With the help of these three natural adversaries, *Scapteriscus* populations diminished by 95% (Frank and

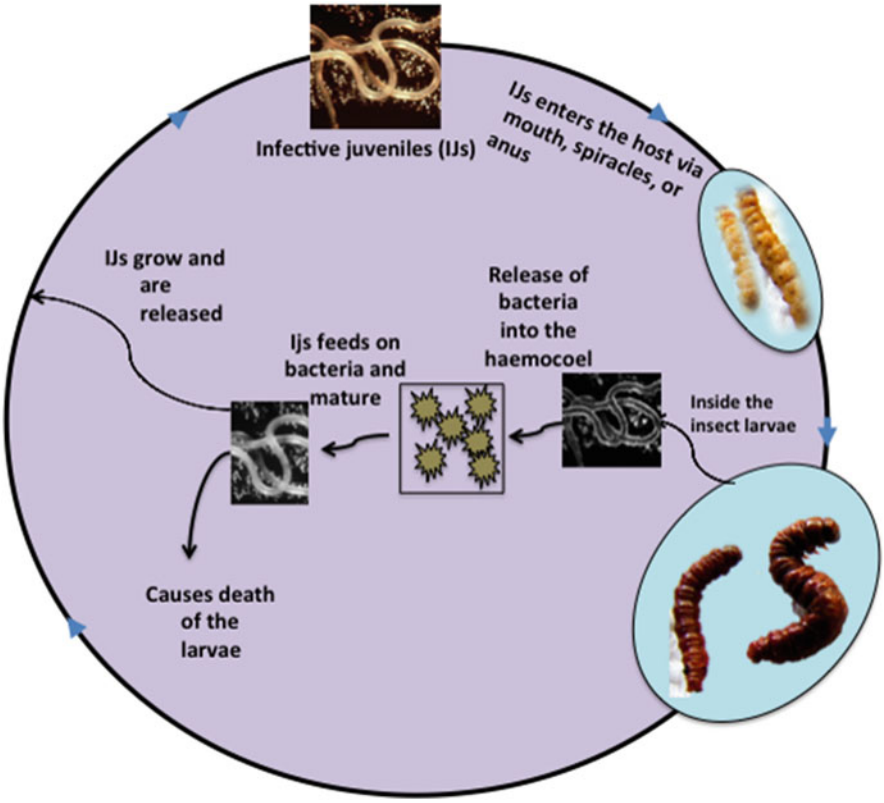

Fig. 19.4 Life cycle of entomopathogenic nematodes

Walker 2006). These EPNs with high successful rate are now applied at various infestation sites in Florida (Frank 2009).

Advantages of Microbial Pesticides over Chemical Pesticides

(a) They are safe to applicators (human) and nonpathogenic to nontarget organisms. They are not even harmful to beneficial organisms like predators and parasitoids.
(b) They are safe to be used in food supply.
(c) They do not persist in the environment.
(d) There are no/very little chances of development of resistance in the pests.
(e) They do not cause any lethal effect or risk to the environment.
(f) Most of them possess good shelf life.
(g) They are easy and inexpensive to mass produce.

(h) They are easy for application as well and do not need any specific equipment.
(i) They are adaptable for genetic modifications.
(j) They are suitable to be used in different types of habitat where use of chemical pesticides might be restricted.

Disadvantages of Microbial Pesticides

(a) Since they target a specific group of microbes, crop plants are still at risk and may be attacked by other pests.
(b) They show slower killing of pests as compared to chemical pesticides.
(c) They need precise timing for application so that they can attack early instars of pests and show better efficacy.
(d) Due to less persistence, many rounds of application may be needed.
(e) Microbial pesticides are sensitive to heat, UV radiation, desiccation, etc.
(f) Some have short shelf life.
(g) There are few constrains in their mass production, formulations, registration, and commercialization.
(h) Its cost of production may be higher except for high-value crops.

19.3 Increasing Trends in Production of Biopesticides

Outburst of secondary pests; growing pest resistance; toxicity of soil, air, water, and food; detrimental effect on humans; and ecological imbalance are some unacceptable effects of continuous and excessive use of chemical-based pesticides. Such emerging issues are of great concern and have led many countries to amend their policies on limiting the use of chemical pesticides and switch over to better biological control methods. Application of new environmentally friendly biopesticides is a better option than conventional chemical control techniques. Under integrated pest management, biopesticides have shown better effectuality compared to synthetic products (Mazid et al. 2011). Growing organic demand and residue free crop product are some of the decisive instigator for biopesticide demand. Eventually, the need for bioinsecticides, fungicides, and bionematicides is increasing exponentially. The US biopesticides market has anticipated that it may rise to approximately $300 million by 2020. In India, only 4.2% of overall pesticide market consists of biopesticide. It is expected to show expansion with annual growth rate about 10% in the near future. Till now, only 20–30 biopesticides have been registered under the Insecticide Act 1968. Considerable biopesticides manufactured and used in India are *Bacillus thuringiensis*, neem-based pesticide, *Trichoderma*, and nuclear polyhedrosis virus (Kumar 2012).

19.4 Policy Measures

Biopesticides do not produce any risk factor; therefore, the Environmental Protection Agency (EPA), USA, promotes its growth and utilization. EPA can register any new biopesticide within a year based on its virulence, constituents and data availability. Regular and continuous inspections are made to regulate the potency of current biopesticide. India has also adopted IPM strategies and considered the use of various biopesticides as its major component. Here, the Ministry of Agriculture employs the usage of pesticides under the Pesticides Management Bill 2008. As a substitute to regular synthetic pesticides, biopesticides do face innumerable challenges such as in their manufacturing, development and application issues.

19.5 Suggestions

Microbial pesticides have been widely used as biopesticides to check pest infestation and improve crop production. Further, the below-mentioned recommendations can be considered for the effective utilization of microbes to restrict pest infestation:

- Efforts should be made for advertisement and acceptance of biocontrol strategies by all the participants in the marketing chain from producer to consumer.
- Outreach activities such as demonstration, promotion, and training programs can be conducted in order to popularize biopesticides among the consumers.
- Further research is needed to figure out what new methods can be applied to overcome limitations that are faced while using microbial pesticides such as their sensitivity to UV light, desiccation, etc.
- Search for new biocontrol agents needs to be continued for future usage in different types of habitats and climates.
- Newer methods of production, formulation, storage, and application need to be established for better efficacy, user friendliness, and cost-effectivity.
- Transgenic plants with microbial genes can be generated for major crops.
- Further research is needed to find out ecology of pest pathogens for their sustainable use.

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Chapter 20
Microbe-Mediated Plant Growth Promotion: A Mechanistic Overview on Cultivable Plant Growth-Promoting Members

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Abstract The global demand for increasing agricultural productivity and declining farming land resource has posed a severe threat to crop production and agroecosystems. The use of chemical and mineral fertilizers has boosted up the agricultural productivity but considerably diminished the soil fertility, soil health, and sustainability. Improvement in agricultural sustainability requires the combined holistic approach integrating optimal use of soil fertilization, soil physical properties, soil biological processes, and soil microbial diversity, combining integrated plant nutrient management. Since past few decades, plant growth-promoting bacteria (PGPB) and plant growth-promoting rhizobacteria (PGPR) have replaced the conventional use of chemical fertilizers and pesticides in horticulture, silviculture, agriculture, environmental remediation, and cleanup strategies, and utilization of such microbial candidates for improving soil health and nutrient availability for plants is a vital practice since antiquity. Apart from the phytostimulatory effects on plants, PGPBs are potent colonizers of plant root or rhizosphere that improve both crop and soil health through various direct and indirect approaches such as nitrogen fixation, phosphate solubilization, quorum sensing, siderophore production, antimicrobials, volatile organically, mineral solubilization, induced systemic resistance, nutrient acquisition, modification of soil texture, soil porosity, etc. Increase in biomass, yield, seedling emergence, root proliferation, and timely flowering are

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the direct benefits that make these microbes most preferred in the agricultural crop production, with a high market demand. Researchers are now moving way forward to decipher their molecular mechanisms of plant beneficiation through genomic comparisons, real-time protein expressions revealing the ecophysiology, and niche adaptation that might facilitate functioning of these beneficial microbes. In this chapter, we have highlighted the status and recent trends of some important plant-beneficial bacterial members, their growth-promoting abilities, and genomic perspectives for sustainable use in crop productivity.

20.1 Introduction

Increasing agricultural productivity per unit of land and ensuring that agricultural growth responds to food security needs are the major concerns in agriculture of today. The fertilizer-based monocropping farming model that we have been following since long is not sustainable as it is harmful for human, plant, and soil health (Kumar et al. 2017a). Day by day, the food demand is increasing in the developing countries dramatically, and production of more food and fiber to feed a growing population and implementation of more efficient and sustainable production methods are challenges in today's era. In the twenty-first century, loss of productivity in the agricultural trade is due to abiotic and biotic environmental stresses (Barnabas et al. 2008). Ecological stresses are the major limiting factors for plant metabolism, growth, and productivity, especially in the arid and semiarid zones of the world. Abiotic stresses associated with soil salinity, drought, pH of soil, environmental temperature, ozone, toxic metals, and low nutrient concentration, singly or in combination, can cause lethal effects in almost all phonological stages of plant, from germination to plant enlargement limiting factors for crop production (Rengasamy 2006; Ladeiro 2012; Ashraf and Harris 2013).

Reports have been revealed the crop yield loss (70%) may be attributed to abiotic stresses, like drought. Drought is one of the major checks in agriculture (Raju et al. 2014). Drought induces changes in physiological processes of plants, together with photosynthesis, membrane integrity, enzyme stability, proline, and ABA (Karim and Rahman 2015). Bacteria, viruses, fungi, nematodes, and herbivore insect-like living organisms are the causal factors of biotic stress (Fisher et al. 2012), and they reduce agricultural yield by 30% globally. They affect the natural habitat ecology. Healthy soil conservation is a strategic element of sustainable agriculture. The noticeable solutions that can yield more agricultural products are land management, use of renewal inputs, usage of transgenic crops, and expanded practice of plant growth-promoting rhizobacteria (PGPR) (Glick 2012). PGPR is a set of soil microbial flora. They abode in the rhizosphere and on the surface of the monocot and dicot plant roots (Vacheron et al. 2013). PGPR has shown the potential to be a promising technique in the practice of supportable agriculture and could play a key role in

the mitigation of drought. The microbes colonize and impart drought by synthesizing exopolysaccharides (EPS), phytohormones, 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Govindasamy et al. 2008), volatile compounds, antioxidants, inducing accumulation of osmolytes, up- or downregulation of stress-responsive genes, and changes in root morphology at the rhizosphere/endo-rhizosphere region of the affected plant roots (Vurukonda et al. 2016). The induced systemic tolerance (IST) system, the physiological state of beneficial microbes, elicits tolerance to drought stresses (Lim and Kim 2013). Inoculation of cytokine-producing PGPR helps on growth and water stress consistence of forest container seedlings under drought condition (Liu et al. 2013). Biotic stresses even can be prevented after the use of PGPR (Gupta et al. 2015).

Based on the colonization abilities of the bacterial members, PGP microbes are broadly classified into extracellular (ePGPR) and intracellular (iPGPR) colonizers. Extracellular *PGP microbes belonging to the genera Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Pseudomonas*, *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Flavobacterium*, *Micrococcus*, *Erwinia*, and *Serratia* reside in the rhizosphere or spaces between cells of the root cortex and in the rhizoplane, while intracellular (iPGPR) bacteria such as species of *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, endophytes, and *Frankia* are mostly associated with the root nodules (Gupta et al. 2015; Gray and Smith 2005). Accepting and enumerating the impact of PGPR on the root system and the whole plant remain challenging (Gupta et al. 2000). Studies have confirmed that PGPR are perhaps plant-specific genotype and cultivar (Bashan 1998; Lucy et al. 2004). The molecular mechanisms of PGPR affect the architecture of the root system and interfere with the plant hormonal pathways (Vacheron et al. 2013). The two-way cross talk between microbes and plant host for plant growth promotion is presented in Fig. 20.1.

20.2 Mechanisms of Plant Growth Promotion

The mechanisms of plant growth differ between species and strains; so, typically, not a single mechanism is accountable for plant growth promotion. PGPR enhances plant growth either by following direct or indirect mechanisms (Glick 1995; Gupta et al. 2000; Kumar et al. 2012, 2016a) or a combination of both (Fig. 20.2) corresponding to siderophore production, biological nitrogen fixation, phosphate solubilization (Richardson et al. 2009; Ortiz Castro et al. 2009; Hayat et al. 2010; Kumar et al. 2017b), rhizosphere engineering, production of 1-aminocyclopropane-1-carboxylate deaminase (ACC), quorum sensing (QS) signal interference and inhibition of biofilm formation, phytohormone production, antimicrobial activity (Yuwono et al. 2005), and volatile organic compound (VOC) production (Bhattacharyya and Jha 2012). Direct mechanisms, facilitating resource acquisition and modulating phytohormone levels, affect the plant's metabolism and balance plant growth regulators by leading to an increase in its adaptive capacity and

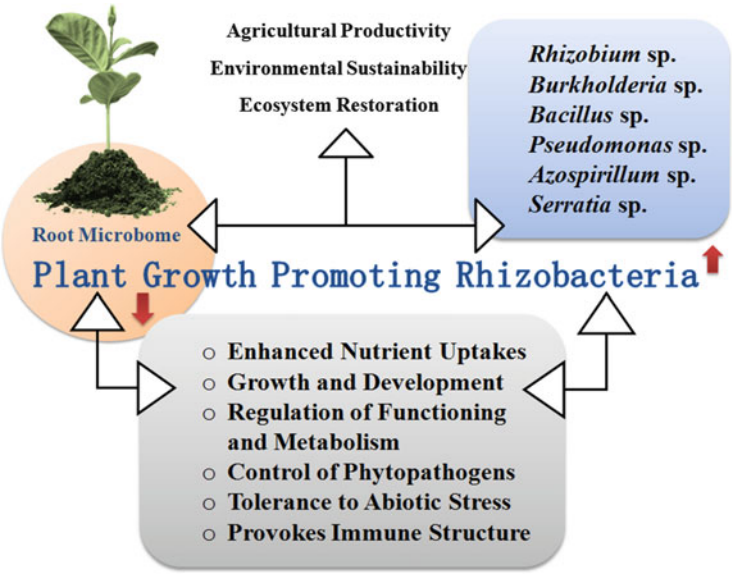

Fig. 20.1 Multifaceted diagram of bidirectional response of PGPR and host for plant growth promotion

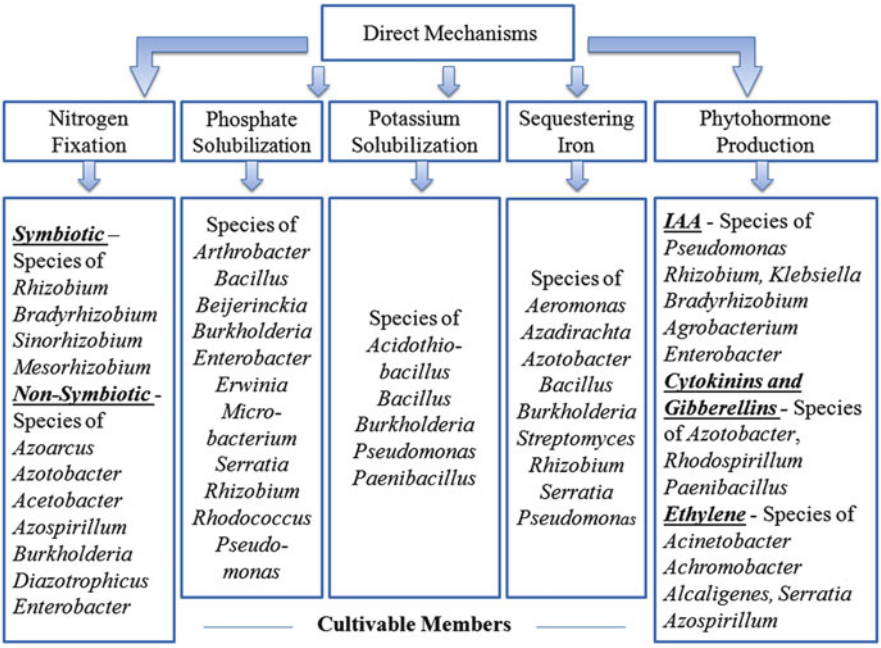

Fig. 20.2 Direct plant growth promotion by bacteria

releasing hormones. Plants and colonization of bacteria have cohabited for millions of years. They live and promote the healthy growth of plant. Facilitating resource acquisition is categorized as nitrogen fixation, potassium solubilization, iron sequestering, and phosphate solubilization (Glick 2012).

20.2.1 Nitrogen Fixation

Nitrogen, being the vital nutrient required for plant growth, and nitrogenase (*nif*) are the key players in providing available N (NH_4^+) to the plant through biological nitrogen fixation. Nitrogenase includes structural genes that are involved in the initiation of the Fe protein, biosynthesis of the molybdenum cofactor, and electron donation and regulatory genes for the synthesis and function of the enzyme. The most critical fixation gene, *Nif*, is typically present in a cluster of around 20–24 kb with 07 operons encoding 20 different proteins (Ahemad and Kibret 2014). Nitrogen-fixing microbes are generally categorized as (a) symbiotic N_2-fixing bacteria like species of *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azoarcus*, *Azotobacter* and (b) nonsymbiotic N_2-fixing bacteria, viz., species of *Azospirillum*, *Diazotrophicus*, *Gluconacetobacter*, *Burkholderia*, *Acetobacter*, and *Enterobacter* (Kumar et al. 2013a; Kumar 2017).

20.2.2 Phosphate and Potassium Solubilization

The phosphate solubilization mechanisms include the release of complexing or mineral-dissolving substances such as organic acid protons, anions, CO_2, hydroxyl ions, and siderophores, the liberation of extracellular enzymes, and the emancipation substrate degradation (McGill and Cole 1981; Sahoo et al. 2017). Species of *Bacillus*, *Burkholderia*, *Microbacterium*, *Rhizobium*, *Enterobacter*, *Rhodococcus*, *Beijerinckia*, *Arthrobacter*, *Serratia*, *Erwinia*, *Flavobacterium*, and *Pseudomonas* are documented as phosphate solubilizers. Members of *Pseudomonas*, *Paenibacillus*, *Burkholderia*, *Acidithiobacillus ferrooxidans*, *Bacillus edaphicus*, and *Bacillus mucilaginosus* (Goswami et al. 2016) are standard potassium (K) solubilizers. These bacterial groups convert insoluble form of K in the soil to soluble forms, through various chemical reactions like exchange reactions, chelation, and acidification (Masood and Bano 2016).

20.2.3 Sequestering Iron (Siderophore)

Iron is an essential element and plays a key role in various physiological processes like DNA synthesis, respiration, and photosynthesis along with key factors of

many enzymes and Fe–S cluster (Dellagi et al. 2009), but the availability of soluble Fe is limited because of its low solubility at neutral pH. Microorganisms secrete high-affinity iron-chelating compounds in low Fe environments which refer to siderophores as the strong iron-chelating agents. These are water-soluble, and extracellular and intracellular siderophores, which have greater affinity for Fe, are synthesized by almost all microbes under iron limitations. Siderophores produced by the same genus are homologous, while others that could utilize those produced by other rhizobacteria of various genera are heterologous siderophores. Loper and Buyer (1991) reported the production of siderophore by different bacterial genera, like pyoverdines by *Pseudomonas* spp., hydroxamates by *Erwinia carotovora* and *Enterobacter cloacae*, catechols by *Agrobacterium tumefaciens* and *Erwinia chrysanthemi*, and rhizobactin by *Rhizobium meliloti*. Species of *Aeromonas*, *Streptomyces*, *Rhizobium*, *Bacillus*, *Azadirachta*, *Burkholderia*, *Serratia*, *Azotobacter*, and *Pseudomonas* are grouped as iron-chelating bacteria. In these rhizobacteria, Fe^{3+} siderophore complex is reduced to Fe^{2+} which is further released into the cell from the siderophore via the inner and outer membrane linking (Parker et al. 2007). The siderophores are destroyed/recycled during the process. The microorganisms producing siderophores have also a major role in the disease suppression of soil-borne disease especially toward fusarium wilts by the action of siderophore-mediated iron competition as well as inducing systemic resistance in plants (Leeman et al. 1996; Meziane et al. 2005).

20.2.4 Modulating Phytohormone Levels

Plant growth-regulating hormones are called phytohormones, namely indole acetic acid (IAA), ethylene, cytokinins, and gibberellins (Glick 2012; Kumar et al. 2013b; Kumar and Mishra 2014). Auxin production is mediated by tryptophan (Trp)-dependent and *Trp*-independent pathways (Wani et al. 2016). Several beneficial effects have been documented for indole acetic acid, viz., regulation in plant cell division and differentiation; stimulatory effects on germination of seed and tuber; development of root and xylem; management of vegetative growth; formation of lateral and adventitious root; effective response to light, gravity, and fluorescence; affects photosynthesis; pigment formation; biosynthesis of various metabolites; and resistance to biotic/abiotic stresses (Glick 2012).

Members of the genera *Pseudomonas*, *Rhizobium*, *Bradyrhizobium*, *Agrobacterium*, *Enterobacter*, and *Klebsiella* are good at IAA production. Ethylene, a gaseous phytohormone, is biosynthesized from methionine via S-adenosyl-L-methionine (AdoMet) and the cyclic nonprotein amino acid ACC (Wani et al. 2016). ACC synthase converts AdoMet to ACC, while ACC oxidase catalyzes the conversion of ACC to ethylene. Species of *Acinetobacter*, *Achromobacter*, *Alcaligenes*, *Azospirillum*, *Ralstonia*, and *Serratia* are ethylene producers. Ethylene also plays a key role in the defense to heat stress. The cytokinins are master regulators during plant growth and development. They increase their endogenous levels via uptake

and enhanced biosynthesis. The gibberellins are tetracyclic diterpenoid carboxylic acids, and few of them function as growth hormones in higher plants, of which GA1 and GA4 are the predominant ones. They are effective counters to seed germination, leaf expansion, stem elongation, flower and trichome initiation, and flower and fruit development. Members of the genera *Azotobacter*, *Pantoea*, *Rhodospirillum*, and *Paenibacillus* are effective cytokinin and gibberellin producers.

20.2.5 Induced Systemic Resistance

The ability of the plant to resist against the disease and develop a defense to overcome it is known as induced systemic resistance (ISR). ISR is directly linked to physiological tolerance with microbial antagonisms in the rhizosphere region as well as production of phytoalexins as a consequence of defense response. Metabolism of jasmonic acid is the major key player in the whole process. PGPR produce antagonistic substances like siderophores, antibiotics (Mageshwaran et al. 2010, 2012), antimicrobial peptides, acyl homoserine lactones, and volatile compounds (acetoin and 2,3-butanediol) that help plant resist against microbial pathogens, thus enhancing plant growth promotion (Weller et al. 2002). Several strains of *Pseudomonas* sp., *Pseudomonas syringae*, and *Pseudomonas stutzeri* have been applied effectively against phytopathogens like *Colletotrichum* and Fusarium wilt diseases (El-Badry et al. 2006). Application of several *Bacillus* species (*B. amyloliquefaciens*, *B. mycoides*, *B. sphaericus*, and *B. subtilis*) is reported to cause significant reduction in disease incidence (Ryu et al. 2004; Govindasamy et al. 2010) in varied field condition experiments. Productions of defense-related enzymes like peroxidase, polyphenol oxidase, β-1,3-glucanase, chitinases, and phenylalanine are the most primary mechanisms of PGPR for inducing SR against *Fusarium oxysporum* and *Rhizoctonia solani* (Dutta et al. 2008). There are reports describing many potential *Pseudomonas* strains (AN-1-UHF, AN-5-UHF, PN-7-UHF, and PN-13-UHF) to produce proteolytic enzymes which have a very pivotal role in plant growth promotion of apple and pear (Ruchi et al. 2008). Combinations of such strains with other biocontrol agents pose a potent synergistic inhibitory effect against pathogens and in the promotion of plant growth.

20.2.6 Volatile Organic Compound Production

Some specific PGPR strains are found to release some mixed chemicals also known as volatile organic compounds (VOCs) which have a noteworthy role in plant growth promotion. These volatile compounds have also an important role in the mechanism for the stimulation of growth of plants by rhizobacteria. These compounds have also a major task in ISR mechanisms (Ryu et al. 2004). Some major volatile compounds mostly produced by PGP microbes belong to the class of

acetaldehyde, ethanol, hydroxyurea, cycloserine, butanal, ethoxyethene, 2-butanol, 1-butanol, 2-methyl,1-propanol, 2-pentanone, 3-hydroxy-2-butanone, 2-ethyl-1-butanol, methoxy-phenyl-oxime, benzaldehyde, dimethyl disulfide, 2-heptanone, dimethyl trisulfide, trimethyl pyrazine, 2-ethyl 1-hexanol, 2-phenyl ethanol, phenyl acetaldehyde, etc. There are some volatile organic compounds, viz., 2,3-butanediol and acetoin, which have been found to be released by certain PGPR strains like *Bacillus subtilis* GB03, *Bacillus amyloliquefaciens* IN937a, and *Enterobacter cloacae* JM22 that have a major role in plant growth promotion of *Arabidopsis thaliana* (Ryu et al. 2003). In *Arabidopsis* against *Erwinia carotovora*, the compounds secreted by these *Bacillus* species have also been able to induce ISR (Ryan et al. 2009). VOCs produced by the rhizobacterial strains can act as signaling molecules in the mediation of plant–microbe interactions as volatiles produced by PGPR colonizing roots are generated at adequate dose to activate the plant responses (Ryu et al. 2003). Some plant volatiles having low molecular weight, viz., jasmonates, terpenes, and green leaf components, as effective signal molecules for living organisms in different trophic levels have also been recognized (Farmer 2001) which have several roles in plant defense mechanisms.

20.2.7 Indirect Mechanisms

Plant growth-promoting microbes indirectly and effectively enhance the plant defense strategies against phytopathogens through several ways (Fig. 20.3), and these processes happen outside the plant, with the involvement of the plants' defensive developments (Goswami et al. 2016). The defensive setups are maintained by the presence of the species of *Bacillus*, *Streptomyces*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Stenotrophomonas*, *Bradyrhizobium*, *Rhizobium*, *Serratia*, and *Streptomyces.* Productions of antibiotics (streptomycin, oligomycin A, butyrolactones, oomycin A, kanosamine, phenazine-1-carboxylic acid, pyrrolnitrin, pyoluteorin, xanthobaccin, viscosinamide, zwittermicin A, and 2,4-diacetylphloroglucinol) prevent the growth of plant pathogens in the vicinity of the plant root (Whipps 2001; Govindasamy et al. 2010; Kumar et al. 2016b), having a broad-spectrum activity. These antibiotics are effective against many phytopathogenic fungi belonging to *Basidiomycetes*, *Deuteromycetes,* and *Ascomycetes*, including *Botrytis cinerea*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* (Kumar et al. 2016b), and *Verticillium dahliae* (Raaijmakers et al. 2010).

Secretion of microbial extracellular lytic enzymes including chitinases, cellulases, β-1,3-glucanases, proteases, and lipases can lyse a portion of the cell walls of many pathogenic fungi of *Fusarium* and *Rhizoctonia* member groups. Production of laminarinase and extracellular chitinase is produced by *P. stutzeri* lyse mycelia of *F. solani*. *Pseudomonas* strains, AN-1-UHF, AN-5-UHF, PN-7-UHF, and PN-13-UHF, were reported to produce lytic enzymes especially proteolytic enzymes which have a significant role in the plant growth promotion of apple and pear (Ruchi et al. 2008). *Bacillus* species isolated from different tomato rhizospheric soil are also

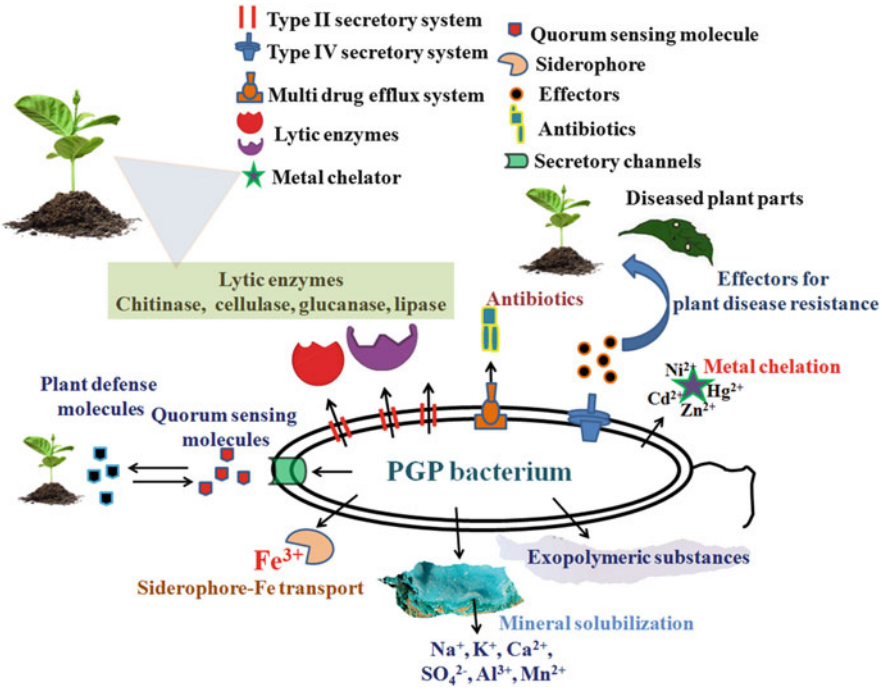

Fig. 20.3 Multifaceted diagram of indirect mechanisms of plant growth promotion by PGP microbe

found to secrete several hydrolytic enzymes such as β-1,3-glucanase, protease, chitinase, and cellulose which have a vital role in plant growth promotion and plant disease management (Kumar et al. 2012). Chitinolytic *Pseudomonas* isolate has also showed a pronounced antifungal activity (Velazhahan et al. 1999). PGP bacteria induce defense systems by inducing systemic acquired resistance and induced systemic resistance (López-Bucio et al. 2007).

The resistance mechanisms reduce the phytotoxic microbial communities and also elicit induced systemic tolerance to abiotic stress (Yang et al. 2009). Solubilization of minerals by PGP microbes (highly specialized lithoautotrophs) is one of the most interesting feature for the availability of inorganic nutrients like K, Na, Ca, and other trace elements by producing inorganic acids (HNO_3, H_2SO_4) as an end product of their metabolism. Members belonging to the genus *Thiobacillus* (S metabolizing) and nitrifiers (*Nitrosomonas* and *Nitrobacter*) are the prominent bacterial members solubilizing rock minerals (K/Ca bearing or PO_4^{3-} minerals). Thiobacilli members (*T. thiooxidans*, *T. ferrooxidans*) are acidophilic or acid tolerant (below pH 1–2), are able to fix CO_2, and use reduced inorganic S compounds. Nitrifying bacteria use urea, ammonium compounds, nitrite, and NO as energy source and some organic compounds for the production of acid on mineral surfaces (concrete, natural stone, glass, feldspar minerals). Some microbial members are potent producers of CO_2 as

the major end product, where CaO, $Ca(OH)_2$, and $CaSiO_2$ react with CO_2 to form $CaCO_3$ in the process of carbonatization, resulting in the decrease of pH from 12.5 to around 8.5 and the subsequent iron/concrete corrosion. The organic acids produced by microbes are having two modes of action of minerals: (a) action of protons and (b) chelation of metal ions. Acids like acetic, gluconic, glucuronic, oxalic, oxaloacetic, succinic, malic, glyoxylic, and others are the most favorable for solubilization processes.

Along with these, other organic acids (amino acids) and polysaccharides are also excreted outside by the microbial cells as a result of unbalanced growth, metabolic bottlenecks, surplus of substrates, or limited supply of nutrients (P, N, K, etc.). Production of organic acids (acetic, butyric, formic, fumaric) and organic solvents (ethanol, butanol, propanol, lactate, acetoin, aldehydes, etc.) as a result of fermentation is also the potential contributor for partial dissolution, swelling, and wear-tear of minerals. Some plant growth-promoting microbes produce exopolymeric substances containing sugars, sugar acids, and amino acids that act as complexing agents and also as metal chelators facilitating reduced metal stress in root rhizosphere. Microbial action of the production of biotic elicitors is also promising in developing defense system of plants, where chemical stimuli activate the production of phytoalexin-type molecules, which elicit morphological and physiological responses in plants in opposition to phytopathogens (Sekar and Kandavel 2010). Compounds like serpentine, ajmalicine, crocetin, picrocrocin, scopolamine, hyoscyamine, and tanshinone are the major stimulatory chemicals produced by PGP microbes for plant defense against pathogenic organisms.

20.3 Taxonomy of Candidate PGP Microbes

Taxonomy, systematics, biosystematics, scientific classification, biological classification, and phylogenetics have allied meanings in records. Classification of small and simple shapes holding bacteria on the basis of morphological characterization is extremely difficult. Besides shape, bacteria are well identified and classified on the basis of their biochemistry and growth conditions. They take account of media, morphology, antibiotic sensitivity, biochemical tests, serological methods, and bacteriophage typing, together constituting the chemotaxonomic and physiological characterization. Recent developments in taxonomic studies including genotypic characters (G+C % content, DNA–DNA homology % based on HPLC and TM methods, whole genome-based average nucleotide identity, average amino acid identity, tetra correlation among nucleotides, pulse-field gel electrophoresis), chemotaxonomic characters (fatty acid methyl esters, cell wall polyamines, cellular sugars, polar lipids, respiratory quinones, cellular amines), characters (pigments, colony properties), numerical taxonomy (computer-assisted characterization like correlation based on Jaccard's coefficient, simple matching coefficient, Spearman coefficient), and genomic (multilocus sequence typing, pan genomics ribosomal protein sequences, genome relatedness from whole genome) have revolutionized

the characterization of many species. The details of the taxonomic markers and their resolution in bacterial systematics are presented in Fig. 20.4. Current strategies of integrating multiple omics technologies like whole genome sequencing (functional and comparative genomics), proteomics (whole-cell and membrane associated), transcriptomics (total RNA pool sequencing), along with matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) have shown high potentiality in evolutionary biology to consider how differently bacteria are associated and evolved (Jia et al. 2015) and their complete physiological as well as genetic cataloging.

20.4 Genus *Rhizobium:* Associative Symbiotic and Free-Living N_2 Fixers

The genera *Azorhizobium*, *Bradyrhizobium*, *Burkholderia*, *Devosia*, *Ensifer*, *Methylobacterium*, *Mesorhizobium*, *Microvirga*, *Ochrobactrum*, *Phyllobacterium*, *Rhizobium*, *Shinella* of *Alphaproteobacteria*, and *Cupriavidus* of *Betaproteobacteria* and some *Gammaproteobacteria* form the set of rhizobia (Berrada and Fikri-

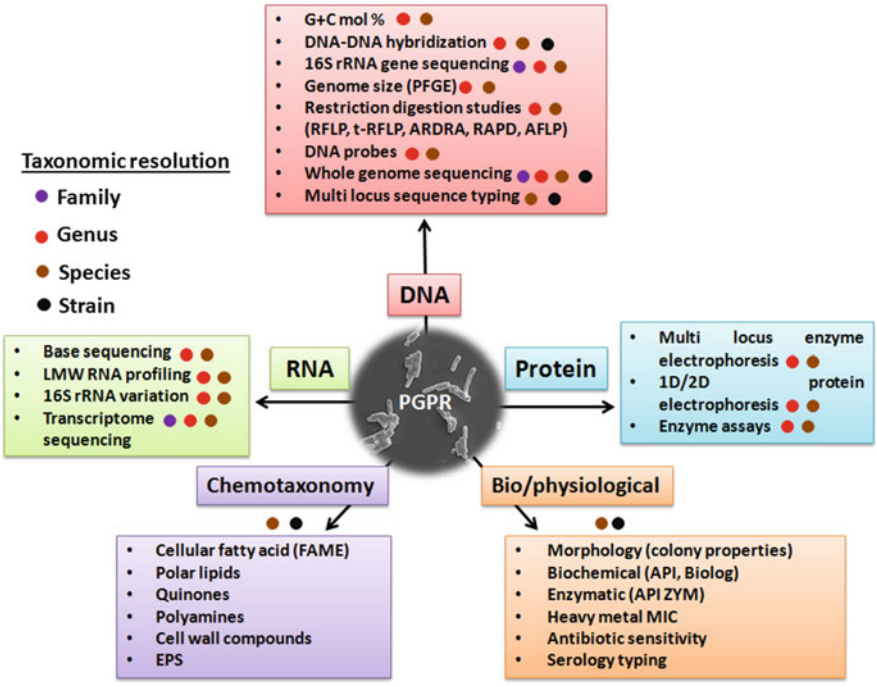

Fig. 20.4 Schematic overview of taxonomic methods used for characterization of microbial candidates and their resolution

Benbrahim 2014). Among all, the members of the genus *Rhizobium* are the most studied for its N_2 fixation ability and supportive plant growth-promoting behavior. Members are Gram-negative, aerobic to facultative anaerobic, nonsporulating, motile rods of 0.5–0.9 × 1.2–3.0 μm (Zakhia and de Lajudie 2001; Willems 2006), mostly attributed to symbiotic N_2 fixation as well as free-living forms (Mohapatra et al. 2016). Since its first description by Frank (1889), 94 validly named species (LPSN, http://www.bacterio.net/) were affiliated to the genus *Rhizobium*. G+C % is on average 59–64 mol%. Colonies are found circular, semitranslucent, raised, and 2–4 mm in diameter within few days of inoculation on solid medium. Turbidity develops in liquid medium after 2 or 3 days. They are chemoorganotrophic in nature. Optimum pH and temperature range between 6–7 and 25–30 °C, respectively. *Rhizobium* is often located in the nodules of beans, peas, and groundnuts. Strains seem host specific in many cases. The bacterial colonization is able to invade the root hairs naturally. In nodules, bacterial clusters fix atmospheric nitrogen into ammonia for plants (Frank 1889). Study shows *Rhizobium* resists chloramphenicol, polymyxin B, erythromycin, neomycin, and penicillin (Cole and Elkan 1979).

On the basis of scientific classification, *Rhizobium* comes under kingdom, *Bacteria*; phylum, *Proteobacteria*; class, *Alphaproteobacteria*; order, *Rhizobiales*; and family, *Rhizobiaceae*. For cultivation and isolation of *Rhizobium* species, yeast mannitol agar and *Rhizobium* medium are used (Gulati 1979). Yeast extract, mannitol, dipotassium phosphate, magnesium sulfate, sodium chloride, and agar are the key components of the medium. *Rhizobium* genus includes *R. galegae* (Mousavi et al. 2014) isolated from the nodules of wild *Galega orientalis* and *Galega officinalis*; *R. gallicum* (Amarger et al. 1997) cultivated in Europe and Tunisia from flat-podded variety of nodulating beans, i.e., *Phaseolus vulgaris*; *R. indigoferae* (Wei et al. 2002) isolated from Indigo fera shrubs; *R. leguminosarum* (Frank 1889; Noel et al. 1996) isolated from canola and lettuce; *R. loessense* (Wei et al. 2003) isolated from nodules of *Astragalus* and *Lespedeza* species; *R. lusitanum* (Valverde et al. 2006) isolated from *Phaseolus vulgaris* and *Leucaena leucocephala*; *R. mongolense* (van Berkum et al. 1996) isolated from Inner Mongolian *Medicago ruthenica*; *R. bangladeshense*; and *R. binae* (Rashid et al. 2015) isolated from root nodules of lentils in Bangladesh. The members are well distributed in soil with immense ecological as well as agricultural significance for their ability to fix nitrogen (N_2) in legume crops for their ability to form root nodules on legumes and fix N_2 (Viteri and Schmidt 1987; Young et al. 2001), with 94 species being in standing nomenclature (http://www.bacterio.net/rhizobium.html). In recent years, new members have been isolated from diverse nonlegume niches including sand dunes, effluent treatment plant, activated sludge, bioreactor, pesticide-contaminated sites, freshwater river, and sea water. New members are also described to degrade various pollutants, heavy metals, and hydrocarbons like naphthalene (*R. naphthalenivorans*; Kaiya et al. 2012), selenite reduction (*R. selenitireducens*; Hunter et al. 2007), exopolysaccharide production (*R. alamii*; Berge et al. 2009), aniline (*R. borbori*; Zhang et al. 2011), use of PAH (*R. petrolearium*; Zhang et al. 2012), and triazophos (*R. flavum*; Gu et al. 2014).

20.5 Genus *Pseudomonas:* Plant Beneficial, Pollutant Degrader

In 1894, the *Pseudomonas* group was depicted as the most assorted and ever-present bacterial genera like Antarctica to the tropics and described to include Gram-negative, strictly aerobic rods that are motile by polar flagella (Skerman et al. 1980). *Pseudomonas* species have been cultured from all kinds of environments worldwide, in sediments, water, soil, the sea, deserts, the plant rhizosphere, fungi, diseased animal specimens, and human clinical samples. *Pseudomonas* strains can linger their constancy in diverse habitats and under very unpleasant circumstances. Over decades, the taxonomy of the *Pseudomonas* genus has been controversial for other bacterial taxa (Peix et al. 2009). Based on the 16S-rRNA similarity, currently there are 140 species belonging to the genus *Pseudomonas* which are termed as *sensu stricto* group I with names that have standing in nomenclature in LPSN (http://www.bacterio.net/pseudomonas.html).

The members are aerobic, Gram-negative, straight or slightly curved rods, 0.5–1.0 μm in diameter, and 1.5–5.0 μm in length. *Pseudomonas* are motile with one or several polar flagella. Some species are found well particular in forming poly-β-hydroxybutyrate as the carbon-storage granule, which appears as sudanophilic inclusions. No resting stages are documented. *Pseudomonas* is not fussy in general. They can grow up on protein hydrolysate, magnesium chloride, and potassium sulfate kind intermediates containing agar media. Species-specific *Pseudomonas* isolation agars also contain cetrimide, nalidixic acid, cephaloridine, penicillin G, pimaricin, malachite green, and glycerol. According to biochemical characterization, *Pseudomonas* shows catalase positive, Voges–Proskauer, and indole and methyl red negative in general. An additional attribute associated with *Pseudomonas* species is that they ooze a yellowish green fluorescence, called pyoverdine, pyocyanin as a blue pigment, a reddish pigment called pyorubin, and pyomelanin as brown function under iron-limiting conditions, as a siderophore, but few secrete quinolobactin as yellow/dark green in the presence of iron. *Pseudomonas* strains are reported to produce IAA, HCN, siderophores, phenazines, cyclic lipopeptides, pyoverdine, and quorum-sensing signaling compounds (Gupta et al. 2014; Kumar et al. 2016b). On the other hand, *Pseudomonas* strains have been executed using MALDI-TOF-MS for excellent identification results (Pineda et al. 2010).

According to the scientific classification, *Pseudomonas* comes under kingdom, *Bacteria*; phylum, *Proteobacteria*; class, *Gammaproteobacteria*; order, *Pseudomonadales*; family, *Pseudomonadaceae*; genus, *Pseudomonas*; and species, *P. fluorescens*, *P. aurantiaca*, and *P. putida. Pseudomonas fluorescens* strains play a major role in plant growth promotion, induction of systemic resistance, and action as bacterial antagonist to control pathogenic bacteria and fungi. It is a potential biopesticide for augmentative biological control of several diseases and bioremediation of various unrefined compounds in agriculture and horticulture (Ganeshan and Kumar 2005). *Pseudomonas aurantiaca s*trains are generally orange-colored soil bacterial members. Rhizosphere soils of sugarcane, soya bean, canola, and potatoes

are the customary habitats of such species. The bacterium produces di-2,4-diacetylfluoroglucylmethan. Di-2,4-diacetylfluoroglucylmethan is a natural phenol compound, which inhibits the growth of phytopathogens and promotes plant growth indirectly. Based on 16S rRNA analysis, *Pseudomonas aurantiaca* is a subspecies of *Pseudomonas chlororaphis* (Peix et al. 2007). *Pseudomonas putida* strains harbor multi-plasmid hydrocarbon-degrading genes (called degradative plasmids). They are the first patented organisms in the world. *P. putida* has been confirmed as a potential biocontrol agent with effectual antagonist activity on damping off diseases such as *Pythium* (Amer and Utkhede 2000) and *Fusarium* (Validov et al. 2007).

20.6 Genus *Bacillus*: Dominant Cum Abundant Members

The majority of *Bacillus edaphicus*, *Bacillus mucilaginosus*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus lipopeptides*, *Bacillus pasteurii*, *Bacillus pumilus*, *Bacillus mycoides*, and *Bacillus sphaericus* are distributed globally with the extensive amount of ability to promote plant growth and have been widely recognized (Govindasamy et al. 2010). The growth promotion includes production of siderophore, phytohormones and antibiotics, solubilization and mobilization of phosphate, inhibition of plant ethylene production, and induction of efficient pathogen resistance (Whipps 2001; Gutiérrez-Mañero et al. 2001; Idris et al. 2007; Richardson et al. 2009). Multilayered chambers of cell wall, secretion of peptide signal molecules and peptide antibiotics, with extracellular enzymes, contribute to survival under unfavorable conservation for extensive periods of time. Repressing capability of plant pathogens by *Bacillus subtilis* and *Bacillus cereus* has been widely recognized. Genus *Bacillus* was named in 1835 by Christian Ehrenberg. By Ferdinand Cohn, *Bacillus* was further characterized as most ubiquitous, spore-forming, Gram-positive, aerobic/facultative anaerobic bacteria. *Bacillus* has expanded to extreme phenotypic variety and heterogeneity. Today, *Bacillus* holds 243 types of species with cultivable isolates (16S rRNA gene sequences >1200 bp) from varied environments (https://rdp.cme.msu.edu/hierarchy/hierarchy_browser/Bacillus), where only 19 types of strains have been reported to be from plants or plant-associated niches.

20.7 Genus *Azotobacter:* Free-Living N_2 Fixers

Azotobacter is a motile, free-living aerobic bacterium with a genomic content of G−C of 63–67.5% (T_m) (Becking 1981). This heterotrophic group of bacteria has thick-walled cysts which may produce large quantities of capsular slime. The particular genus plays an important role in nitrogen cycle as nonsymbiotic nitrogen fixer and acts as PGPR. The bacterial group makes possible the root expansion, improves nutrient uptake potentiality, protects from plant diseases, and increases

biomass production in the rhizosphere region of nearly every one of the crops (Kasa et al. 2015). They are distributed in soils, water, and sediments. *Azotobacter chroococcum*, an oval or a spherical kind of Gram-negative bacterium, was revealed and explained by Martinus Beijerinck in 1901 for the first time (Beijerinck 1901; Mrkovacki and Milic 2001). Lipman stated about *Azotobacter vinelandii* in 1909 and in 1904 on the subject of *Azotobacter beijerinckii*, which he named in the admiration of Beijerinck. In 1949, Russian microbiologist Nikolai Krasilnikov identified the species of *Azotobacter nigricans. Azotobacter nigricans* was divided into two subspecies—*Azotobacter nigricans* subsp. *nigricans* and *Azotobacter nigricans* subsp. *achromogenes* in 1981 by Thompson Skerman. Again, in the year 1981, Thompson and Skerman described *Azotobacter armeniacus.*

In 1991, Page and Shiv Prasad informed concerning *Azotobacter salinestris*—a micro-aerophilic and air-tolerant bacterium. According to the taxonomical division, *Azotobacter* comes near the domain, *Bacteria*; phylum, *Proteobacteri*a; class, *Gammaproteobacteria*; order, *Pseudomonadales*; and family, *Pseudomonadaceae/Azotobacteraceae* (Becking 1999), with most members reported to be described as *A. vinelandii* or *A. chroococcum.* Morphological similarity and biochemical uncertainty with FNFB like *Derxia*, *Azomonas*, and *Beijerinckia* are the difficulties in characterizing *Azotobacter* species. In 2004, a phylogenetic study has shown that *Azotobacter vinelandii* evolved from *Pseudomonas aeruginosa*. After years, in 2007, the genera *Azotobacter*, *Azomonas*, and *Pseudomonas* were publicized as allied or might be synonyms.

20.8 Genomic Insight and Behavior of Some Plant Growth-Promoting Microbes

Of today, 20,584 eubacterial and 907 archaebacterial candidates have been described, out of which 9966 non-type bacterial, 3890 type bacterial, and 210 archaebacterial genomes have been sequenced. The use of genome sequencing through next-generation sequencing (NGS) approach with massively parallel sequencing capacity, high depth coverage, and cost-effective features has moved the basics of bacterial species designation, taxonomy, and phylogeny to a next level termed as "taxonogenomics or phylogenomics." Complete genome projects are enabling the researchers to study the genetic and functional relatedness between organisms at the whole-cell level, thus far beyond conventional 16S rRNA-based phylogeny system. Genetic events such as horizontal gene transfer (HGT), gene rearrangements, plasmid functions in species evolution, and niche adaptation, have become a newer attraction for the geneticists with the high affordability and accessibility to general microbiology laboratories. Completed genome projects with genome features of some candidate PGPR strains are presented in Table 20.1. Recently, NGS has been used to study genomes of different PGPR (free-living and endophytic strains) mainly isolated from crop species such as rice, maize, wheat,

potato, sugarcane, barley, coffee, tea, soybean, etc. and are presented in Table 20.2, with their plant-beneficial properties.

The microbiology of the rhizosphere has been thoroughly studied for more than 100 years, but study on endosphere and the organisms associated (endophytes) remains largely unexplored. Endophytic microbes reside within various tissues of the host plant in a commensal or beneficial manner, and endophytic microbiome is known for its antagonistic activity against pathogens (Berg et al. 2013). They are found to be the promising source of natural metabolites with potential benefits to plant as well as other animals because of their significant bioactivities and medical importance (Kaul et al. 2012; Premjanu and Jayanthy 2012; Mousa and Raizada 2013; Kusari et al. 2014). Endophytes are also beneficial for the host plants with biotic and abiotic stress tolerance, nutrient acquisition, and plant growth promotion (Rodriguez et al. 2008; Kumar et al. 2013c). Genome sequencing has revealed the genetic inventory of these organisms with capability for various plant growth-promoting properties like nitrogen fixation, production of phytohormone (IAA, GA, etc.), mineral acquisition (Fe, P, K), biotic/abiotic stress tolerance, and other nutrient cycling processes (Fouts et al. 2008; Firrincieli et al. 2015; Martinez-Garcia et al. 2015). Recent studies have provided greater understanding on the mode of endophytism in plant root and other plant hosts through gene coding for *N*-acyl homoserine lactone synthases, hydrolases, adherence factors, and fusaric acid resistance in *Pantoea ananatis* (Megias et al. 2016). Genomes of such entophytes (*Gluconacetobacter diazotrophicus* Pal5, *Stenotrophomonas maltophilia* R551-3, *Pseudomonas fluorescens* PICF7, *Kosakonia oryzae* K0348, *Raoultella terrigena* R1Gly, *Bacillus thuringiensis* KB1, *Pseudomonas putida* W619, *Azospirillum* sp. B510, *Variovorax paradoxus*, *Herbaspirillum seropedicae* strain SmR1, *Burkholderia phytofirmans* strain PsJN, *Burkholderia* sp. strain KJ006, *Pseudomonas poae* RE*1-1-14, *Paenibacillus* sp. P22, *Pantoea agglomerans*, *Pseudomonas* sp. strain RIT288, *Janthinobacterium lividum*) are served to be the model systems for studying entophytic plant–microbe interactions. The concept of PGPR-mediated plant growth promotion is gaining worldwide importance and acceptance and has been applied on a wide range of crops including cereals, pulses, vegetables, oilseeds, and plantation crops. Combination of the use of these microbes in plant disease management and the solutions of soil nutrient management might provide ample advantages to agriculture.

20.9 Conclusions and Future Prospects

To avert the lack of sufficient amount of one or more nutrient sources such as nitrogen, iron, and phosphorus and also to obtain higher crop yields, it would obviously be advantageous if efficient biological resources of providing nitrogen, iron, and phosphorus to plants could be commercialized to substitute inexpensive chemical nitrogen, iron, and phosphorus that are currently used. Plant growth-promoting bacteria (PGPB) modulates plant stress indicators under environmental

Table 20.1 Genomic properties of PGPR bacteria as obtained through whole genome sequencing from JGI-IMG database

Genomes	GOLD ID	Size (/10)	Genes	GC	CDS	RNAs	rRNA	tRNAs	COGs	KOG	Pfams	Enzymes	SP	TMH	HTG
R. populi CCTCC AB 2013068	Gs0129175	52.73	5118	0.7	5052	66	3	47	3861	919	4405	1287	399	1170	370
R. leguminosarum bv. trifolii WSM1325	Gs0011842	74.18	7292	0.6	7232	60	9	51	5011	1279	5945	1641	652	1633	132
R. subbaraonis DSM 24765	Gs0129175	65.82	6367	0.6	6289	78	4	48	4332	1024	5084	1412	524	1450	241
R. miluonense HAMBI 2971	Gs0110196	68.08	6493	0.6	6426	67	5	47	4883	1221	5567	1591	557	1510	83
R. rhizosphaerae MH17	Gs0135582	55.34	4924	0.7	4852	72	3	53	3618	874	4077	1235	377	1139	25
R. flavum CCTCC AB 2013042	Gs0129175	46.42	4596	0.6	4528	68	3	47	3346	860	3846	1179	428	1118	45
R. etli 8C-3	Gs0128632	73.09	7131	0.6	7030	101	10	57	4902	1185	5719	1573	586	1582	52
R. giardinii bv. giardinii H152	Gs0014878	68.10	6691	0.6	6618	73	2	48	4585	1171	5471	1668	556	1491	101
R. leguminosarum bv. phaseoli 4292	Gs0014878	73.47	7193	0.6	7109	84	9	53	5163	1289	5983	1677	622	1615	10
R. leguminosarum bv. Trifolii	Gs0000556	72.06	6766	0.6	6755	79	3	68	5122	1227	4988	1433	654	1025	35
R. yantingense CCTCC AB 2014007	Gs0129175	58.16	5580	0.6	5507	73	3	51	4098	950	4621	1313	498	1348	85
R. acidisoli FH23	Gs0117713	73.44	7111	0.6	7028	83	3	53	4948	1234	5794	1605	607	1562	35
R. selenitireducens ATCC BAA-1503	Gs0015051	49.77	4780	0.6	4714	66	6	46	3586	882	4107	1211	429	1109	210
R. favelukesii LPU83	Gs0112019	75.70	7785	0.6	7687	98	9	56	4824	1167	5933	1568	582	1581	23
R. taibaishanense DSM 100021	Gs0129175	54.02	4925	0.6	4856	69	4	47	3716	913	4167	1282	376	1148	59
R. alamii LMG 24466	Gs0129175	74.12	7299	0.6	7219	80	2	52	5164	1263	5950	1564	686	1681	41
R. smilacinae CCTCC AB 2013016	Gs0129175	60.06	5775	0.6	5702	73	3	53	4280	984	4867	1399	509	1423	75
R. tropici CF286	Gs0103573	71.42	6824	0.6	6744	80	2	53	4940	1223	5686	1616	609	1606	79
R. hidalgonense FH14	Gs0135555	72.55	7079	0.6	7001	78	3	51	4856	1209	5700	1541	627	1605	12
R. rhizoryzae DSM 29514	Gs0129175	48.62	4616	0.6	4546	70	3	50	3506	886	3929	1235	377	1062	29
R. marinum MGL06	Gs0111130	50.62	4965	0.6	4900	65	3	43	3634	882	4162	1251	432	1187	111

(continued)

Table 20.1 (continued)

Genomes	GOLD ID	Size (/10)	Genes	GC	CDS	RNAs	rRNA	tRNAs	COGs	KOG	Pfams	Enzymes	SP	TMH	HTG
R. yanglingense LMG 19592	Gs0129175	71.76	7032	0.6	6945	87	4	55	5030	1269	5798	1603	601	1593	22
R. paknamense DSM 100301	Gs0129175	53.09	4799	0.6	4718	81	4	54	3577	901	4052	1242	380	1109	87
R. pisi DSM 30132	Gs0129175	69.36	6780	0.6	6705	75	2	51	4828	1226	5580	1556	585	1546	22
R. vignae CCBAU 05176	Gs0111133	63.43	6140	0.6	6071	69	5	45	4487	1069	5139	1459	545	1418	77
R. anhuiense C15	Gs0135555	70.67	6808	0.6	6732	76	3	49	5051	1248	5753	1609	615	1594	18
R. nepotum 39/7	Gs0119505	53.25	5068	0.6	4996	72	3	46	3902	913	4356	464	442	1217	32
R. mesoamericanum DSM 28449	Gs0129175	63.53	6267	0.6	6193	74	3	48	4306	1101	5016	1437	509	1416	31
P. mediterranea CFBP 5447	Gs0030206	63.09	5737	0.6	5536	201	3	54	4025	1107	4759	1399	569	1227	37
P. pertucinogena DSM 18268	Gs0015051	30.65	2928	0.6	2863	65	4	48	2194	628	2527	940	280	738	251
P. pelagia CL-AP6	Gs0030222	46.42	4348	0.6	4260	88	3	46	3042	835	3681	1131	429	1066	966
P. taiwanensis SJ9	Gs0030418	62.53	5600	0.6	5518	82	12	68	3288	867	4361	1277	512	1094	44
P. flexibilis JCM 14085	Gs0115713	37.48	3553	0.7	3467	86	6	58	2695	766	3120	1044	358	862	30
P. guariconensis LMG 27394	Gs0114533	50.79	4741	0.6	4618	123	14	60	3547	981	4085	1300	510	1079	18
P. amygdali pv. lachrymans 107	Gs0117564	73.09	7087	0.6	6945	142	2	84	3901	968	4795	1400	682	1407	0
P. asturiensis sp. nov LMG 26898	Gs0114533	61.74	5666	0.6	5540	126	7	58	3961	1046	4731	1430	569	1266	45
P. paralactis DSM 29164	Gs0118325	60.14	5615	0.6	5348	267	10	56	3880	1007	4554	1346	606	1217	19
P. furukawaii KF707	Gs0030226	66.78	6211	0.7	6111	100	27	73	4295	1208	5171	1499	599	1314	169
P. xinjiangensis CCTCC 207151	Gs0114533	35.37	3352	0.6	3278	74	9	50	2544	720	2909	990	338	851	213
P. rhodesiae	Gs0113582	60.50	5553	0.6	5420	133	9	58	3939	1010	4640	1363	602	1267	46
P. thivervalensis DSM 13194	Gs0119845	65.80	5971	0.6	5731	240	13	58	4304	1120	4988	1458	622	1301	0
P. otitidis LMG 23769	Gs0114533	63.45	5879	0.7	5779	100	6	54	4157	1094	4943	1400	721	1292	51
P. aeruginosa Pae221	Gs0117356	62.83	5869	0.7	5751	118	3	57	4431	1176	5070	1487	699	1393	0
P. benzenivorans DSM 8628	Gs0114533	57.43	5305	0.7	5188	117	6	60	3794	1054	4549	1397	553	1202	254

P. aeruginosa ATCC 700888	Gs0030008	67.95	6423	0.7	6367	56	3	53	4388	1145	5404	1593	693	1429	43
P. aeruginosa JD322	Gs0118288	61.77	6523	0.7	6435	88	5	27	4030	1005	5385	1635	665	1460	1
P. alkylphenolia KL28	Gs0000556	0.08	7	0.6	7	0	0	0	4	1	5	1	0	2	0
P. composti CECT 7516	Gs0114533	53.92	5040	0.6	4928	112	8	56	3731	946	4328	1289	591	1205	43
P. oryzihabitans H72	Gs0120401	53.16	5005	0.7	4897	108	14	58	3641	952	4214	1313	496	1138	8
P. simiae DSM 18861	Gs0114533	62.40	5814	0.6	5687	127	12	54	4264	1107	4932	1444	660	1321	21
P. azotoformans LMG 21611	Gs0114533	67.27	6256	0.6	5997	259	16	68	4542	1201	5261	1533	747	1423	24
P. batumici UCM B-321	Gs0115688	65.93	5979	0.6	5833	146	6	53	4199	1127	4987	1475	602	1356	163
P. aeruginosa CIG1	Gs0030008	65.36	6078	0.7	6025	53	3	50	4233	1082	5204	1528	675	1382	55
P. amygdali pv. tabaci str. ATCC 11528	Gs0116387	61.28	5587	0.6	5465	122	8	57	3799	983	4587	1350	554	1218	0
P. avellanae BPIC 631	Gs0030107	58.47	4789	0.6	4757	32	3	29	3230	825	4089	1268	434	1026	22
P. aeruginosa RW72	Gs0120424	64.78	6047	0.7	5922	125	3	55	4511	1184	5174	1478	721	1409	1
P. taetrolens DSM 21104	Gs0118325	49.20	4582	0.6	4479	103	8	59	3485	991	3945	1316	429	1055	0
P. pseudoalcaligenes CECT 5344	Gs0030225	46.56	4378	0.6	4314	64	3	61	3104	801	3789	1170	384	960	187
P. antarctica LMG 22709	Gs0114533	63.77	6038	0.6	5796	242	18	67	4150	1075	4918	1419	641	1321	50
A. beijerinckii DSM 1041	Gs0103574	50.84	4951	0.7	4824	127	8	53	3150	889	4033	1326	397	893	361
A. beijerinckii DSM 282	Gs0103574	49.15	4872	0.7	4756	116	6	54	3076	854	3928	1271	386	893	362
A. beijerinckii DSM 373	Gs0103574	50.72	4987	0.7	4870	117	5	53	3183	895	4084	1318	396	911	419
A. beijerinckii DSM 378	Gs0103574	49.40	4719	0.7	4598	121	4	54	3117	868	3906	1295	391	905	357
A. beijerinckii DSM 381	Gs0103574	49.23	4865	0.7	4748	117	6	53	3096	868	3928	1277	378	903	358
A. chroococcum DSM 2286	Gs0131304	48.60	4631	0.7	4515	116	6	55	3107	853	3840	1231	399	965	95
A. chroococcum NCIMB 8003	Gs0001478	51.92	4871	0.7	4728	143	18	67	3269	854	3992	1283	413	964	278
A. vinelandii CA	Gs0001480	53.66	5147	0.7	5048	99	18	64	3485	955	4150	1326	395	976	4
A. vinelandii CA6	Gs0001481	53.23	5105	0.7	5006	99	18	64	3453	952	4113	1320	388	962	4
A. vinelandii DJ, ATCC BAA-1303	Gs0001479	53.65	5133	0.7	5051	82	18	64	3441	954	4149	1325	395	976	1140
A. vinelandii DSM 279	Gs0103574	54.85	5230	0.7	5099	131	11	55	3538	954	4402	1353	433	1026	116
A. vinelandii NBRC 13581	Gs0001482	51.30	4872	0.7	4761	111	5	50	3399	934	4099	1294	423	989	34

(continued)

Table 20.1 (continued)

Genomes	GOLD ID	Size (/10)	Genes	GC	CDS	RNAs	rRNA	tRNAs	COGs	KOG	Pfams	Enzymes	SP	TMH	HTG
B. acidiproducens DSM 23148	Gs0015051	33.20	3425	0.4	3278	147	18	73	2249	675	2792	969	135	839	68
B. aerophilus C772	Gs0115005	37.53	3917	0.4	3808	109	19	61	2683	737	3315	1081	186	1062	3
B. aidingensis DSM 18341	Gs0015051	44.20	4531	0.5	4413	118	13	57	2982	834	3662	1178	255	1131	170
B. akibai JCM 9157	Gs0105906	47.40	5021	0.4	4861	160	19	94	2791	751	3871	1279	280	1367	47
B. alcalophilus ATCC 27647	Gs0001513	42.18	4095	0.4	4063	32	2	30	2697	702	3388	1076	166	1105	157
B. altitudinis 41KF2b	Gs0001515	36.79	3800	0.4	3745	55	3	52	2643	719	3259	1070	257	1059	3
B. alveayuensis 24KAM51	Gs0113108	67.02	6828	0.4	6685	143	13	88	4542	1319	5681	2001	162	1627	72
B. amyloliquefaciens 11B91	Gs0120820	40.24	4025	0.5	3904	121	15	75	2730	792	3386	1084	186	1029	3
B. amyloliquefaciens plantarum	Gs0104006	39.92	4170	0.5	4060	110	3	80	2664	766	3452	1146	185	1062	3
B. andreraoultii KW-12	Gs0002052	40.44	3897	0.4	3766	131	25	88	2400	684	3083	915	88	999	0
B. anthracis 2,000,031,006	Gs0118987	54.35	5856	0.4	5756	100	8	66	3272	886	4612	1242	235	1726	0
B. aquimaris	Gs0002052	44.23	4533	0.5	4386	147	19	84	2799	782	3604	1092	182	1242	35
B. aryabhattai B8W22	Gs0001585	50.95	5351	0.4	5232	119	6	78	3363	983	4292	1296	318	1516	13
B. atrophaeus 1013-1	Gs0001587	41.26	4213	0.4	4115	98	3	66	2783	817	3474	1153	262	1105	0
B. aurantiacus DSM 18675	Gs0015051	40.25	4094	0.4	3991	103	17	71	2583	731	3231	1037	232	1160	57
B. australimaris NH7I_1	Gs0120219	36.44	3740	0.4	3675	65	2	37	2645	739	3232	1067	184	1024	3
B. azotoformans LMG 9581	Gs0001602	42.23	4255	0.4	4226	29	4	25	2652	704	3487	1090	266	1128	386
B. badius DSM 30822	Gs0115022	40.64	4210	0.4	4068	142	22	89	2640	747	3328	1038	158	1065	20
B. bataviensis LMG 21833	Gs0001603	53.71	5236	0.4	5207	29	6	23	3534	1013	4453	1387	282	1448	317
B. beveridgei MLTeJB	Gs0113225	35.82	3469	0.5	3363	106	22	67	2451	657	2890	972	117	900	53
B. bingmayongensis FJAT-13831	Gs0001891	54.72	5667	0.4	5546	121	11	83	3331	922	4528	1265	335	1521	24
B. bogoriensis ATCC BAA-922	Gs0015051	50.02	4950	0.4	4822	128	27	71	3027	772	3869	1180	291	1427	148
B. bombysepticus Wang	Gs0110388	58.74	5884	0.4	5724	160	39	95	3487	964	4688	1310	267	1690	7
B. boroniphilus JCM 21738	Gs0105907	43.65	5420	0.4	5294	126	7	77	2148	514	3957	1404	246	1346	4
B. butanolivorans AFS003229	Gs0133685	58.68	5756	0.4	5652	104	4	64	3646	1107	4581	1412	192	1460	9
B. camelliae 7578-1	Gs0135640	49.46	5109	0.4	5023	86	4	56	3054	830	3932	1159	148	1277	68

B. campisalis SA2-6	Gs0116372	51.82	5300	0.5	5062	238	81	116	3410	1026	4239	1380	235	1380	66
B. caseinilyticus SP	Gs0110196	58.73	5337	0.5	5199	138	12	101	3352	774	4164	1299	223	1517	175
B. cecembensis DSM 21993	Gs0116134	47.32	4834	0.4	4735	99	4	46	2840	765	3679	1004	198	1303	36
B. cellulasensis NIO-1130	Gs0119568	36.13	3779	0.4	3684	95	14	53	2637	723	3228	1063	176	1043	0
B. cellulosilyticus N-4, DSM 2522	Gs0018994	46.82	4443	0.4	4327	116	30	81	2673	691	3431	1054	237	1316	340
B. cereus #17	Gs0118079	58.39	5996	0.4	5834	162	38	92	3567	934	4745	1320	263	1713	2

Abbreviations: *R.—Rhizobium*; *P.—Pseudomonas*; *A.—Azotobacter*; *B.—Bacillus*; SP—signal peptides; TMH—transmembrane helices; HTG—horizontally transferred genes

Table 20.2 Genomic perspective of some plant-beneficial PGP microbes

PGPR	Genome size (Mb)	Host plant	PGP traits
Azoarcus sp. BH72	4.37	Rice	N_2 fixation
Azospirillum lipoferum 4B	6.85	Rice, maize, wheat	N_2 fixation, phytohormone
Azospirillum sp. B510	7.6	Rice	N_2 fixation, phytohormone
Burkholderia phytofirmans PsJN	8.2	Potato, tomato, maize, barley	IAA synthesis, ACC deaminase
Burkholderia sp. KJ006	6.6	Rice	ACC deaminase, antifungal action
Enterobacter cloacae ENHKU01	4.7	Pepper	Unknown
Enterobacter sp. 638	4.67	Poplar	Siderophore, IAA, acetoin and 2,3-butanediol synthesis
Gluconacetobacter diazotrophicus PaI5	3.9	Sugarcane, rice, coffee, tea	N_2 fixation, auxin synthesis
Klebsiella pneumoniae 342	5.9	Maize, wheat	N_2 fixation
Pseudomonas putida W619	5.77	Poplar	IAA synthesis, ACC deaminase
Pseudomonas stutzeri A1501	4.5	Rice	N_2 fixation
Serratia proteamaculans 568	5.5	Soybean	IAA synthesis, ACC deaminase, acetoin and 2,3-butanediol synthesis
Stenotrophomonas sp. KA1	4.57	Poplar	IAA synthesis, ACC deaminase
Stenotrophomonas maltophilia R551-3	4.67	Poplar	IAA synthesis, ACC deaminase
Rhizobium leguminosarum	5.5	Pea	N fixation, phytohormone
Citrobacter freundii	5.9	Rice	Phytohormone, IAA synthesis

Source: Ashraf et al. (2004), Krause et al. (2006), Yan et al. (2008), Taghavi et al. (2009), Kaneko et al. (2010), Weilharter et al. (2011), Liu et al. (2013)

stresses. PGPB helps in mounting niche in the expansion of organic agriculture. The benefits done by PGP bacteria to the agriculture are enormous. Numerous genetically engineered PGP bacteria are already being used successfully in a number of countries in the developing world commercially as adjuncts to agricultural practice. The use of detailed molecular techniques and next-generation OMICS-based tools is still to be implemented to study elaborate biochemical and molecular functions of the plant-beneficial microbes. Integrated use of genomics, proteomics, transcriptomics, metabolomics, and secretomics might help biologists to gain better insight into the ecophysiological aspects and niche adaptation strategies of PGP microbes. In spite of all odds, commercialized and more efficacious strains of *Azotobacter*, *Bacillus*, *Paenibacillus*, *Pseudomonas*, and various *Rhizobia* sp. are showing promising development in the field of inoculation. So, study on microbes and their interaction

with plants on commercial scale is still required to make PGPB an efficient technique in agricultural sustainability and intensive production practices.

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Chapter 21
Mycorrhizae and Tolerance of Abiotic Stress in Citrus Plants

Chun-Yan Liu, Ying-Ning Zou, De-Jian Zhang, Bo Shu, and Qiang-Sheng Wu

Abstract Many environmental factors such as soil water, soil salinity, and low or high temperature confer strong inhibition in tree growth and fruit quality of citrus. Soil arbuscular mycorrhizal fungi (AMF) can establish arbuscular mycorrhizal symbiosis with terrestrial plants. It is documented that citrus plants are heavily dependent on arbuscular mycorrhizal symbiosis. Generally, these negative abiotic stresses dramatically inhibit both AMF infection in citrus roots and extraradical hyphae development in rhizosphere soils. Nevertheless, studies indicated the mitigated effects in citrus plants subjected to short-term or long-term adverse environments. Under abiotic stresses conditions, AMF still significantly promotes citrus plant growth performance and subsequently considerably enhances the tolerance of abiotic stresses. Many studies had shown the underlying mechanisms of AMF-enhanced tolerance of abiotic stresses in citrus plants: (1) greater plant growth performance and root architecture; (2) enhanced water and nutrient absorption by extraradical hyphae; (3) massive accumulation of osmolytes and enhancement of antioxidant-protected systems; (4) changes in phytohormones and signaling substances; and (5) upregulation expression of relevant stressed genes. Future perspectives in this field are proposed. Such benefits of mycorrhizal symbiosis can provide the approach as biofertilizers to sustain agriculture and environments.

21.1 Introduction

Citrus is a global fruit tree grown in tropical and subtropical regions. Recently, citrus-planting area in the world has increased steadily from 876.73 hm^2 to 1343.27 hm^2 from 2000 to 2015 (FAO). The world's citrus production increased from 11517.8 million tons to 17848.2 million tons. Currently, citrus cultivation in the

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world is mostly concentrated in Asia, accounting for 52.90% of the total area, 24.50% in Americas, 16.60% in Africa, and 6% in Europe and Oceania. China, India, and Morocco are the countries with the fastest growing area of citrus in recent years. Among the major producing countries, China and India have the fastest growth in output, increasing from 9.2358 million tons and 4.41 million tons in 2000, respectively, to 35.4693 million tons and 11.466 million tons in 2014, followed by Egypt, Turkey, and South Africa, which increased by 85.69%, 70.26%, and 56.93%, respectively, from 2000 to 2014. In many cases, based on the impact of climate change, citrus plants are persistently challenged with numerous abiotic stresses in the field, such as temperature stress (heating and chilling) (Zhu et al. 2010, 2011), soil water deficit stress (Zou et al. 2017; Zhang et al. 2018), salt stress (Wu and Zou 2013; Zhang et al. 2016), nutrient stress (P and Fe deficiency stress) (Shu et al. 2012; Chen et al. 2017a, b; Liu et al. 2018a), heavy metal stress (As, Pb, and Ni stress), and waterlogging stress (Wu et al. 2013c; Zou et al. 2014b). These adverse effects due to abiotic stresses seriously obstruct citriculture.

Arbuscular mycorrhizal fungi (AMF), the most closely related beneficial microorganisms in plant rhizosphere, widely exist in natural conditions, which can form a symbiotic relationship with approximately 80% of terrestrial plants (Wu et al. 2013a). In general, arbuscular mycorrhizae (AMs) include hyphae, entry points, inter- and extra-hyphae, arbuscules, vesicles, and spores. External mycelium colonizes root systems through entry points and then establishes a symbiotic relationship between roots and AMF, where the typical structure of arbuscule is formed in cortical cells. After plant roots establish symbiosis with AMF, AMs absorb a large amount of water and mineral nutrients (such as P and N) from soils to host plants. Host plants supply photosynthate to AMF for its growth (Asrar et al. 2012). Mycorrhizal roots for water and nutrient uptake can be enhanced. Studies indicated that under abiotic stress conditions, AMF could enhance the tolerance of host plants by regulating plant water and nutrient uptake efficiency, photosynthetic rate, osmotic regulation capacity, reactive oxygen metabolism, plant hormone synthesis, and molecular changes (Ruiz-Lozano et al. 2008; Wu et al. 2013b). In this chapter, we simply outline the responses of arbuscular mycorrhizae to abiotic stress in citrus plants.

21.2 Citrus Mycorrhizae

Citrus plants can form arbuscular mycorrhizal association under field cultivation. In 1922, Peyrone first observed and documented the presence of mycorrhizae in Italian citrus orchards. In 1933, Rayner from Citrus Test Station of the University of California, Riverside, USA, successfully observed the mycelia and large vesicles existed in young roots of *Citrus sinensis* and *C. aurantium*. In 1935, Reed and

Fremont found that mycorrhizal fungi were abundant in unfertilized soils, while no mycorrhizal fungi existed in soils after $NaNO_3$ application. In the same year, Rayner (1935) also proposed that mycorrhizal fungi were an important factor in citrus nutrition metabolism and played a very important role in citrus production. Since the 1990s, citrus mycorrhizal works have developed rapidly. AMF have been proved to be involved in regulating water and carbon metabolisms in citrus, promoting nutrient uptake by host plants (Smith and Read 2008; Cozzolino et al. 2010), and strengthening the resistance of host plants to abiotic stress and disease resistance (Zou et al. 2017). We had observed the different AM structures (vesicles, arbuscules, entry points, extra- and intraradical mycelium, and spores) in roots of citrus plants grown in pots or field (Fig. 21.1).

In citrus orchards, the most common AMF species are *Funneliformis mosseae*, *Diversispora versiformis*, *Rhizoglomus intraradices*, and *Paraglomus occultum*. Although these AMF species can establish a beneficial symbiotic structure with citrus roots, AM development is also influenced by internal and external factors, including AMF species (Yao et al. 2009), host plant genotypes (Li et al. 2013a; Table 21.1), soil moisture and nutrient status (Khalvati et al. 2005; Egerton-Warburton et al. 2008; Miransari 2010; Wu et al. 2013a), and soil pH value (Wang et al. 2008a). Li et al. (2013a) inoculated *Diversispora spurca* on four different citrus genotypes and observed that root mycorrhizal colonization of the four different citrus genotypes was ranked as kumquat > lime > trifoliate orange > red tangerine in the decreasing order. Somchit et al. (2009) collected a mixed AMF inoculum from *Citrus* sp. rhizosphere and then inoculated on lime, pomelo, sweet orange, and a hybrid citrange or Troyer. They found that AM colonization in the root ranged from 75% to 96% and spore densities of rhizosphere were 14–28 spores/10 g soil. Nevertheless, there was no difference in mycorrhizal development among citrus genotypes. Possibly, spore production does not correlate with root mycorrhizal colonization, but depended on the inherent nature of AMF in various soil conditions (Youpensuk et al. 2006).

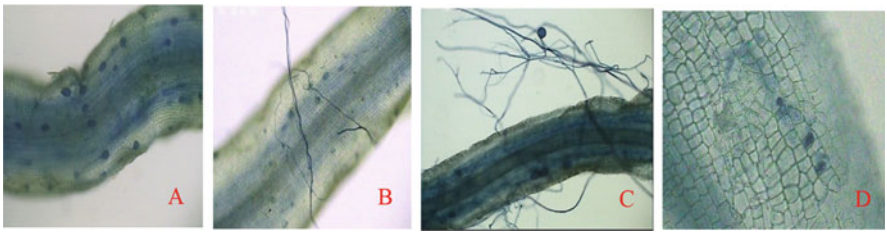

Fig. 21.1 Mycorrhizal structures in citrus roots. (**a**) Intraradical hyphae and vesicles in red tangerine roots. (**b**) Extraradical hyphae and entry points in kumquat roots. (**c**) Extraradical hyphae and spore in trifoliate orange roots. (**d**) Arbuscules in trifoliate orange roots

Table 21.1 Root mycorrhizal development and extraradical hyphae length in four citrus genotype plants colonized by *Funneliformis mosseae*

Citrus genotype	Extraradical hyphae length (cm/g)	AMF colonization (%)	Vesicle (#/cm)	Arbuscules (#/cm)	Entry points (#/cm)
Citrus tangerina	36.78 ± 3.83a	43.7 ± 1.7a	9.06 ± 0.92b	4.46 ± 0.23b	5.95 ± 0.51d
Fortunella margarita	27.51 ± 2.08b	21.1 ± 1.87c	3.36 ± 0.37d	1.87 ± 0.16d	7.20 ± 0.84c
Citrus junos	35.29 ± 2.82a	30.9 ± 1.93b	5.47 ± 0.62c	3.10 ± 0.35c	10.91 ± 1.02b
Poncirus trifoliata	29.6 ± 2.6b	42.09 ± 0.78a	11.87 ± 0.60a	6.89 ± 0.27a	20.01 ± 1.28a

Note: Data (mean ± SD, $n = 4$) followed by different letters indicate significant differences ($P < 0.05$) in different citrus genotypes

21.3 Citrus Mycorrhizae in Response to Abiotic Stress

Several studies have demonstrated that drought stress significantly inhibited the mycorrhizal infection rate and mycelial length in rhizosphere soils of citrus plants because drought stress usually restrains mycelial growth and spore germination (Zhang et al. 2018; Huang and Wu 2017). The inhibition of mycorrhizal growth was related to the decrease of carbohydrates in plants under drought stress (Wu et al. 2013a). As reported by Wu et al. (2013c), soil waterlogging stress notably reduced root mycorrhizal colonization by 43% in *D. spurca*-colonized *C. junos* seedlings, whereas the entry points and vesicles were dramatically increased by 241% and 78%, respectively. In *Diversispora spurca*-colonized trifoliate orange, waterlogging treatment showed 29% lower root colonization and 78% lower number of vesicles than normal water supply treatment, but had 95% higher entry point numbers (Zou et al. 2014a). Interestingly, under waterlogging conditions, intercropped with *Paspalum notatum* significantly increased the hyphal density and root colonization of trifoliate orange seedlings colonized by *Gigaspora margarita* (Matsumura *et al.* 2008). As early as 1986, Duke et al. (1986) revealed the reduction of the root AM colonization of split-root citrus plants under salt stress. With an increasing salt stress (0, 50, 100, and 150 mM NaCl), root mycorrhizal colonization in Karna Khatta and Troyer citrange was heavily reduced (Murkute et al. 2006). In *G. mosseae*-infected *C. tangerine* seedlings, salt stress (100 mM) reduced root AMF colonization (Wu et al. 2010a). Possibly, salinity seriously inhibited hyphal development, thus resulting in the decline in AMF colonization (Juniper and Abbott 2006). Nevertheless, Wu et al. (2010b) also found that soil salinity did not affect the root AMF colonization of red tangerine seedlings colonized by *P. occultum*. A similar result was also obtained on sweet orange and sour orange inoculated with *G. intraradices* (Hartmond et al. 1987). The two distinct responses of AMF to salt stress are due to the origins of AMF strains, as reported by Carvalho et al. (2004) in *P. occultum* and *G. mosseae* originated from a saline soil and a nonsaline soil.

Soil nutrient levels, especially P levels, can affect the infection of AMF to host plant roots. Numerous studies indicated that mycorrhizal colonization of plants usually decreases with the increase of soil phosphorus levels (Lekberg and Koide 2005; Gabriel-Neumann et al. 2011). The influence of soil P levels on spore germination ability and extraradical mycelial development indirectly determines the mycorrhizal colonization on the root system (Trindade et al. 2006; Leifheit et al. 2014). Mycorrhizal colonization in trifoliate orange was substantially decreased with increasing substrate P levels (Wu et al. 2015a). In addition, Liu et al. (2018a) further confirmed that low P (0.1 mM) treatment dramatically elevated the infection of *F. mosseae* in the root system of trifoliate orange compared to an appropriate P level (1 mM).

Temperature is another important factor affecting root mycorrhizal development. Suboptimum temperature can adversely affect AM development (Tommerup 1983; Daniels Hetrick and Bloom 1984). As reported by Wu and Zou (2010), under low temperature treatment, the positive effect of mycorrhizal infection for host plants

(e.g., *Citrus tangerina*) almost disappeared. Root mycorrhizal colonization and entry point number were higher under 25 °C than under 15 °C and 35 °C in trifoliate orange colonized by *G. mosseae* (Wu 2011). Interestingly, root AM colonization and entry point numbers of *G. mosseae*-infected trifoliate orange root were also maintained 30% and 99% higher under 35 °C than under 15 °C, respectively (Wu 2011). In trifoliate orange seedlings, the number of vesicles and arbuscules was significantly reduced under 15 °C, but arbuscule number was reduced under 35 °C (Wu 2011). These results revealed that compared with high temperature treatment, the more susceptible influence on mycorrhizal establishment was showed in *G. mosseae*-colonized trifoliate orange seedlings under low temperature conditions, which may relate with the spore response of AMF at suboptimum temperature (Tommerup 1983).

21.4 Mycorrhizal Functioning on the Tolerance of Drought Stress

Drought stress severely limits plant growth and crop production. Nearly one-third of agricultural soils in the world are subjected to drought stress (Calvo-Polanco et al. 2016). As the global climate is deteriorating, drought has become a worldwide environmental problem in the arid and semiarid areas (Compant et al. 2010). Drought stress always leads to inferior soil water content and plant cell dehydration, which affect cell division and differentiation, leaf morphology, stem elongation, root system architecture, gas exchange, water/nutrient transportation, and its use efficiency (Kaushai and Wani 2016). In the process of plant growth and development, new mechanisms have evolved to adapt drought stress, as observed in mycorrhizal plants (Khoyerdi et al. 2016).

21.4.1 Morphological Adaptation of Roots

In order to analyze the adaptability of the root system to drought stress, Liu et al. (2016) conducted an experiment on trifoliate orange seedling colonized by *F. mosseae*. The result showed that *F. mosseae* inoculation stimulated root morphology and also increased the lateral root numbers irrespective of water situations, as compared with non-AMF seedlings (Fig. 21.2). The improvement of root morphological adaptability caused by mycorrhizal infection can enhance the potential function of the root system to absorb water and nutrients in soils (Comas et al. 2013), thus improving the drought tolerance of host plants. Under drought stress, mycorrhizal colonization induced better root morphology adaptation, which is possibly related with root indole-3-acetic acid (IAA), methyl jasmonate (MeJA), nitric oxide (NO), and calmodulin (CaM) changes (Zou et al. 2017). Furthermore, AMF can

Fig. 21.2 Plant growth of trifoliate orange seedlings inoculated with *Funneliformis mosseae* under 0.1 mM ($P_{0.1}$) and 1.0 mM (P_1) P level conditions

improve the ecological adaptability of citrus roots by promoting the occurrence of root hairs. In trifoliate orange seedling, AMF promoted the root hair growth by activating auxin synthesis genes (*PtYUC3* and *PtYUC8*), upregulating auxin-species influx carrier genes (*PtABCB19* and *PtLAX2*), and downregulating auxin-species efflux carrier genes (*PtPIN1* and *PtPIN3*) under drought stress (Liu et al. 2018b). Moreover, AMF-modulated root morphological changes may also be related to polyamine metabolism and hormone levels (Wu et al. 2012; Liu et al. 2016). It is suggested that AMF can improve root adaptability to drought stress by improving hormone changes and metabolism in host plants.

21.4.2 Water Uptake of Mycorrhizal Hyphae

Under drought stress, AMF could accelerate the water uptake efficiency in host plants by increasing the biomass of extraradical mycelia (Marulanda et al. 2003). Mycorrhizal hyphae colonize plant root epidermis through entry points. Mycorrhizal hyphae could transport the water to arbuscules directly through the intraradical hyphae, thus forming a special way to uptake water and shorten its transport distance (Zhu et al. 2015a, b). Querejeta et al. (2003) analyzed the water movement under drought stress by using separated root chambers and fluorescent dyes. The results

showed that water is transported from soils to plants by the stomatal opening in daytime and exudes it into soils through the top of extraradical mycelia at night when the stomatal opening is closed. It is assumed that AMF can flexibly regulate the transport pathways of apoplast and intercellular water according to plant needs. Li et al. (2013b) cloned aquaporin (AQP) genes from mycorrhizal fungi to provide the evidence regarding uptake of water by AMF in plants. In fact, AQP is a class of small molecular transmembrane proteins that efficiently transports water in plant tissues, which are located in specific nuclear membrane regions in plants (Ran et al. 2016). AQP plays vital effects in regulating plant development and transmembrane transport of water. AQP is strongly expressed and abundant in tissues with high water transmembrane transport, such as in fast-growing areas (e.g., buds and leaves) of plants and in the main water-absorbing areas (roots) (Otto and Kaldenhoff 2000). Mycorrhizal roles on AQP expressions have been verified in many plants, such as *Glycine max* (Porcel et al. 2006), *Lycopersicon esculentum* (Ouziad et al. 2006), and *Medicago truncatula* (Roussel et al. 1997; Uehlein et al. 2007), but there are few reports regarding the water uptake modulated by AQP expression in citrus plants colonized by AMF. Recently, He et al. (2019) reported the expression patterns of root tonoplast intrinsic protein (*TIPs*) in trifoliate orange seedlings inoculated with *F. mosseae* under ample water and drought stress conditions. They found that the expressions of *PtTIP1;2*, *PtTIP2;1*, *PtTIP4;1*, and *PtTIP5;1* were increased by mycorrhization but the expressions of *PtTIP1;1* and *PtTIP2;2* were reduced under well-watered conditions. Under drought stress, the changed pattern regarding *TIPs* expression under mycorrhization was as follows: *PtTIP1;2*, *PtTIP1;3*, and *PtTIP4;1* expressions were upregulated and roots *PtTIP2;1* and *PtTIP5;1* were down-regulated. It seems that there were diverse responses of root *TIPs* to mycorrhization under drought stress, indicating different mechanisms regarding AMF mechanisms in drought tolerance.

21.4.3 Physiological Responses

Previous studies have demonstrated that AMs enhanced plant water uptake as well as mineral element uptake, especially P. Interestingly, under drought stress, the effect of mycorrhizal fungi on nutrient uptake is more important than under sufficient water conditions. As reported by Wu and Zou (2009), AMF-increased mineral nutrient concentrations were higher under soil water deficit than under ample water in trifoliate orange. As stated by Wu et al. (2011), mycorrhizal mycelium also sustained better nutritional (especially P) uptake and water absorption in trifoliate orange seedlings though drought stress seriously decreased the active, functional, and total hyphal activities. Hence, the key physiological mechanism of mycorrhizal fungi in improving drought resistance of host plants is that AMF extraradical mycelium enhances nutrient uptake of host plants.

Photosynthesis is the most basic physiological response of plants. It is the principal way for plants to assimilate carbon, thus providing energy and nutrients

for plant growth. Alleviating the adverse photosynthetic reactions which is induced by stress is an essential mechanism for improving the resistance to plants. Many previous studies have demonstrated that AMF colonization increased chlorophyll content, gas exchange, and water use efficiency in leaves while decreasing intercellular CO_2 concentration of plants regardless of water status (Huang et al. 2011; Zhu et al. 2011; Gong et al. 2013). In *Citrus tangerina*, Wu and Xia (2006) reported that under drought stress, inoculation with *G. versiforme* remarkably increased leaf water potential, transpiration rates, photosynthetic rates, stomatal conductance, and relative water content, but decreased leaf temperature. In *F. mosseae*-infected trifoliate orange seedlings, the photosynthetic rates, stomatal conductance, and transpiration rate were evidently increased by mycorrhization under well-watered and drought stress conditions (Wang et al. 2017). It seems that AM symbiosis conferred higher capacity of gas exchange in plants by reducing stomatal resistance and increasing transpiration rates (Zhu et al. 2011). In addition, AM trifoliate orange seedlings presented lower intercellular CO_2 concentration than non-AM seedlings, irrespective of soil water status (Wang et al. 2017), indicating that AM citrus plants have a fairly higher CO_2 assimilation capacity. Possibly AMF inoculation has the capacity to reduce the drought damage of photosynthetic apparatus. In addition, AMF inoculation enhanced plant tolerance in response to drought stress by increasing carbon storage of host plants, thereby stimulating plant growth (Ludwig-Müller 2009).

21.4.4 Biochemical Responses

Besides physiological responses, biochemical mechanisms regarding AMF roles in drought tolerance of host plants are involved. *F. mosseae*-colonized trifoliate orange seedlings showed significantly higher levels of IAA, ABA, MeJA, and ZR in roots, irrespective of soil water status (Liu et al. 2016). Liu et al. (2018b) reported that *Funneliformis mosseae* markedly increased root IAA concentration in trifoliate orange under well-watered and drought stress conditions, respectively. Another study in trifoliate orange seedlings showed that AMF inoculation stimulates the ABA, IAA, and ZR accumulation in leaves under both well-watered and drought stress conditions (Wang et al. 2017).

Recently, Huang et al. (2014) conducted an experiment on trifoliate orange seedlings with *Funneliformis mosseae* application under drought stress. They observed that AMF-increased Cu/Zn-SOD and Mn-SOD activities were associated with AMF-increased calmodulin (CaM) synthesis. It was speculated that AMF might activate antioxidant protective systems by promoting the synthesis of CaM signal substance. In addition, *F. mosseae* inoculation induced relatively higher net H_2O_2 effluxes in trifoliate orange roots under drought stress, especially in the root meristem zone (Zou et al. 2015). Such behavior of H_2O_2 effluxes under mycorrhization conditions is related with the fact that AQPs in mycorrhizal hyphae transport both H_2O and H_2O_2.

21.4.5 *Mycorrhizal Improvement in Soil Structure*

In the process of mycorrhizal hyphae and their spore germinations or development, a glycoprotein is produced, named as glomalin (Wright et al. 1996). Glomalin is characterized by its stable performance and high preserved in the soil. In general, glomalin is defined as glomalin-related soil protein (GRSP) in soils according to the Bradford protocol (Rillig 2004). GRSP can bind soil particles with a "super glue" ability, which can promote the soil aggregate formation and stability. Therefore, GRSP is seen as a stabilizer of soil structure formation, which can change soil moisture status (Spohn and Giani 2010). AMF secrete GRSP into soils to improve soil structure (Wu et al. 2008). Drought stress substantially increased total GRSP concentrations in rhizosphere soil compared with well-watered condition, and *G. mosseae*, *G. diaphanum*, and *G. versiforme* notably increased total GRSP levels in rhizosphere soil and improved the stability of soil structure under drought conditions (Wu et al. 2008). In order to analyze the relationships between GRSP and water potential, an experiment conducted by Zou et al. (2014b) showed that soil and leaf water potential in trifoliate orange were significantly and negatively correlated with only total GRSP, indicating that total GRSP is more active under drought stress than easily extractable GRSP (Zou et al. 2016). As suggested by Nichols (2008), AMF released the GRSP covered on fungal hyphae and formed a hydrophobic layer on the surface of soil aggregates, and water loss within mycorrhizal soil aggregates was reduced. As a result, in mycorrhizal soils, extraradical mycelia secreted the GRSP to maintain superior soil structure under drought stress, which resulted in higher soil available water content than poorly structured non-mycorrhizal soils (Augé 2001).

21.5 Mycorrhizal Functioning on the Tolerance of Waterlogging Stress

Waterlogging, an abiotic stress, often results in anoxic respiration as its hypoxic conditions (Elzenga and van Veen 2010; Tanaka et al. 2011). As a result, plants grown in waterlogging have bad root hydraulic conductivity, stomatal aperture, photosynthetic capacity, and nutrient availability (Kozlowski and Pallardy 1997; Ashraf 2012; Yin et al. 2012). Several researches indicated that AM citrus plants presented greater plant growth performance and plant biomass than non-AM plants under waterlogging stress. Under waterlogging stress, plant height of *Citrus junos* (Wu et al. 2013c) and *Poncirus trifoliata* (Zou et al. 2014a) seedlings was significantly increased by *Diversispora spurca* inoculation. In addition, the root system architecture and morphology of the two citrus species (*C. junos* and *P. trifoliata*) were also improved by AMF inoculation under waterlogging conditions (Wu et al. 2013c; Zou et al. 2014a). Meanwhile, activity of antioxidant enzymes (SOD and CAT) in *Diversispora spurca*-colonized *C. junos* plants (Wu et al. 2013c) and

P. trifoliata plants (Zou et al. 2014a) was also significantly increased under waterlogging. It seems that AMF enhanced waterlogged tolerance of citrus plants by morphological adaption and biochemical mechanisms. However, more information regarding AMF effects on waterlogging stress in citrus plants needs to be concerned.

21.6 Mycorrhizal Functioning on the Tolerance of Salt Stress

AM symbiosis, established between plant roots and beneficial fungi, is an important way to improve salt resistance of host plants (Murkute et al. 2006). Mycorrhizal plants had better growth performance and produced more plant biomass than non-mycorrhizal plants under salt conditions (Abdel Latef and He 2011; Cantrell and Linderman 2001; Evelin et al. 2011; Kumar and Sharma 2011; Porcel et al. 2016). Salt stress considerably inhibited mycorrhizal formation in trifoliate orange seedlings, while inoculation with AMF still enhanced salt tolerance by activating antioxidant-protected systems (Wu et al. 2010b).

In citrus seedlings, mycorrhizal fungi application significantly promoted plant growth under salinity stress (Khalil et al. 2011). Wu et al. (2010a) conducted an experiment on *C. tangerina* Hort. ex Tanaka under salt stress and observed greater stomatal conductance, net photosynthetic rate, and transpiration rate of AM citrus. Inoculation with *D. versiformis* significantly improved growth behavior of trifoliate orange, ameliorated root morphological traits, and induced the GRSP secretion under non-salt stress and salt stress conditions, respectively (Zhang et al. 2016). Besides, mycorrhizal soils recorded greater water-stable aggregate distribution and mean weight diameter in rhizosphere soils of trifoliate orange seedlings irrespective of salt stress or non-salt stress, indicating better soil aggregate stability in AMF rhizosphere (Zhang et al. 2016). Furthermore, Wu and Zou (2013) carried out qualitative and quantitative analysis of root H^+ effluxes of trifoliate orange seedling under salt stress. They found that *F. mosseae* inoculation induced more H^+ effluxes from roots to plant rhizosphere, which established a more acidic environment in the rhizosphere of AM seedlings for improving salt tolerance. In addition, *F. mosseae* inoculation markedly increased the ratio of K^+/Na^+ of trifoliate orange under non-salt and salt stress conditions (Wu et al. 2013b). Moreover, the selective absorption of K^+ versus Na^+ in roots was increased under salt stress by AMF inoculation, while the selective transport of K^+ versus Na^+ from roots to leaves was reduced by mycorrhizal treatment under salt stress. It can be concluded that in citrus plants, mycorrhizal symbiosis enhances salinity tolerance through selective absorption of K^+/Na^+ but not selective transport of K^+/Na^+. More information in molecular levels needs to be studied.

21.7 Mycorrhizal Functioning on the Tolerance of P Stress

Phosphorus (P) is one of the most important and essential macronutrients in plants. Generally, approximately 80% of P in soil exists in the form of insoluble, thus leading to P deficiency of plants in high frequency. Citrus plants have evolved a series of strategies to adapt to P stress, the most important way of which is to establish symbiosis with soil beneficial AMF to absorb water and nutrients from soil (Achatz and Rillig 2014; Xie et al. 2014). Under 0.1 mM and 1 mM P treatment, *F. mosseae* inoculation significantly promoted the plant growth performance of trifoliate orange seedlings (Fig. 21.2), and AM seedlings presented higher concentration of P in roots than non-AM seedlings (Liu et al. 2018a). The result is consistent with Wu et al. (2015a) and Shu et al. (2012) in trifoliate orange seedlings under different P treatments. As early as 1994, Smith et al. (1994) reported that P uptake and transport rate by mycorrhizae were 6–10 times faster than that by root hairs. N, K, Cu, and Zn were strongly increased by AMF in host plants (Smith and Read 2008; Cozzolino et al. 2010). Wu et al. (2016) inoculated four different AMF species, *C. etunicatum*, *D. versiformis*, *F. mosseae*, and *R. intraradices*, respectively, on trifoliate orange seedlings. The results showed considerably greater concentrations of root P in AM seedlings than in non-AM seedlings. Meanwhile, significant correlation was observed between P levels and AM formation and root hair growth ($P < 0.01$) (Wu et al. 2016). Chen et al. (2017a) reported that mycorrhizal seedling showed higher P concentration in trifoliate orange colonized by *R. irregularis*. Further transcriptome analysis revealed that *Rhizophagus irregularis* was involved in citrus P metabolism (Chen et al. 2017a). P metabolism was the key pathway involved in regulating lateral root formation under mycorrhization (Chen et al. 2017a). Subsequently, another study conducted by Chen et al. (2017b) in trifoliate orange found that under different substrate P levels (20 and 50 mg/Kg), inoculation with *Rhizophagus irregularis* significantly promoted the lateral root development, which was closely related to expressions of lateral root-related genes. A positive and significant correlation was found between lateral root development and the expression of lateral root-related genes and *TIR1* (Chen et al. 2017b). The results by Liu et al. (2018a) showed that AMF trifoliate orange seedlings had greater root hair density under 0.1 mM P levels but lower under 1 mM P levels (Fig. 21.3). Such results are possibly related with mycorrhiza-induced *expansins* expression levels under 0.1 mM P levels. However, under 1 mM P levels, mycorrhizal fungi

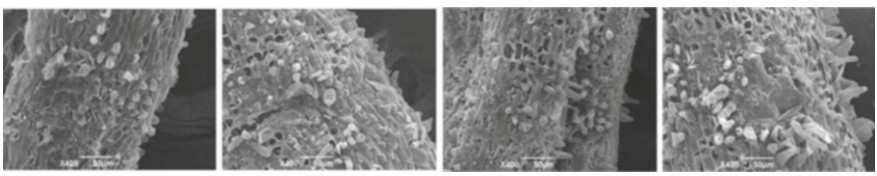

Fig. 21.3 Root hair morphological status of trifoliate orange seedlings colonized by *Funneliformis mosseae* (Fm) under 0.1 mM ($P_{0.1}$) and 1.0 mM (P_1) P level conditions (Liu et al. 2018a)

colonization mainly induced greater root hair length and diameter by stimulating IAA accumulation (Liu et al. 2018b).

The main function of plant roots is to absorb P nutrients from soils, but P can be effectively utilized by plants, depending on the transport efficiency of P in plants. P transfer in AMs is found in arbuscules (Javot et al. 2007a; Pumplin and Harrison 2009), where phosphate transporters of AMF, e.g., *PT4* and *PT11*, are released into the plant cell (Javot et al. 2007b; Shu et al. 2012; Yang et al. 2012; Zhang et al. 2016). Shu et al. (2012) conducted an experiment to research the effect of five different AMF species on P transport in trifoliate orange seedling under P deficiency. The results indicated that the most suitable fungus type for the plant behavior varied in accompany with soil Pi levels. Soil P levels and root AM colonization were also participated in the expressions of the seven *Pht1* phosphate transporter genes (Shu et al. 2012). Pi starvation upregulated most members of the *Pht1* family except *PtPT6*. Nevertheless, transcript levels of *PtPT1*, *PtPT2*, *PtPT3*, and *PtPT7* were lower in mycorrhizal roots. Liu et al. (2017) found that *F. mosseae* dramatically increased root acid phosphatase activities and relative expression of root acid phosphatase gene *PtPAP1* under 1 mM P levels. The AMF inoculation dramatically decreased the relative expression of leaf (*PtPAP1* and *PtPAP3*) and root (*PtPAP3*) acid phosphatase genes and leaf P transporter gene (*PtPT5* and *PtPT6*), but markedly increased the transcript level of root P transporter genes (*PtPT3*, *PtPT5*, and *PtPT6*) (Liu et al. 2017). It implies that mycorrhizal inoculation enhanced expression of P transport genes, thus collectively improving P absorption in citrus plants.

21.8 Mycorrhizal Functioning on the Tolerance of Fe Stress

Iron (Fe) plays a role in plant physiological performance, especially in activating a variety of enzymes to improve photosynthetic performance (Hewit 1983; Malkaouti and Tehrani 2005). The forms of Fe in soil are rich and varied, mainly in the forms of exchangeable, carbonate-bound, iron-manganese oxide-bound, organic matter-bound, amorphous iron-bound, crystalline iron-bound, and residual iron (Jiang et al. 1990). The main factors affecting exchangeable Fe content in soil are soil pH and soil redox capacity (Wang et al. 2009). Fe solubility increases in acidic soils (Cao et al. 2002), while in arid or semiarid alkaline soils (pH $>$ 7.0), Fe deficiency is more serious. Citrus is a kind of Fe-deficient sensitive fruit trees, which is prone to Fe-deficiency (Wang et al. 2008b). AMs are known to improve plant growth performance and health by enhancing mineral nutritions, including Fe (Caris et al. 1998). Many studies had shown the positive effect of AMF on plant growth behavior in trifoliate orange under Fe deficiency (Li et al. 2015; Wang et al. 2007, 2008b), which is closely related to the excessive production of secondary metabolites induced by AMF (Li et al. 2015). Under Fe deficiency conditions, *G. versiforme*-colonized *Poncirus trifoliata* seedlings showed higher phenolic synthesis capacity, indicating that AMF inoculation mitigates the damage to plants

caused by Fe deficiency. AMF presence could promote Fe uptake in trifoliate orange and red tangerine seedlings colonized by *G. versiforme* by activating Fe (III)-chelate reductase activity (FCR) (Wang et al. 2008a). In addition, mycorrhizal symbiosis with *G. versiforme* reduced the contents of exchangeable, organic-bound, and residual Fe in soils (Wang et al. 2009). Moreover, the mycorrhizal colonization was positively correlated with residual Fe in soils, indicating that AMF could activate mineral elements in soil and promote the increase of available Fe content by changing the contents of various forms of Fe in soil (Wang et al. 2009).

21.9 Mycorrhizal Functioning on the Tolerance of Temperature Stress

Among many abiotic stresses, temperature is also one of the important environmental factors affecting plant growth and productivity development. In crop-growing season, temperature stress, including high temperature and low temperature, can negatively affect crop growth and play a decisive role in yield (Wahid et al. 2007). AM symbiosis represented potential functioning on tolerance of temperature stress in host plants (Ruotsalainen and Kytöviita 2004; Zhu et al. 2010, 2011, 2015a, b). Superior net photosynthetic rate in mycorrhizal plants indicated greater carbon dioxide assimilation ability by mycorrhization. Therefore, although AMF consumes a lot of carbohydrates for their own growth, the infection of AMF can significantly promote plant growth. However, contradictory results were obtained for *C. tangerine* (Wu and Zou 2010). Under low temperature (15 °C) stress, AM-colonized citrus tangerine seedlings showed lower net photosynthetic rate compared with non-AM seedlings (Wu and Zou 2010). In addition, AMF inoculation did not alter the content of K, Mg, Fe, Cu, Mn, and Zn, but markedly increased Ca content under low temperature stress (Wu and Zou 2010). Possibly, low temperature severely inhibits mycorrhizal growth and development in roots and soils, thereby reducing AMF functionings on mitigating low temperature damage. However, the AMF effects were reversed under suitable temperature (e.g., 25 °C) and high temperature (e.g., 35 °C) conditions (Wu 2011; Wu and Zou 2010). As a result, it is suggested that the positive mitigation effect of mycorrhizae on citrus plants under high temperature and moderate temperature conditions was weakened under low temperature conditions.

21.10 Application of AMF as Biofertilizer into Citriculture

Citrus is a world fruit tree, which has a strong adaptability and widely plant range. Under natural cultivation conditions, citrus plants highly rely on AMs as it can promote water and nutrient absorption. Several experiments had

been conducted in greenhouse and fields to evaluate the effects of AMF on mineral nutrient concentration and plant growth responses of citrus. Ortas et al. (2002a) conducted an experiment on *C. sinensis* plant colonized by five different AM fungal species from *Glomus* sp. The five AM fungal species were propagated by clover and maize. Then the respective mycorrhizal fungi were inoculated into *C. sinensis* L. in greenhouse. Among all, *G. clarium* was the most effective promotion AM fungus for *C. sinensis* growth, including improvement in plant growth, nutrient levels, biomass, and leaf area (Ortas et al. 2002a). The authors also observed maximum plant height, total root length, and mycorrhizal infection in *G. clarium*-inoculated plants. In sour orange, mycorrhizal inoculation with *G. clarium* induced tenfold increase in total plant biomass compared with non-inoculated seedlings under three levels of P_2O_5 and three levels of Zn conditions (Ortas et al. 2002b). In container, mycorrhizal citrus plants had the best responses with *G. mosseae* in andesitic tuff + peat + soil (4:5:1, v/v) substance (Ortas and Ustuner 2014). It seems that the *G. clarium* exhibited the considerable role in *C. sinensis* for growth and will be considered using in citriculture.

Recently, Wu and his team used *G. mosseae* and mixed-AMF inoculum to inoculate into rhizosphere of *C. reticulate* Blanco var. *ponkan* cv. Jinshuigan in fields. After 8 months, they found a slight increase in fruit transverse diameter and significant increase in fruit color (Fig. 21.4). In addition, Wu et al. (2015b) applied exogenous easily extractable GRSP (EE-GRSP, a secondary metabolite of arbuscular mycorrhizae) into a 27-year-old Satsuma mandarin grafted on trifoliate orange in the field for 5 months. The results indicated strongly positive effects on

Fig. 21.4 Tree growth and fruit status in *Citrus reticulata* Blanco var. *ponkan* cv. Jinshuigan in fields after 8 months of inoculation with single *Glomus mosseae* and mixed-AMF (*Glomus mosseae*, *G. intraradices*, and *G. versiforme*) inoculums

soil organic carbon, water-stable aggregate stability, and soil phosphatase activity. Wang et al. (2015) further selected an exogenous 1/2 strength EE-GRSP solution that represented the best stimulated effects on plant growth performance and soil structure in trifoliate orange. Subsequently, Chi et al. (2018) revealed that trifoliate orange seedlings with exogenous EE-GRSP exhibited better growth performance, gas exchange, leaf Fe-SOD and root Mn-, Cu/Zn-, and Fe-SOD activities, and leaf ABA, IAA, and MeJA levels under drought stress. In short, exogenous application of mycorrhizal secondary metabolite, e.g., EE-GRSP, can be considered as a plant and/or soil regulator to regulate plant growth, physiological activity, soil structure, and soil fertility in citrus plants. Recently, Wu and his team developed a suitable protocol regarding indigenous AMF propagation of citrus rhizosphere utilizing colonized root segments. Such works will accelerate the AMF application as biofertilizer into citriculture, though many difficult problems are still pending.

21.11 Conclusions and Outlook

AMF has the capacity to mitigate passive effects of abiotic stress in citrus plants, including drought, salinity, waterlogging, P and Fe deficiency, and high temperature. The AM potential effects, at least in citrus plants, are described in Fig. 21.5: (1) promotion in plant growth performance and root development of mycorrhizal plants; (2) increase in water and nutrient uptake by extraradical hyphae; (3) greater balance of phytohormones and higher signaling substance levels in mycorrhizal plants; (4) the increased antioxidant protected systems and more accumulation of osmolytes in AM plants; (5) higher chlorophyll levels in mycorrhizal plants; and (6) better soil structure and fertility in mycorrhizosphere by hyphae and glomalin.

A small number of field works had tried to apply both AMF and AMF-secondary metabolite (EE-GRSP) into citrus plants in fields for consideration. Even so, it still has lots of works needed to be highlighted:

1. Exploiting RNA-seq technique and metabolomics to comprehend AMF-induced diversification in metabolic pathways of citrus plants under abiotic stress and to establish the whole-gene network
2. Detecting the expression of AQP in citrus roots and mycorrhizae under abiotic stress and further analyzing the relation of both AQP gene expression and hyphae/plant water absorption
3. Selecting a combination of AMF and plant growth-promoting rhizobacteria (e.g., phosphate-solubilizing bacteria) on citrus plants under abiotic stress
4. Conducting more field studies to confirm mycorrhizal effects on citrus plants

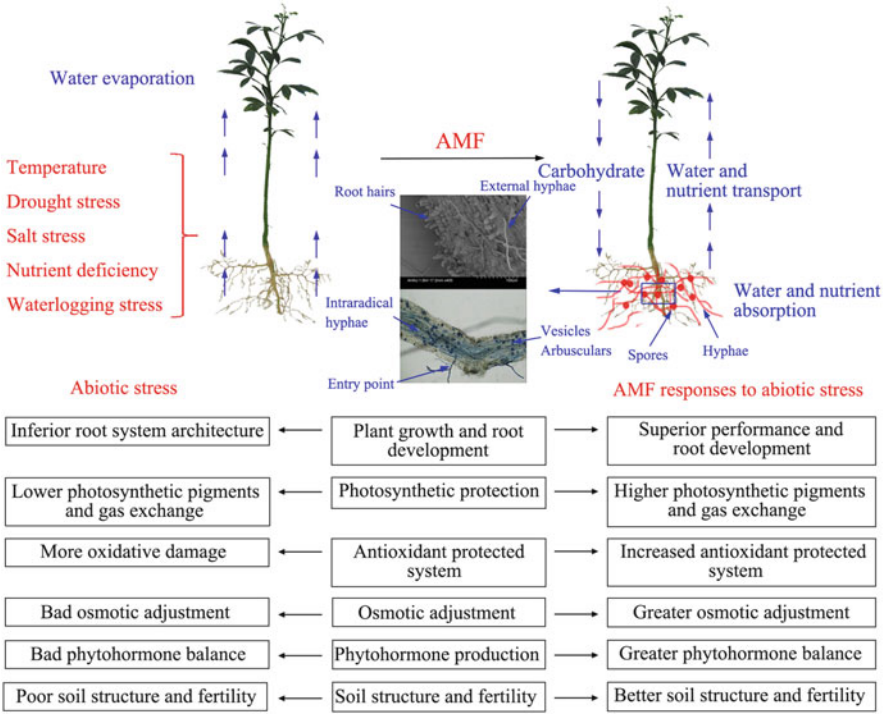

Fig. 21.5 The responses of mycorrhizal and non-mycorrhizal citrus plants to abiotic stress. Here, citrus is colonized by AMF and subsequently forms extraradical hyphae to absorb water and nutrients from soils to hosts, and mycorrhizal presence also promotes root hair growth. Under abiotic stress conditions, mycorrhizal plants show the improvement in plant growth performance and root system architecture, increased water and nutrient uptake by extraradical hyphae, greater phytohormone balance and higher signaling substance levels, increased antioxidant-protected systems and more accumulation of osmolytes, higher chlorophyll levels, better soil structure, and fertility in mycorrhizosphere by glomalin and hyphae. On the other hand, non-mycorrhizal plants under abiotic stress face water and nutrient deficiency, bad soil structure and fertility, more oxidative damage, and inferior osmotic adjustment and plant growth

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Chapter 22
Arbuscular Mycorrhizal Fungi (AMF) from Heavy Metal-Contaminated Soils: Molecular Approach and Application in Phytoremediation

Sanjeev Kumar and Saurabh Saxena

Abstract Discharge of effluents from textile industry into river and agricultural land is one of the major global problems. The discharge of dye-containing effluents directly into the water makes it toxic for environment and living organisms. Currently available physical and chemical processes do not remove toxic chemicals, dyes, and detergents completely from the environment. It is now known that biological organisms like Arbuscular mycorrhizal fungi (AMF), in association with different plant species grown under contaminated soils, enhance uptake of heavy metals. However, very limited knowledge is available with community composition of tolerant mycorrhizal species/strains associated with heavy metal accumulator plants. Therefore, the present chapter deals with identification of novel approaches for diagnosis of mycorrhizal species from complex environmental soil. Furthermore, this chapter suggests more sustainable approaches for reclamation of heavy metals by AMF associated with the heavy metal accumulator plants.

Keywords Contaminated soils · Environmental soil · Heavy metal · Arbuscular mycorrhizal fungi

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22.1 Introduction

Textile industry is a major source of water and soil pollution due to effluent discharge in cultivated land. Effluents originated by textile industry bring harmful dyes, dye additives, and a wide range of detergents, some of which are nonbiodegradable, toxic, mutagenic, and carcinogenic. It imposes a major threat to flora and fauna in the affected area. Water bodies contaminated with industrial wastewater loaded with these toxic chemicals become deprived of biological oxygen and chemical oxygen demand (BOD/COD) shown in Fig. 22.1. The toxic elements include numerous inhibitor compounds (interfering effective biological wastewater treatment), active compounds, and organic halogens (e.g., chlorine compounds) with higher concentration of salts. Since most of the textile factories do not have an efficient recycling treatment technology, they discharge their effluents (viz. dyes having heavy metal contaminants) into the agriculture land. Detoxification and recycling of these toxic heavy metals using chemical and physical treatments are not feasible on large scale due to constraints in cost, process, and environmental concern. However, toxic effluents' adsorption by (living or dead) microbial biomass or bioremediation systems provides cost-effective raw material as compared with other methods (Kumar et al. 2016). Moreover, use of biological organisms may provide holistic and efficient technology for complete degradation of toxic chemicals, mainly heavy metals collected from textile effluent sites. Phytoremediation is a cost-effective sustainable alternative approach of remediation technology, which may be applicable for a wide range of cultivated land contaminated with heavy metals.

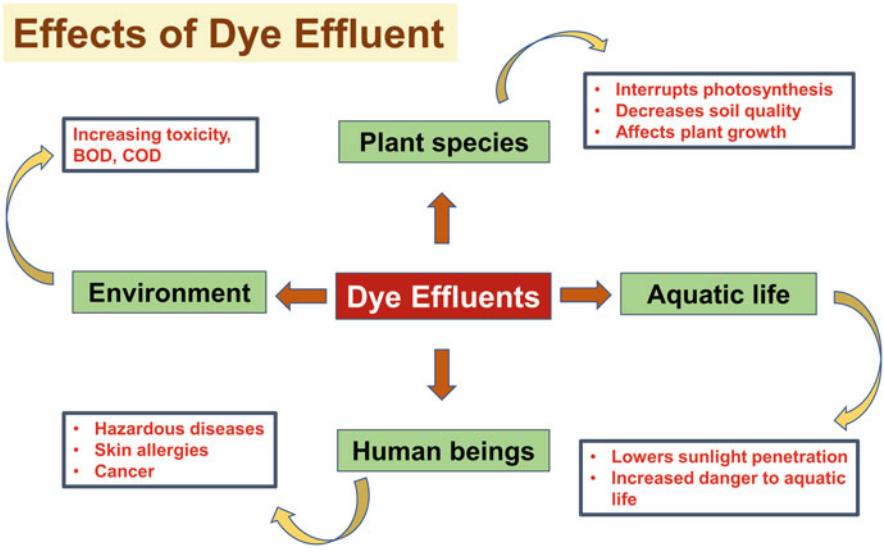

Fig. 22.1 Diagrammatic representation of effect of textile effluent on environment

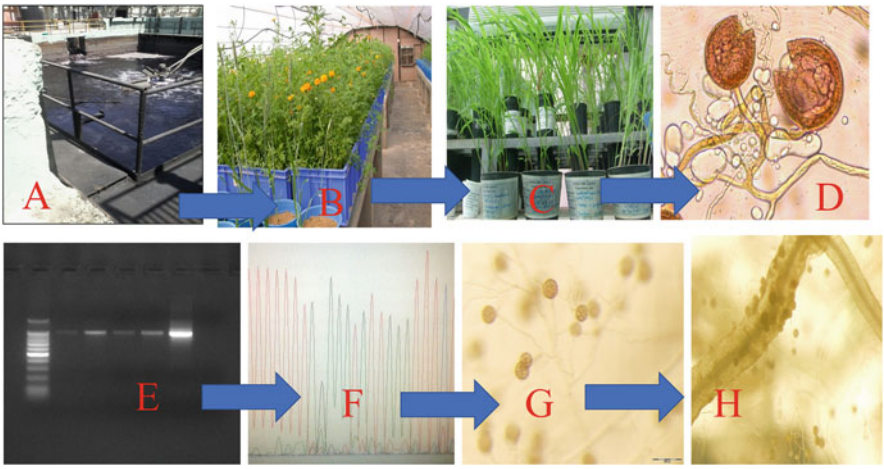

Fig. 22.2 (**a**–**h**) Schematic representation indicating the process of phytoremediation using in vitro-grown tolerant mycorrhizal fungi. (**a**) Collection of tannery effluents from outlet of textile industry or industrial wasteland soil. (**b, c**) Multiplication of mycorrhizal fungi under trap culture condition originated from textile effluent sites. (**d**) Mycorrhizal spore under PVLG+ Melzer's reagent isolated from textile effluent sites. (**e, f**) Molecular identification and sequencing of AM fungi using of r-RNA gene. (**g, h**) In vitro multiplication of mycorrhizal spore under root organ culture

Arbuscular mycorrhizal fungi associated with more than 80% of terrestrial plants enhance phytoaccumulation of heavy metals, viz., zinc (Zn), cadmium (Cd), arsenic (As), and selenium (Se), in plants as suggested by many authors (Leyval et al. 1997; Liu et al. 2003). Several studies indicated that mycorrhizal species create selection pressure of soil contaminated with heavy metals (Rashid et al. 2009; Kumar and Adholeya 2018). In that view, the aim of the present chapter is to suggest consortia of tolerant mycorrhizal species associated with textile effluent dumping areas and to propose the development of a robust in vitro cultivation system for multiplication of tolerant species/strains of AMF collected from heavy metal-contaminated soil, which can be used for bioremediation program (Fig. 22.2).

22.2 Heavy Metal and Reclamation by Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi (AMF) are soilborne symbiotic fungi, the majority of which are associated with the roots of higher plants (Barea and Jeffries 1995). They form a positive interaction with 80% of the terrestrial plant species in all ecosystems (Brundrett 2002). AMF are able to tolerate a diverse range of metal concentrations in soils. Different signaling processes within AMF to retain metal homeostasis have been observed (Gonzalez-Guerrero et al. 2008). Interestingly, AMF can tolerate

harsh conditions and increase immobilization of heavy metal within soil by translocating metals into hyphae and roots. Moreover, AMF reduces movement of metals from plants to soil and root to shoot translocation (Bever et al. 1996; Dehn and Schüepp 1990; Kaldorf et al. 1999). AMF have several mechanisms contributing to adaptation to environmental stresses, including action of cell wall's chitin (Joner and Leyval 1997), extraradical hyphae, and release of certain proteins such as siderophore, metallothioneins, and phytochelatins (Kapoor and Viraraghavan 1995). AMF can be affected by heavy metal toxicity and the presence of other mycotrophic plants growing in soils contaminated with heavy metals (Leyval et al. 1997). Many reports have demonstrated their metal tolerance ability in soil contaminated with heavy metals (del Val et al. 1999; Hildebrandt et al. 1999). AMF exhibit ability of sequestering and accumulate heavy metals in their biomass as well as in the roots of host plant (Joner et al. 2000; Joner and Leyval 2001; Gadd 2005). Indeed, these fungi stimulate plant resistance, reduce heavy metal toxicity impact, and promote plant growth under metal stress (Gaur and Adholeya 2004; Prasad et al. 2017).

Intracellular and extraradical mycelium of AMF and ectomycorrhizal (ECM) fungi have shown potential for metal absorption (Joner et al. 2000). Turnau and Haselwandter (2002) found that in Zn-contaminated soil, approximately 70% of *Fragaria vesca* roots were colonized by *Funneliformis mosseae*. Furthermore, Gonzalez-Chavez et al. (2002) reported the accumulation of Cu by the extraradical mycelium (ERM) of different species of *Glomus*. They demonstrated that ERM of AMF from polluted soils accumulated Cu in the mucilaginous outer hyphal wall zone, cell wall, and inside the hyphal cytoplasm. AM isolates from heavy metal-polluted soils are more metal-tolerant than the isolates from nonpolluted soils (Pawlowska and Charvat 2004). Recently, Arias et al. (2010) using transmission electron microscopy (TEM) micrographs showed the presence of *Septoglomus deserticola* within roots of Prosopis. X-ray mapping demonstrated higher Cr and Pb deposition in xylem and phloem cells. Thus, they suggested that interaction with *Septoglomus deserticola* improves metal tolerance/accumulation in Prosopis. Regvar et al. (2003) observed different SSU rDNA sequences of *Rhizophagus intraradices* detected from metal-contaminated and noncontaminated sites. None of the sequences obtained from the metal-contaminated sites were identical to any other *Rhizophagus intraradices* sequences retrieved from other locations, indicating slightly different sequences from habitat to habitat (Clapp et al. 2001). Furthermore, a study by Wubet et al. (2003) concluded that arbuscular mycorrhizal propagules play a major role in the successful establishment of re-vegetation program in any ecological habitat. Their study revealed that AM fungal ecotypes specifically adapted to heavy metals may exist at such locations.

Arbuscular mycorrhizal fungi can successfully colonize within the roots of some hyper-accumulator plant species and enhance heavy metal tolerance mechanism and accumulation (Gaur and Adholeya 2004). For example, AMF can establish symbiotic association with Ni-hyper-accumulator *Berkheya coddii* (Turnau and Mesjasz-Przybylowicz 2003), As-accumulator *Pteris vittata* (Al Agely et al. 2005; Leung et al. 2006), and *Cynodon dactylon* (hyper-accumulator for many heavy metals).

Table 22.1 List of AM fungi associated with different plant species used for process of phytoremediation

Plant species	AM fungal species	Heavy metals	References
A. capillaris, *Zea mays*, *Legeum spartum*	*Rhizophagus intraradices*, *Funneliformis mosseae*, *Glomus macrocarpum*	Pb	Diaz et al. (1996)
Berkheya coddii, *A. porrum*, *Sorghum bicolor*	*Gigaspora* sp., *G. caledonium*	Ni	Turnau and Mesjasz-Przybylowicz (2003)
Trifolium repens, *Hordeum vulgare*, *Trifolium subterraneum*, *Viola calaminaria*	*Funneliformis mosseae*, *Glomus* sp., *Gigaspora* sp.,	Cd	Joner and Leyval (1997), Weissenhorn et al. (1993)
Trifolium repens, *Festuca rubra*	*Glomus* sp., *Glomus constrictum*, *Glomus ambisporum*	Zn	Zhu et al. (2001), Kaldorf et al. (1999)
Festuca and Agropyron	*Rhizophagus intraradices*, *Funneliformis mosseae*, *Claroideoglomus etunicatum,* and *Gigaspora gigantea*	Zn, Cd, As, and Se	Giasson et al. (2006)

Recently, Hassan et al. (2011) assessed AM community in roots of Plantago plants growing on sites polluted with trace metal using PCR-DGGE method. They recorded *Funneliformis mosseae* in metal-polluted sites and suggested the tolerance of trace metal stress by this species. Many reports described that AMF enhance efficiency of plants for the removal of heavy metals from toxic environment (Regvar et al. 2003; Turnau and Mesjasz-Przybylowicz 2003) (Table 22.1). Further, Nazir and Bareen (2011) investigated the synergistic effect of *Rhizophagus fasciculatus* and *Trichoderma pseudokoningii* on *Helianthus annuus* for decontaminating toxic metals from tannery sludge. They showed that combination of these fungi can also be exploited for decontamination of heavy metals from tannery sludge. AM fungi were also recorded from tannery effluent polluted soil in Tamil Nadu, India, by Sambadan et al. (1991). Raman et al. (1993) described and identified *Glomus* and *Gigaspora* spp. in the mycorrhizosphere of 14 plant species collected from magnesite mine spoil in India. Raman and Sambandan (1998) and Khade and Adholeya (2009) recognized consortia of tolerant mycorrhizal species from tannery sludge-contaminated soils of Kanpur, Uttar Pradesh. Kumar et al. (2016) recorded *Rhizophagus fasciculatus* and *Septoglomus deserticola* from trap culture originated from sludge-contaminated field soil as shown in Fig. 22.3.

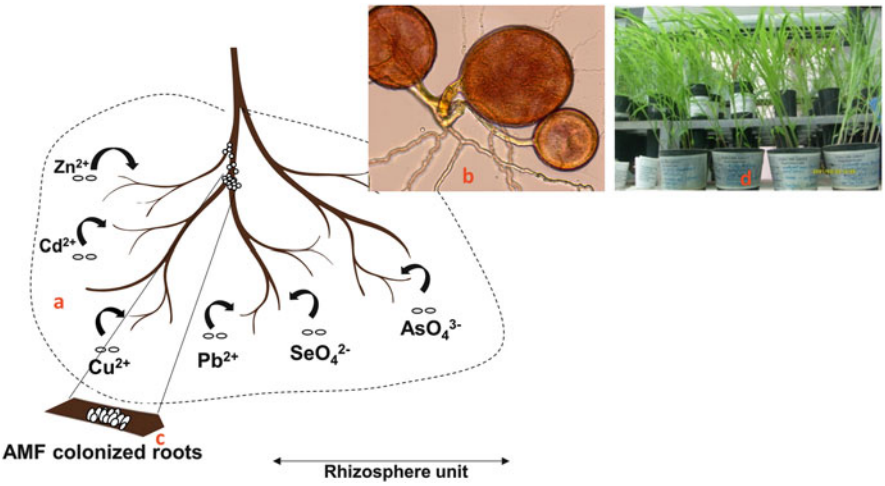

Fig. 22.3 Rhizospheric soil and AMF interaction with heavy metals. Modified from Giasson et al. (2008); (**a**) Mycorrhizosphere region of plant roots traps heavy metals and transfers to root zone. (**b**) Metal-tolerant AMF species (*Septoglomus deserticola*) with hyphae. (**c**) AM fungi exude compounds to dissolve heavy metals d. Propagation of AM fungi under greenhouse trap culture

22.3 AM Fungi Heavy Metals' Tolerance Mechanism

Heavy metals (HM) like Cd, Pb, and Hg are mainly found in terrestrial or aquatic ecosystem (Mertz 1981); however, these are not essential for plant growth. AM fungi are able to tolerate a wide range of HM concentration and other adverse conditions in soil (Bagyaraj 1995; Kamal et al. 2010). It was suggested by many authors that altered concentration of heavy metals in cultivated land creates selection pressure for development of tolerant AM fungal species/strains. Göhre and Paszkowski (2006) reported that AM fungi induce immobilization of HM within soil (phytostabilization) and also enhance the uptake of heavy metal by root and phytoextraction (root to shoot transport). This indicates that cleaning of contaminated soil, induced by association with *mycorrhiza*, depends on the plant–fungus HM combination and is also affected by soil structure and activity.

Organic acids and glomalin exuded from plants and fungi, respectively, play an important role in immobilization of heavy metals in the soil. AMF-colonized plants release organic acids which promote heavy metal sequestration and sorption. Organic acids precipitated as polyphosphate granules chelate and immobilize HM in the soil (Gaur and Adholeya 2004). Nutrients and metal can be exchanged between the fungi and the host plants through arbuscule structure inside the cortex of host roots. Secretion of these compounds can result in up to 85% reduction in heavy metal incorporation, as demonstrated in ectomycorrhizal fungus *Paxillus involutus* (Bellion et al. 2006). Hildebrandt et al. (2007) identified four major genes responsible for HM tolerance including a Zn transporter, a metallothionein,

glutathione S-transferase, and 90-kDa heat shock protein mainly expressed in intra- and extraradical mycelium of AMF sporulation in zinc-contaminated soil. Gonzalez-Guerrero et al. (2008) observed that various active and passive molecular processes are employed by these fungi to maintain metal homeostasis in plants. Passive process mainly involved is binding of metals to fungal cell wall and is responsible for little percentage of metal uptakes from the soil. Meanwhile, metallothionein- and glutathione-like chelators present in the cytosol actively bind to heavy metals. Heavy metal transporters collaborate with the intercellular chelators to actively reduce metal toxicity by pumping metal out of cytosol. Lanfranco et al. (2002) found that metallothionein-like polypeptides bind to HM for sequestration, which leads to detoxification of heavy metals like Cd and Cu in AM fungal cells.

AM fungi are able to modify their development pattern to avoid unfavorable conditions. Pawlowska and Charvat (2004) observed that AM fungi tend to hold germinating phase probably to avoid metal stress conditions. However, it is observed that in some cases, mycelium continue functioning in the presence of toxic substances. In the study by Gonzalez-Guerrero et al. (2008), it was found that spores of *Glomus intraradices* from HM stress environments contain higher levels of metals than rest of fungal colony. These metals were mainly found either bound to cell wall or compartmentalized in vacuoles in various fungal structures. *Desmostachya bipinnata* colonized with mycorrhizal fungi showed higher degree of Cd accumulation and lower root-to-shoot ratio as compared to nonmycorrhizal plant. A study has suggested that naturally growing tolerant mycorrhizal fungi have comparatively higher potential to solubilized toxic heavy metal than nontolerant AM strains (Wei et al. 2015). Tolerant mycorrhizal fungi dissolve toxic metals by producing organic acids in soil (Finlay 2008). Turrini et al. (2018) identified a new species of AMF, *Rhizoglomus venetianum,* from heavy metal-contaminated sites of Sacca San Biagio Island, downtown Venice, Italy. Furthermore, many authors suggested that sporulation of AM species/strains depends upon type of host plant grown under specific selection pressure condition (Hart et al. 2003).

22.4 Mechanism of AM Fungi Community Structure by Influence of Soil Activity

Soil manipulation practices reduce the sporulation and colonization potentials of mycorrhiza by disrupting the extraradical mycelium network (McGonigle and Miller 1999). The disruption of hyphal network reduces its surface area (Mozafar et al. 2000). In order to avoid stress condition in a heterogeneous environment, the mycorrhizal fungi develop more extensive mycelium (Bago et al. 1998). In another report, Gonzalez-Chavez et al. (2004) observed that secretion of organic acids as chelators, a glycoprotein exuded by AM fungi and glomalin, plays an important role in metal immobilization. The broad range of metal sequestered by glomalin may be used for biostabilization (Khan 2005).

22.5 Insight of Molecular Diagnostic Use for Mycorrhizal Fungi Grown Under Heavy Metal-Contaminated Soil

Several reports revealed that industrial wasteland soils and farming practices influence mycorrhizal abundance and species composition. Merryweather and Fitter (1998) suggested poor resolution of Glomeromycota due to overlapping of spore morphology. In addition, AMF species/strain-level screening fails most of the time due to overlapping of spore morphology and lack of skilled taxonomists (Kumar and Adholeya 2013). The integration of molecular and morphological studies originated from textile effluent sites leads to clear separation of unidentified taxa of AM fungi, which is a well-established approach. Moreover, recent developments in molecular phylogeny are all equally important in understanding the evolution and genetics of AM fungi. Identification of mycorrhizal diversity in colonized roots mainly involves nested PCR amplification of 18S-ITS rDNA region and separation on denaturing gradient gel electrophoresis (Dematheis et al. 2013). Many authors suggested that diagnosis of AMF in field soils flawed due to availability of limited mycorrhizal spore biomass used as starting material for molecular analysis (Dematheis et al. 2013).

Moreover, reports indicate that species/isolate-level resolution in AM fungi is a difficult task due to the presence of numerous ribosomal variants, both in conserved and variable region of ribosomal DNA (Pawlowska and Charvat 2004; Kuhn et al. 2001). Krüger et al. (2009) concluded that r-DNA primers claimed by many authors to be AMF specific are not able to resolve genetically diverse species of Glomeromycota. This can be overcome by rapid development of molecular identification tools based on 454 pyrosequencing, which could be a suitable alternative for identification of AM fungi to species/isolate in different ecological habitats or niche (Stover et al. 2018). Hiiesalu et al. (2014), using 454-pyrosequencing approach, proposed that AM species richness were positively correlated with plant richness. Several authors have used next-generation sequencing platform and recognized unidentified species/strains with greater potential to unravel missing or rare AM species originating from complex environmental soil (Medinger et al. 2010).

22.6 Conclusion

The present chapter suggested that use of biochemical and physical process does not efficiently detoxify detergents and dye completely from textile effluents. In contrast, use of AMF leads to sustainable and reproducible approach to complete removal of dye and toxic elements from textile effluents. Inoculation of AM fungi, which grow naturally in textile effluent discharge sites, into plant roots has enormous potential to enhance phytoaccumulation of heavy metals. The present chapter deals with identification and screening of specific indigenous AM fungal consortia, which may be potentially beneficial for reclamation of wasteland-affected site. Moreover,

development of sustainable and cost effective in vitro technology can fulfill demand of soil health by complete recycling of industrial wasteland. In future, dissemination of mycorrhizal-based in vitro technology with molecular diagnostic tool may successfully solve the problem of reclamation of industrial wasteland-affected soils.

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Chapter 23
The Role of Arbuscular Mycorrhiza in Sustainable Environment and Agriculture

Xiongfei Guo

Abstract Arbuscular mycorrhizal (AM) fungi are a class of beneficial microorganisms that are widely distributed in soil ecosystems and can form symbiotic associations with more than 90% of terrestrial higher plants. They play an important role in promoting plant growth, improving plant disease resistance and stress resistance, and maintaining the sustainable development of agricultural ecosystem. In addition, mycorrhizal fungi can degrade residual organic pollutants such as pesticides and herbicides in soil and also improve the health of heavy metal-contaminated soils and therefore play a major role in the bioremediation of polluted soil environment. The role of AM fungi in agricultural development and environmental remediation was explored from the perspectives of crop yield, water use efficiency, pest control, improvement of crop quality, remediation of agricultural nonpoint source pollution, remediation of refractory organic pollution, and remediation of heavy metal pollution. This paper focused on the latest advances and summarized the two important functions to test mycorrhizal fungi to promote agricultural production and environmental restoration and prospected the future development trend.

Keywords Soil pollution · Soil health · Environment restoration · Symbiotic association

23.1 Introduction

Mycorrhiza is a complex absorption organ formed by the symbiosis of fungi and plant roots in soil. Mycorrhizal fungi widely exist in nature. They can occur in various ecological environments and form a symbiotic system with most higher

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plants (more than 80% terrestrial plants) (Smith and Read 1997). The existence of this symbiotic system can effectively enhance the plant absorption and the utilization of nutrient and improve plant stress tolerance (Shen et al. 2005; Sun et al. 2012) and resistance to pests and diseases (Ahemad 2014). Therefore, previous studies on mycorrhizal fungi have focused on the promotion of crop growth and yield in the field of agricultural production (Antunes et al. 2006; Dodd et al. 2002), which has become the development direction of new environmentally-friendly agricultural technology. In recent years, with the serious decline of global environmental quality, the important role of mycorrhizal symbiotic system as a new type of bioremediation in the process of remediation of polluted or damaged environment is attracting widespread attention (Straker et al. 2007). Previous studies have shown that the mycorrhizal-rhizosphere microorganism-plant system formed by the core interface of mycorrhizal fungi can improve the ability of degradation and transformation of pollutants through co-metabolism, reduce soil pollution, and improve environmental quality (Jautris and Corinne 2003; Lenoir et al. 2016). On the other hand, it can alleviate the stress of unhealthy environment by improving plant nutritional status and ensuring plant growth in damaged or polluted environment, significantly improve the success rate of restoration and reconstruction of damaged and degraded ecosystems, shorten the repair cycle, and ensure the stability of the restoration effect (Guda et al. 2014). The unique physiological and ecological functions of the mycorrhizal fungi system are expected to become an effective means to promote the sustainable development of agriculture and cope with the current complex and serious environmental problems and will be the core direction of the future development of environmental restoration technology.

23.2 Promoting Effect of AM Fungi on Sustainable Agricultural Development

23.2.1 Improving Crop Yields

The main function of AM fungi is to improve the mineral nutrition of plants. It is a very important “biological fertilizer” in sustainable agricultural development. It was found that AM fungi could promote plants to absorb P from soil and increase the total absorption and utilization of P (Koide et al. 2000). The content of available P in plant growth environment is the main controlling factor for the coexistence of plant and AM fungi, which may be closely related to promoting plant growth and increasing plant yield. AM fungi play an important role not only in promoting P uptake, but also in promoting host plants to absorb other nutrients. The results showed that the formation of AM symbiosis could promote host plants to absorb N, K, Zn, Cu, Ca, and other mineral elements in soil (Kaya et al. 2009). Under certain conditions, the availability of these elements could also regulate the formation and development of AM symbiosis (Ryan and Angus 2003). Therefore, the interaction between AM fungi and host plants can improve plant nutrition and increase plant yield.

23.2.2 Increasing Water Utilization Rate

At present, there are more and more arid areas in the world, and the arid climate occurs frequently. Therefore, more attention is being given to the effect of AM fungi on plant water use efficiency under drought stress. At present, studies have shown that AM fungi can promote water uptake and utilization by plant roots, improve water metabolism, and enhance drought resistance (Yang et al. 2014; Zhang et al. 2014). Under drought stress, AM fungi can also improve the water status of plants, and its effect is more significant than that under normal water supply. AM fungi can enhance their drought resistance and promote plant growth (Egerton-Warburton et al. 2007). Asrar and Elhindi (2011) planted *Tagetes erecta* under different drought stress conditions to study the effects of AM fungi on the growth, pigment content, and P element content of *Tagetes erecta*. The results showed that, under drought stress, inoculating AM fungi could promote all plant growth parameters and the formation of photosynthetic pigments, and the total pigment content in mycorrhizal plants was significantly higher than that in non-mycorrhizal plants. In the research of Gholamhoseini et al. (2013), *Helianthus annuus* was used as an experimental material to study the effects of drought stress on growth, nutrient uptake, yield, oil content, and water use efficiency of sunflower under AM fungi inoculation. The results showed that, under drought stress, sunflower plants inoculated with AM fungi had higher seed setting rate and oil content than those without AM fungi inoculation. In addition, some other studies have yielded similar results, such as AM fungi can alleviate the effects of drought stress on *Fragaria virginiana*, *Zea mays*, and *Solanum lycopersicum* (Bárzana et al. 2012; Borowicz 2010). Therefore, AM fungi can effectively alleviate the damage caused by drought stress on plants, which can be popularized and applied in arid areas of western China to develop sustainable agricultural production.

23.2.3 Prevention and Control of Pests and Diseases

Modern sustainable agriculture does not advocate the use of chemical pesticides. Therefore, it is urgent to find green and environmentally-friendly control technologies for crop diseases and insect pests. More than 30 AM fungi have been proved to be able to inhibit plant-fungal diseases such as *Fusarium oxysporum*, *Verticillium dahliae*, *Rhizoctonia solani*, *Phytophthora nicotianae*, *Sclerotium cepivorum*, *Aphanomyces euteiches*, and so on (Hernández-Montiel et al. 2013; Zhang et al. 2012; Garmendia et al. 2005; Lioussanne et al. 2009; Kjøller and Rosendahl 1997; Torres-Barragán et al. 1996). It can control *Heterodera glycines*, *Meloidogyne incognita*, *Meloidogyne javanica*, *Radopholus similis*, *Pratylenchus coffeae*, *Heterodera avenae*, and other nematode diseases (Zhang et al. 2008a, b; Castillo et al. 2006; Elsen et al. 2008; Tylka et al. 1991).

AM fungi are the most common, biomass-maximizing, and significant beneficial fungi in plant rhizosphere. The infection of AM fungi can effectively control plant diseases and insect pests, which is conducive to the expansion of planting area of sustainable agriculture.

23.2.4 Improving Crop Quality

Studies have confirmed that the inoculation of AM fungi can significantly improve the quality of multiple crops (Baum et al. 2015). The characteristics affecting the quality of crops mainly include the contents of bioactive substances (thioglycolate, carotenoids, and cellulose), basic nutrients (protein, vitamins, mineral elements), and sensory properties, such as appearance (shape, size, color) and texture (Baum et al. 2015). Li et al. (2005) found that the inoculation of *Glomus mosseae* and *Glomus versiforme* could increase crude protein, soluble sugar, and the total content of 16 kinds of amino acids in *Cucumis sativus* fruits, soluble sugar content in *Citrullus lanatus*, and starch and amino acid content in *Colocasia esculenta*. Mena-Violante et al. (2006) also showed that AM fungi inoculation could increase fresh weight, fruit size (length, width, and pedicel length), fruit color, chlorophyll content, and carotenoid content in *Capsicum annuum* and significantly improve fruit quality.

23.3 Role of AM in Environmental Remediation

23.3.1 Application of Mycorrhizal Technology in Agricultural Nonpoint Source Pollution Rehabilitation

In agricultural production, in order to obtain high yield of crops, excessive fertilizers and pesticides are often applied. The actual utilization rate of these chemicals is only 20%–30%. Most of them enter the environment through different loss ways and are lost in soil, water, and air. Under the effect of leaching and migration of irrigation and precipitation, serious nonpoint source pollution is formed (Carpenter et al. 1998).

The excellent characteristics of AM fungi can play an important ecological role in the source and diffusion of nonpoint source pollution. Firstly, green and clean agricultural system, based on mycorrhizal technology, can reduce the application of agricultural nonpoint source pollutants at source. One of the main functions of AM fungi is to improve mineral nutrition of plants (Carpio et al. 2005). Using mycorrhizal technology instead of traditional fertilizer application can effectively alleviate the symptoms of plant nutrition deficiency in poor soil, promote plant growth, and reduce the use of chemical fertilizer. AM fungi can also play an

important role in the restoration of nonpoint source pollution. Strengthening the purification function of vegetation system has always been the main means of nonpoint source pollution control. On the diffusion path of nonpoint source pollutants, AM fungi mycelium can enlarge the specific surface area of vegetation root intercepting and contacting pollutants; strengthen the absorption capacity and rate of pollutants in vegetation system; accelerate the degradation rate of nonpoint source pollutants through the co-metabolism of AM fungi and vegetation system, which is an effective method to inhibit and reduce the diffusion of nonpoint source pollution; and then achieve the goal of transformation and removal of nonpoint source pollution (Requena et al. 2007).

Of course, at present, the project of preventing nonpoint source pollution based on mycorrhizal technology is still difficult to achieve in a large scale. The wide distribution and complexity of nonpoint source pollution cause the difficulty of application of mycorrhizal technology. Existing evidence shows that the effect of mycorrhizal inoculation in laboratory is always better than that in field. This is because the effectiveness of mycorrhizal inoculation is always influenced by host type, soil characteristics, composition of indigenous microorganisms, and many other ecological factors, especially the competition between indigenous mycorrhizal fungi and artificially inoculated mycorrhizal fungi, which tends to reduce the effect of artificial reinforcement. Therefore, the selection, application, and optimization of fungi strains still need further exploration and research. Screening and adding efficient indigenous mycorrhizal fungi and strengthening the functional advantages of indigenous fungi by improving environmental conditions may be more feasible technical means.

23.3.2 Application of Mycorrhizal Technology in Rehabilitation of Refractory Organic Pollution

The results showed that the degradation of organic pollutants in rhizosphere of mycorrhizal plants was significantly higher than that in rootless soils (Jia et al. 2004). AM fungi have been tried in the research of Binet et al. (1998) for the remediation of polycyclic aromatic hydrocarbon (PAH)-contaminated soil, Sarand et al. (1998) for the remediation of petroleum-contaminated soil, Donnelly and Fletcher (1995) for the remediation of polychlorinated biphenyl (PCB)-contaminated soil, Meharg et al. (1997a, b) for the remediation of chlorophenol- and explosive (TNT)-contaminated soil, Menendez et al. (1999) for organic pesticides, and Wang et al. (2003) for the degradation and remediation of plastic film plasticizer-phthalate esters (PAEs). The application of biotechnology has achieved good results. The results showed that the presence of AM fungi could accelerate the decomposition of these refractory organic pollutants and make them inorganic and harmless. In this process, the tolerance and degradation ability of AM fungi to pollutants is the basis of bioremediation of polluted environment. Evidence has shown that AM fungi can

effectively degrade and transfer complex organic pollutants in contaminated soils by increasing plant survival (Leung et al. 2007), stimulating root exudates (Buee et al. 2000), and enhancing the activity of other microorganisms (Nichols et al. 1997), thus reducing pollution levels and achieving bioremediation of contaminated soils (Giovanni and Simon 1998).

The process of absorption, transfer, and enrichment of soil pollutants in soil-microorganism-plant system and the contribution, potential, and mechanism of mycorrhizal fungi to pollutant degradation are becoming the focal points of scientists in related fields. Remediation of contaminated sites by higher plants and enhancement and acceleration of pollutant recycling and transformation by symbiotic mycorrhizal fungi are the latest research directions.

23.3.3 Application of Mycorrhizal Technology in Rehabilitation of Heavy Metal Pollution

With the large-scale application of chemical fertilizers and pesticides and the rapid development of industry, soil pollution is becoming more and more serious (Marques et al. 2011). Many pollutants in the soil, such as heavy metals, cannot be decomposed by soil microorganisms, but can only migrate, transform, and accumulate in the environment, leading to serious damages to the sustainable development of natural environment. When the concentrations of heavy metals in the environment reach certain limits, they will impose a toxic effect on the soil-plant system, thus will endanger human life and health through the food chain (Tao et al. 2017). Therefore, soil pollution has become one of the major environmental issues of global concern. The remediation and treatment of soils that have been contaminated by heavy metals have become one of the hotspots and difficulties in environmental science and ecology research.

Currently, there are three methods for repairing contaminated soils: physical restoration, chemical restoration, and bioremediation (González et al. 2002; Khalid et al. 2017). Physical restoration is one of the earliest repair techniques, most of which employ thermodynamics, electrodynamics, thermal desorption, and other methods (Mei et al. 2010). Chemical restoration is to transform soil contaminants into insoluble materials by adding modifiers to reduce their ability to migrate in the soil, including chemical leaching, solution leaching, etc. (Wuana and Okieimen 2011). Even though in physical repair and chemical repair, some methods are efficient, effective, and reasonable, they still have problems such as poor stability after repair and easy to cause secondary pollution (Bbosa et al. 2012). Bioremediation is a new technology that has been widely used in recent years to treat polluted soils with broad application prospects. Bioremediation refers to the use of plants, animals, and microorganisms to absorb, degrade, transform, and convert pollutants in soil and water under certain conditions to reduce the concentration of contaminants in the environment to an acceptable level (Wang et al. 2001). This method has

attracted widespread attention from soil biologists, botanists, and environmental scientists because of the high efficiency, low consumption, convenience and simplicity, and the ability to conserve water and soil and beautify environment.

Arbuscular mycorrhiza (AM) is a reciprocal symbiosis in soil ecosystem with both plant root and microbial properties (Nottingham et al. 2013). They can establish a symbiotic relationship with more than 90% of the terrestrial vascular plant roots on the earth, forming a "mycorrhiza" structure. The formation of mycorrhizal symbionts can promote the absorption of mineral elements such as P, N, K, Zn, Fe, Cu, and Ca by the host plants and improve the nutritional status, plant yield, and product quality (Grunwald et al. 2009). Besides, they can improve the water use efficiency of plant roots in arid and saline-stressed habitats (Lu et al. 2012). AM fungi can promote the growth and development of host plants and improve the ability to resist stress, making the mycorrhizal plants have a comparative advantage in growth and survival rate compared with non-mycorrhizal plants (Zhang et al. 2009; Gianinazzi et al. 2010). Studies have shown that AM fungi can significantly increase the tolerance of host plants in heavy metal-contaminated soils (Hildebrandt et al. 2007; Feddermann et al. 2010; Miransari 2011). Plants that have been growing in a stressful environment for a period of time will gradually have the ability to tolerate the stress, which plays an important role in the growth and development of plants. AM fungi can promote the resistance of host plants to heavy metal stress and reduce the damage caused by heavy metals to plant growth. Therefore, it is of great theoretical and practical value to study the use of AM fungi to improve the tolerance of plants to heavy metal stress and to optimize the bioremediation of heavy metal-contaminated soil with AM fungi. Based on this, this paper reviewed the relationship between AM fungi and bioremediation at home and abroad, and the repair mechanism of AM fungi on heavy metal-contaminated soil, and prospected for the future application of AM fungi in bioremediation. It can play a major role in resources, environment, and sustainable development.

23.3.4 Effects of AM on Tolerance to Heavy Metal Stress of Host Plants

In 1981, Bradley et al. (1981) reported for the first time in "Nature" that ectomycorrhizal fungi can reduce the excessive absorption of Cu and Zn in plants. Since then, the researches on the repair of heavy metal pollution with mycorrhizal fungi and on the tolerance of host plants have been increasing, among which AM fungi have attracted the most interest. When contaminated by heavy metals, AM fungi can help host plants reduce the absorption of heavy metals to avoid damage or adapt to heavy metal stress by promoting the tolerance to heavy metals (Zhang et al. 2010; Słomka et al. 2011). The application of AM fungi in the improvement of soil polluted by heavy metals involves the physiology, ecology, and cellular and molecular biology of mycorrhiza. It was found that under such conditions, AM fungi

colonization can reduce the contents of heavy metals in the plants (especially the aerial parts), which was conducive to the normal growth of plants. Słomka et al. (2011) found that AM fungi could help *Viola tricolor* reduce the absorption of heavy metals and protect the tissues and organs of plants. Lins et al. (2006) also found that the Cu concentration in the aboveground parts of *Leucaena leucocephala* inoculated with *Glomus etunicatum* was lower than that of *Leucaena leucocephala* without inoculation treatment. After inoculation of AM fungi, the contents of Cu, Zn, Pb, and Cd in the roots of *Sesbania rostrata*, *Sesbania cannabina*, and *Medicago sativa* were significantly higher than those in the aerial parts (Lin et al. 2007). The contents of heavy metals in mycorrhizal *Vetiveria zizanioides* were also significantly reduced (Wong et al. 2010). The above results indicate that AM fungi can fix and segregate heavy metals and reduce the transfer of heavy metals to the aerial parts. Playing its role reasonably will make it possible for the crops to be planted safely in heavy metal-contaminated areas, supporting more sustainable agriculture worldwide.

Besides, some studies have found that under heavy metal pollution conditions, AM fungi infection will not reduce the absorption of heavy metals, but can increase the tolerance of plants to heavy metals, thus helping plants survive at higher concentrations of heavy metals. Studies on different plants and heavy metals have found that inoculation of AM fungi can promote plant morphogenesis and increase the tolerance to heavy metal stress from Zn (Hildebrandt et al. 2006), Pb (Zhang et al. 2010; Sudová et al. 2007), Cu (Andrade et al. 2010), As (Trotta et al. 2006), and Cd (Andrade et al. 2008). At the same time, AM fungi can significantly increase the tolerance of hyperaccumulators under heavy metal stress, *Cajanus cajan* (Garg 2012), *Lotodes repens*, and *Lolium perenne* (Dong et al. 2008), and promote the biomass of aboveground and underground parts of plants. And AM fungi can also promote the growth of heavy metal hyperaccumulators such as *Elsholtzia splendens* (Wang et al. 2005) and *Pteris vittata* (Leung et al. 2010) and further enhance their ability to withstand heavy metal stress.

Jamal et al. (2002) studied *Glycine max* and *Lens culinaris* in heavy metal-contaminated soil and found that inoculation with AM fungi increased the absorption of Zn and Ni and therefore proposed the concept of mycorrhizoremediation. A mixed inoculum of *Glomus clarum*, *Gigaspora margarita*, and *Acaulospora* sp. promote the survival of *Coffea arabica* at high concentrations of Cu and Zn as well as the uptake of Cu (Andrade et al. 2010). After inoculation with *Glomus claroideum*, the accumulation of Zn in the roots, stems, and leaves of *Solanum nigrum* was increased by 58%, 44%, and 120%, respectively. And after inoculation with *Glomus intraradices*, the accumulation of Zn in the roots, stems, and leaves of *Solanum nigrum* was increased by 54%, 39%, and 122%, respectively (Marques et al. 2007). Split-compartment cultivation has found that the content of As in the leaves and roots of *Pteris vittata* was significantly increased after inoculation with *Glomus mosseae* (Liu and Chen 2007). Leung et al. (2006) also found that indigenous AM fungi can promote the absorption of P and As in *Pteris vittata* and maintain normal growth. In the plant tissue of *Canavalia ensiformis* inoculated with *Glomus etunicatum*, the Zn content, biomass, and the number of nodules were increased (Andrade et al. 2009). By directly increasing the tolerance of host plants to heavy

metal stress, or by promoting host plant growth, increasing its biomass, and reducing the concentration of heavy metals in the plants, AM fungi help host plants adopt to heavy metal stress, which will increase the utilization of the polluted land distribution in arable land and the productivity of crops and improve the environment of farmland.

Some studies have found that AM fungi have no significant effect on plant growth and heavy metal uptake under heavy metal toxicity. Jankong and Visoottiviseth (2008) reported that the inoculation of mixed fungi composed of *Glomus mosseae*, *Glomus intraradices*, and *Glomus etunicatum* did not affect the growth of hyperaccumulator plant, *Pityrogramma calomelanos*, and non-hyperaccumulator plant, *Tagetes erecta*, nor their absorption of As. Besides, AM fungi inhibited the growth of host plants when the concentration of heavy metals was high. For example, Chen et al. (2006) found that the inoculation with AM fungi inhibited the growth of *Pteris vittata*, and it had no effect on the concentration of As in the tissue.

The protective effect of AM fungi on plants depends on the species of AM fungi, physiological and biochemical characteristics of host plants, heavy metal species, heavy metal ion forms and concentrations, growth matrix (pH, redox status, texture, organic matter content, root exudates, rhizosphere microorganisms, minerals, etc.), and external environmental conditions. In general, AM fungi not only have the ability to tolerate heavy metal toxicity but also can affect the growth of host plants and the absorption and transport of heavy metals and improve the tolerance of host plants to heavy metal toxicity by direct or indirect effects (Leyval 2005), which contributes to the development of agroforestry in polluted land.

23.3.5 Remediation Effect of AM on Heavy Metal-Contaminated Soils

Studies indicated that AM fungi can play a role in plant extraction of cadmium. Extra-organic hyphae can enlarge the nutrient absorption area, and therefore, it is possible to absorb cadmium and transmit it to plants. When contaminated by heavy metals, inoculation with AM fungi can promote the transfer of Cd, Ni, and Cr from the underground parts of cannabis sativa to the aerial parts (Citterio et al. 2005a, b). In addition, though the inoculation with AM fungi has no effect on the growth of *Canavalia gladiata*, it increases the cadmium content in the aerial parts and roots, which is beneficial to plant extraction (Andrade et al. 2005). *Cannabis sativa* is a fast-growing and biomass-producing plant. Although it is not a hyperaccumulator, it is highly resistant to heavy metals and can accumulate heavy metals in the roots. Inoculation with *G. mosseae* can promote the transfer of heavy metals from the roots to the aerial parts, which is of great significance for the application of *cannabis sativa* in plant extraction of heavy metals (Citterio et al. 2005a, b). The mycorrhized *Helianthus annuus* also accumulates more Cr than the control plants (Davies et al.

2002). Inoculation with AM fungi can increase the extraction of heavy metals by *Salix babylonica* (Sommer et al. 2002). AM fungi can also play an important role in plant extraction for hyperaccumulators. Inoculation with AM fungi can increase the biomass and Ni concentration of aerial parts of Ni hyperaccumulator, *B. coddii*, which is related to the tolerance of AM fungi and plant-fungal symbiosis properties (Turnau et al. 2010). In the study of the effect of AM on the absorption of Pb by both transgenic and non-transgenic tobaccos, it was found that for non-transgenic plants, AM increased the Pb content in the roots and promoted the transport of Pb from the root to aerial parts. However, for the transgenic plants, this effect was not obvious (Sudová and Vosátka 2007). Usman and Mohamed et al. (2009) studied the effects of AM and EDTA on the absorption of Pb, Zn, Cu, and Cd in *Helianthus annuus*. The results showed that AM increased the accumulation of heavy metals in plants, but the amplitude was smaller than that of EDTA.

The above studies indicate that mycorrhiza increases the absorption of heavy metal ions by host plants in various ways. However, some studies have concluded that, compared to root cells, external hyphae, vesicles, and arbuscular structure of AMF have larger specific surface area and are more bioabsorbable to heavy metals, so they can immobilize more heavy metals and restrict them from entering plant cells (Zheng et al. 2015). This is the "filtration effect" of AMF on heavy metals, which effectively reduces the accumulation of heavy metals in plants. For example, Zhang et al. (2010) showed that AMF could fix Pb in soil through hyphae, cell wall, and plasma membrane, which effectively reduced the toxicity of Pb^{2+} to *Zea mays*. Since AMF in heavy metal-contaminated soils generally promotes plant growth and increases plant biomass, the reduction in heavy metal concentrations in plants is also considered to be a kind of "growth dilution" effect (Chen et al. 2007).

23.3.6 AM Remediation Mechanisms for Heavy Metal-Contaminated Soils

23.3.6.1 Direct Effects

Heavy metal ion exchange and formation of chelates (Ernst et al. 1992) on the surface of AM fungi mycelium, passivation and fixation of heavy metal by fungi cell wall components such as chitin (González-Chávez et al. 2004), and precipitation of fungi inorganic acid or inorganic acid ions with heavy metals (Clemens 2001) can solidify heavy metals in the soil and weaken their mobility, thus effectively reducing the toxicity of heavy metals to host plants. Studies have shown that Pb in the mycorrhized *Zea mays* seedlings mainly exists in the mycelial cell wall, mycelial cell membrane, mycelial cavity, and vacuolar endoluminal membrane, so the Pb content in the plant is reduced, and the toxicity of Pb to *Zea Mays* seedlings is alleviated (Zhang et al. 2010).

The outer surface of the AM fungi hyphae is the first barrier to restrict heavy metals from entering the hyphae. Mycelium has a strong biosorption potential for

heavy metals and exhibits different adsorption specificities for different metal elements, which has a "filtration effect" on the entry of heavy metal ions into the host plant, thus avoiding excessive heavy metal ions entering the plant roots, balancing mineral element absorption, and improving the comprehensive tolerance of host plants to heavy metals. Chen et al. (2005) applied the glass bead split-compartment cultivation system to study the adsorption characteristics of ex vivo fungi mycelium on metal ions such as Cd, Mn, and Zn and found that the fungi mycelium had significant adsorption capacity for various metal ions. The weight of Cd, Mn, and Zn that mycelium can adsorb was 13.3%, 1.6%, and 2.8% of the dry weight of mycelium, respectively. Turnau et al. (2010) also believed that there were polyphosphates in the hyphae that can bind heavy metals, which can reduce the transport of heavy metals into plants. This effect is the "filtration mechanism." In addition, AM fungi can secrete a specific glycoprotein-glomalin-containing metal ions, which can effectively complex heavy metals in the soil and reduce the heavy metal content in the rhizosphere soil (Sudová et al. 2008).

AM fungi cell walls and plasma membranes are the second barrier to reduce the toxicity of heavy metal ions. Mycorrhizal cell walls and protoplasmic membrane components such as melanin, chitin, cellulose, and their derivatives can combine with heavy metals, and chitin can bind 90% of exogenous heavy metals. In heavy metal-contaminated environment, AM fungi can fix heavy metals in roots or extracellular mycelial cell walls and plasma membranes to mitigate the harmful effects of heavy metals (Redon et al. 2009).

23.3.6.2 Indirect Effects

The indirect effects of AM on heavy metals are mainly delivered by affecting host plants. The underlying mechanisms are as follows:

AM Fungal Infection Alters the Root Morphology of The Host Plants

AM fungi infection can enhance the lignifications of root cell wall, increase the epidermal thickness of the root tip of the host plant and the number of cell layers, promote the growth and branching of roots, and change the morphological structure of the roots, thus affecting the progress of heavy metals entering the root system. For example, the root length of the mycorrhized *Prosopis juliflora* can be increased by 44%–76% (Solísdomínguez et al. 2011). In the soils with high concentration of Cu (150 mg/kg), the biomass and root length of *Zea mays* can be remarkably increased by 108.14% and 58.18%, respectively, after mycorrhization (Shen et al. 2005). Inoculation with *Glomus mosseae* can promote growth of *Vicia faba*, increase the root length by more than 145%, and significantly affect the absorption and transfer of heavy metals (Zhang et al. 2008a). Chen et al. (Chen et al. 2005) found that AM fungal infection can change the biosorption characteristics of roots to a certain

extent, enhance the ability of roots to retain heavy metals, and strengthen the "isolation" of heavy metals at the level of host plant organs.

AM Improves the Absorption of Mineral Nutrients by Host Plants

The conclusion that inoculation with AM fungi can improve plant phosphorus uptake and mineral nutrition is unquestionable. The large mesh of AM fungal mycelium intertwined in the soil not only expands the absorption range of nutrients and water by the roots, but also redistributes the nutrients and water between different plants, thus creating another effective nutrient and water transport pathway for host plants to some extent (Zeng et al. 2005). In addition, AM fungi can also enhance the stability of soil structure by secreting extracellular enzymes, glomalin, etc. and promote the absorption of nutrients by host plants (Rillig and Mummey 2010). Studies have shown that under heavy metal stress, inoculation with AM fungi can enhance plant nutrient and water absorption and photosynthesis, promote plant growth, and increase plant biomass (Madejón et al. 2010). The mechanism is consistent for *Astragalus sinicus* (Chen and Zhao 2009), *Calopogonium mucunoides* (Souza et al. 2012), and *Zea mays* (Zhang et al. 2010). Studies have shown that the contents of P, K, S, Mn, Ca, Mg, and other elements in the leaves of *Coffea arabica* grown in Zn- and Cu-contaminated soil increase after mycorrhization (Andrade et al. 2010). Heavy metal ions such as Cu^{2+}, Mn^{2+}, Cd^{2+}, Zn^{2+}, and Pb^{2+} can react with phosphate (HPO_4^{2-} and $H_2PO_4^{-}$) to reduce the effectiveness of phosphate in soil solution, making it difficult for plants to absorb P. However, the mycorrhized plants can take advantage of the large underground mycelium network to improve the absorption of P. Andrade et al. (2008) demonstrated that AM fungi in heavy metal-contaminated soils played a significant role in improving the uptake of P in host plants. At the same time, Hu et al. (2010) believed that the infection of AM fungi can alleviate the incidence of some soilborne diseases and protect the host plant roots from pathogens and damage, thereby promoting root growth and nutrient absorption (Karasawa et al. 2012; Bouwmeester et al. 2007).

AM fungi can increase the chlorophyll content in host plants, improve the stomatal conductance and transpiration rate of leaves, and thereby increase the net photosynthetic rate of plants. AM fungi can affect stomatal opening by altering the content of endogenous hormones, especially cytokinins, in plants (Liu and Chen 2007). In soils contaminated by Pb, inoculation with *Glomus mosseae* can significantly increase the content of chlorophyll and small molecule thiol in *Chrysopogon zizanioides*, enhancing photosynthesis and increasing plant biomass (Punamiya et al. 2010). The improvement in the absorption of mineral nutrients by host plants by using AM can increase the crop fertilizer use efficiency.

AM Fungi Change the Physical and Chemical Status of the Host Plant's Rhizosphere Environment

Studies have shown that soil pH value of roots of *Zea mays* inoculated with *Glomus caledonium* was significantly increased, while bioavailable Cu concentration was significantly reduced (Shen et al. 2005). Rhizosphere microorganisms greatly promote the release of plant root exudates, and in return, root exudates also provide energy and photosynthetic products for rhizosphere microorganisms. Denaturing gradient gel electrophoresis (DGGE) analysis on small subunit RNA of rhizosphere microorganisms and mycorrhiza of *Prosopis juliflora* grown in heavy metal-contaminated soil showed that inoculation with AM fungi can change the rhizosphere microorganisms and community structure and increase the biodiversity of bacteria and AM fungi, etc. (Solísdomínguez et al. 2011). The microbial cell wall or extracellular mucoid of the rhizosphere and root surface has a certain adsorption effect on heavy metals. Toxic heavy metals are stored in different parts of the microbial cells or incorporated into the extracellular matrix, which are metabolized or sequestered by metabolism to avoid excessive heavy metals from entering the plant. Studies have shown that mycorrhiza can significantly increase the number of Mn-oxidizing bacteria in the rhizosphere of host plants and inhibit the production of Mn-reducing bacteria (Nogueira et al. 2004). The decrease in the number of Mn-reducing bacteria or the increase in the number of Mn-oxidizing bacteria in the rhizosphere will lead to a decrease in the reducing ability of Mn as well as the absorption of Mn by plants, mitigating the toxic effects of Mn on plants. However, some studies have drawn the opposite conclusion. Nogueira et al. (2007) found that the number of Mn-oxidizing bacteria in the mycorrhized roots of plants inoculated with AM fungi was 45% lower than that in the rhizosphere of plants without being inoculated with AM fungi. The balance between Mn-reducing bacteria and Mn-oxidizing bacteria in the rhizosphere or non-mycorrhizal rhizosphere is affected by the characteristics of soil, AM fungi, and host plants (Qiu et al. 2017; He et al. 2013).

AM fungi can improve plant nutrition and promote plant growth because of their direct or indirect effects, such as expanding the absorption area of plant roots, accelerating the transport rate of nutrients and water, and secreting activated substances. Inoculation with suitable AM fungi in heavy metal-contaminated soil can effectively reduce the toxicity of heavy metals to plants and increase the absorption of heavy metal elements in the aerial parts of plants or accumulation in roots, thus promoting the bioremediation of heavy metal-contaminated soil. Bioremediation technology is one of the important development directions of environmental science in the future. It has great potential and broad prospects, especially in the polluted soil area where the ecological environment is weak due to mining and smelting of mineral resources, which contribute to safe planting of agricultural products. However, due to the unique nature of AM fungi, such as the inability of fungi for pure culture and the unsatisfactory research methods, it is not clear to what extent AM fungi affect plant tolerance to heavy metals and absorption and distribution patterns of heavy metals. In addition, the application of AM fungi for bioremediation is an

emerging field of environmental science with very short research period. Many basic theories and practical applications are in urgent to be solved. China has abundant plant resources and mycorrhizal resources. It is an important direction to fully take this advantage to apply mycorrhizal technology to the study of heavy metal hyperaccumulators and to cultivate heavy metal hyperaccumulators with stronger tolerance and higher accumulation efficiency. To play better role in resource depletion and environmental pollution, it is important to solve the problems encountered in popularization and application of AM fungi.

The reciprocal and mutually beneficial relationship between plant and mycorrhizal symbiosis makes it superior to a single organism in the remediation function of heavy metal-contaminated soil. It has great application potentiality in the purification process of heavy metal-contaminated soil.

23.4 Conclusions and Outlook

It can be seen that the use of AM fungi can not only increase crop yields and reduce the risk of crop diseases, thus reducing the input of chemical pollutants, but also promote the degradation and removal of pollutants, maintain a healthy soil system, and improve environmental quality while saving agricultural costs. Mycorrhizal fungi are the most abundant, widely distributed, functional, and niche-occupying superorganisms in terrestrial ecosystems. They can significantly affect plant diversity, community structure, interspecific interaction, resource allocation, and system productivity. Based on the important role of mycorrhizal fungi in the relationship between plants and environment, countries around the world have high expectations for their application in comprehensive environmental management. The research on their mechanism and practical application has been paid more and more attention. Therefore, mycorrhizal technology will be the development direction of green agriculture in the future and one of the effective ways to realize the sustainable development of agriculture.

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Chapter 24
Microbe-Mediated Removal of Heavy Metals for Sustainable Agricultural Practices

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Abstract Both environment and agriculture have been immensely affected by the sustaining humankind on Earth. Anthropogenic sources and natural calamities have increased toxic metal contents in the environment. This has also resulted in toxic metal accumulation within the food chain at an alarming concentration. The recalcitrant nature of these metals has threatened the living world. Thus, reclamation of the contaminated soils has become a global concern. Considering the cost involved and the production of hazardous by-products by the existing physiochemical techniques for cleanup of the polluted environment, newly emerged eco-friendly, cost-effective, and sustainable technologies are gaining attention. Use of indigenous microbes, bacteria prevalent in the rhizosphere, or plant-mediated removal of toxic metal is gaining attention as these processes are cost-effective and eco-friendly. Although there is an immense possibility to use bioremediation as a successful cleanup technology, it is yet to be extensively evaluated in the field conditions. Most of the studies aimed at the investigation of mechanistic details of bioremediation, relying mostly on the greenhouse-based laboratory results. Considering the hazard and complexity of toxic metal remediation, further studies on selecting suitable rhizosphere microbes along with exploring multidisciplinary approaches would provide new opportunities with promising success.

24.1 Introduction

Metals are abundant in the Earth's crust. Some of them act as essential trace elements, but most are toxic due to their non-biodegradable nature and potential for bioaccumulation (Hu et al. 2017). At higher concentrations, most of the heavy metals form nonspecific complexes in the cell and thus increase the risk of toxicity.

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The potential for heavy metal ion toxicity has forced life to evolve and develop metal-resistant determinants (Nies 1999). These very specific, mostly plasmid-mediated systems have been found in all studied eubacterial groups (Silver and Misra 1984; Ji and Silver 1995). Extensive environmental stress can destroy the ability of microbial communities to adapt for the sake of survival. Among different microorganisms, bacteria, which can grow in the presence of toxic metals, play a key role in the biogeochemical cycling of metal ions and therefore could be used in different bioremediation technologies (Spain and Alm 2003). The highly toxic metal-polluted environment is a natural resource of well-adapted bacteria. These bacteria are instrumental in the biotransformation of different metals and therefore capable of regulating their homeostasis in the environment (Merroun 2007). To thrive under stress conditions, bacteria depend on either biochemical transformation or genetic determinants (Silver and Misra 1984).

The genetic basis of metal resistance in bacteria is an active area of research in the field of environmental conservation (Trevors et al. 1985). Genes involved in transformation/mobilization of heavy metals can be localized both on bacterial chromosomes and on extrachromosomal genetic elements (plasmid). So far, most of the resistance systems have been detected in plasmids (Silver and Phung 1996). There are some elemental differences between chromosomal and plasmid-based metal resistance systems. Resistance properties that take care of essential metals are usually chromosome-encoded and more complex than plasmid-based systems. On the contrary, plasmid-encoded systems are often dependent on toxic ion efflux mechanisms. The plasmid-borne ion efflux systems facilitate easy transfer of the resistance cassettes to other organisms (Silver and Walderhaug 1992; Bruins et al. 2000). Comparative genomics study reveals that in nature, horizontal gene transfer is one of the major forces driving the adaptive evolution of microbial genomes and thus plays a role in spreading of heavy metal resistance (Ianeva 2009; Hemme et al. 2016).

Most of the studies carried out so far to investigate the basis of metal resistance revealed presence of an active mechanism involved in driving the efflux of metal ions into the surrounding environment by the bacterial cells. Other mechanisms include exclusion by permeability barrier, extra- and intracellular precipitation, complexation, enzymatic oxidation/reduction, and adsorption (Turpeinen 2002). Chemical speciation directs bioavailability, toxicity, and reactivity of metals. Thus, it is important to have better knowledge about the major factors that dictate correlation between the microbial activity and the biogeochemistry of metals (Banerjee et al. 2011). Microorganisms can interact with metals using different mechanisms, some of which might be applicable as potential bioremediation techniques (Ahemad 2012).

Decontamination of the metal-polluted environment has therefore been considered as a technical challenge to the ecologists and agricultural scientists. Considering the ongoing contamination of sediment and crop fields, development of effective measures for bioremediation of heavy metals is one of the prerequisites. Heavy metals are very difficult to be removed from the environment. Lower success rates and higher cost of available physiochemical techniques for removing heavy metals allowed researchers to look for new eco-friendly and cost-efficient technologies

(Alkorta and Garbisu 2001; Mallick et al. 2015). Bioremediation depends on the application of living organisms especially bacteria, fungi, and plants to detoxify environmental pollutants.

This review gives a glimpse of extended metal pollution worldwide, various mechanisms of metal resistance in plant growth promoting microorganisms and future possibilities of different bioremediation strategies for eco-friendly and cost-effective biotechnological applications.

24.2 Metals and Microbes

Metals play an important role in the physiology of microorganisms. Some metals are essential micronutrients. They play important roles in the redox-cycling, in stabilization of molecules via electrostatic interactions, in various enzymatic functions as co-factors, and in regulation of osmotic pressure. However, many metals have no biological function. These metals are nonessential (Bruins et al. 2000) and often potentially toxic to microorganisms (Turpeinen 2002). It has been documented that the genes involved in detoxification or mobilization of both essential and nonessential metals have evolved parallel to the pathways metabolizing sugar and different carbon sources (Ji and Silver 1995). In nature, metal ions do not undergo chemical or biological degradation for alteration or reduction of toxicity; microorganisms can only change their chemical properties (Alkorta and Garbisu 2001). For regulation and resistance of metals, changing their ionization states through oxidoreduction is necessary. This is achieved utilizing the electron transport and the enzyme-mediated reduction systems (Wakatsuki 1995).

Microbiological mechanisms for the detoxification of metals from the environment mainly include adsorption on the cell surface, intracellular uptake, and chemical transformations (Silver and Phung 1996). Adsorption is a process where metal ions are sequestrated either within the negatively charged microbial cell surface through electrostatic interaction or within the exopolysaccharides secreted by the bacteria. From surface, metal ions are transferred inside the cell with the help of membrane transporters and are bioaccumulated. Inside microbial cells, upon reduction, metal ions are adsorbed either to iron (Fe) oxides or to organic colloids and become immobilized (Sinha et al. 2009). Microorganisms generally take up necessary ions for cellular activities. Some toxic metal ions mimic the structure of essential ions, but evolution has equipped microorganisms with effective mechanisms to discriminate between toxic and nontoxic metal ions. However, synthesis of specific ion uptake system is required to exclude nonessential metal ions in cases where metal ions are in excess. For example, a specific phosphate transporter is being synthesized to exclude arsenate [As(V)] during the uptake of essential phosphate ions. The cell manages to uptake less As(V) by inducing a more specific and efficient phosphate uptake system with 100-fold greater specificity than the regular transport mechanisms (Nies and Silver 1995).

Microbes have developed two types of uptake systems to overcome a situation described for transport of phosphate ions. One is the selective, substrate-specific

uptake system which requires cellular energy in the form of ATP and is only synthesized by the cells in the presence of high concentration of As(V) in the extracellular milieu. The other transport system is the substrate nonspecific rapid system that transports metal ions across the cell membrane of the bacteria using a chemiosmotic gradient without the need for any energy (Nies 1999). Although the mechanisms to uptake metal ions are highly selective, translocation of nonspecific metal ions through the same membrane complex is common in all biological systems. Even highly evolved substrate-selective transport mechanisms might not always prevent entry of toxic metal ions into the cells (Gadd 2010). Thus, intracellular accumulation of toxic metals occurs at a very high concentration (Brierley 1982). Some metals are less soluble and less toxic in the reduced state than in an oxidized state, such as chromium (Cr). In microbial cells, reduction of toxic metal ions can occur by the dissimilatory reduction process, where during anaerobic respiration, microbes utilize metals as a terminal electron acceptor. However, to achieve reduction of toxic metal ions, the redox potential of a given metal ion should fall between that of the hydrogen/proton and oxygen/hydrogen ion pairs. After reduction, a metal compound either diffuses out of the cell or might be re-oxidized. Thus, if the cell decides to detoxify a metal ion by reduction, an efficient efflux system should be in place to export the reduced form of the metal (Nies 1999). In certain cases, metal-reducing bacteria can also contribute to the mobilization of insoluble forms of metal ions (Ramasamy and Parwin Banu 2007). Solubilization might have adverse consequences when mobilized forms are more toxic. Thus, a wide range of microbial protection strategies of microorganisms are available to mobilize and detoxify potentially toxic metal (ions) and can be adopted to develop cost-effective and eco-friendly bioremediation technologies (Nies 1999).

24.3 Mode of Resistance to Different Metals

24.3.1 Metal Exclusion by Permeability Barrier

Metal exclusion by permeability barrier can be explained by modifications in the cell surface structures (membrane, wall, or envelope) of microorganisms. Such mechanism protects metal-sensitive essential cellular macromolecules. For example, in *E. coli* B, exclusion of Cu(II) is achieved by the altered synthesis of the porin, a membrane channel protein (Rouch et al. 1995). This is generally mutation(s) in one single gene, and the resulting mutant has altered permeability of the membrane to metal ions (Ji and Silver 1995). Another example is where nonspecific binding of metals to the outer membrane or envelope results in protection against toxic metal ions due to saturation of the binding sites (Sinha et al. 2009; Nies and Silver 1995). There is a controversy about copper resistance through periplasmic binding of some forms (Mergeay 1991; Silver and Ji 1994). Periplasmic sequestration of Cu(II) has been studied in *Pseudomonas* sp., where the metal resistance is attributed to the

expression of an operon consisting of four genes: *copA*, *copB*, *copC*, and *copD*. CopA and CopC proteins are localized between the inner and outer membranes, while CopB is found in the outer membrane. The cellular localization of these proteins supports the hypothesis that copper resistance occurs due to either extracellular sequestration or periplasmic binding (Silver and Walderhaug 1992; Ji and Silver 1995). An example of conformational changes in the membrane resulting in an alteration of the permeability for metal ions is observed in some species of *Staphylococcus aureus* and *Alcaligenes* sp. (Novick 1967; McEntee et al. 1986). In *Staphylococcus aureus*, it has been shown that the penicillinase-containing plasmid can result in alteration of the membrane permeability and therefore the resistance towards Cd(II) (Novick 1967).

24.3.2 Efflux

Various types of efflux transporters are present in the microbial system. Most of the transporters are nonspecific as they transport different types of molecules across the membrane. Originally these transporters have been identified as multidrug transporters. They transport metals, organic substances, and many other unrelated compounds. The P-type ATPase is an efflux protein that causes exclusion of Cd in *S. aureus* (Silver and Phung 1996). ABC transporter proteins lead to efflux of Mn in *Streptococcus gordonii* (Kolenbrander et al. 1998) and Zn (Patzer and Hantke 1998) and Ni in *E. coli* (Navarro et al. 1993). RND (resistance, nodulation, cell division) transporter proteins are mainly found in Gram-negative bacteria (Saier 1994; Saier et al. 1994) where an RND pump interacts with a membrane fusion protein (MFP) and a proteinaceous outer membrane factor (OMF) to form a transenvelope pore (Paulsen et al. 1997). Besides being multidrug resistance factors, RND transporters are also involved in metal transport nonspecifically. However, metal transportation occurs in the form of organic metal conjugates rather than free metal ions. The CzcCBA efflux pump, consisting of the RND transporter (CzcA), the MFP protein (CzcB), and the OMF protein (CzcC), is involved in detoxification of Zn^{2+}, Co^{2+}, and Cd^{2+} (Rensing et al. 1997). On the contrary, the HoxN protein was found to be essential for Ni^{2+} uptake for the synthesis of hydrogenase enzyme in a strain of *Ralstonia eutropha* (Wolfram et al. 1995) for assimilating molecular hydrogen. In *R. eutropha*, HoxN is used for the uptake of Ni^{2+} with high affinity, while *E. coli* recruits an ABC transporter for the same function (Navarro et al. 1993). HoxN is driven by the chemiosmosis that favors the uptake of divalent cations. Lately, a number of HoxN family members are reported and are found to be involved in Ni^{2+} or Co^{2+} uptake (Komeda et al. 1997). The CHR family is the member of another small family, which is involved in chromate efflux in bacteria and archaea (Nies et al. 1998). The mechanism of transport by the CHR family is unclear; however, in bacteria, anion efflux is always energetically favored. Members of the ChrA family are either chromate or sulfate transporters. The CorA protein of the MIT (inorganic metal

transport) family from *S. typhimurium* is a fast and nonspecific uptake system for Mg^{2+} and other divalent cations (Snavely et al. 1989; Smith and Maguire 1995; Smith et al. 1998). Another protein family, the CDF family, is involved in metal (Zn^{2+}, Cd^{2+}, Co^{2+}) transport in microorganisms and is found in bacteria, archaea, yeasts, worms, mammals, and plants. The CDF transporters are often of six transmembrane alpha helices and large hydrophilic regions populated with histidine residues (Nies and Silver 1995; Paulsen et al. 1997). An example of more specific adaptation is the expression of arsenate [As(V)] efflux pump encoded by the *ars* operon (Sato and Kobayashi 1998). In general, the *ars* operon encodes five genes: *arsR*, *arsD*, *arsB*, *arsA,* and *arsC* (Rouch et al. 1995). Some bacteria contain just three genes, except the regulatory genes *arsD* and *arsR*. The *arsB* encodes for an arsenite efflux pump that is involved in arsenite transport across the inner membrane, while the *arsC* encodes for an enzyme that reduces arsenate to arsenite (Silver and Misra 1984; O'Halloran 1993).

24.3.3 Enzyme-Catalyzed Transformation

Another detoxification mechanism involves redox transformation (Lloyd 2002). Usually, the biotransformation of metals occurs by enzyme-catalyzed redox conversion of inorganic forms (Tebo et al. 1997). In dissimilatory metal reduction, microbes utilize metals with higher ionization states such as Fe(III), Mn(IV), U(VI), Cr(VI), Se(VI), and As(V) as terminal electron acceptors for anaerobic respiration (Lovley and Coates 1997; Lloyd 2002) or might possess uncoupled reduction mechanisms to confer metal resistance. Aerobic and anaerobic reductions of Cr(VI) to Cr(III) (Fude et al. 1994; Cifuentes et al. 1996), Se(VI) to elemental Se (Lloyd and Lovley 2001), U(VI) to U(IV) (Chang et al. 2001), and Hg(II) to Hg(0) (Brim et al. 2000; Lloyd 2002) are some of the widespread detoxification mechanisms documented in microorganisms. Microbial Hg resistance is considered to be a model for enzymatic detoxification. Hg(II) resistance has been well documented both in Gram-positive and in Gram-negative bacteria (Misra 1992). The enzyme-catalyzed transformation abilities of microbes can either solubilize or immobilize metals, resulting in an alteration of their bioavailability and cytotoxicity. Thus the transformation mechanisms play a key role in the maintenance of the biogeochemical cycling of metals (Lovley and Coates 1997; Lloyd and Lovley 2001). For example, U(VI) is highly soluble and mobile, but in the reduced form, U(IV) is highly insoluble. Metal-reducing bacteria can reduce highly toxic soluble chromate [Cr(VI)] to less toxic and less soluble Cr(III) ion (Lloyd 2002). On the contrary, acidophilic iron and sulfur-oxidizing bacteria can leach Cu, As, Cd, Co, and Zn at high concentrations from a contaminated environment (White et al. 1997).

24.3.4 Cell Surface Adsorption

Biosorption is a rapid physicochemical process that occurs naturally in biomass including both live and dead organisms. This has been considered as a promising biotechnological approach for removal and/or recovery of metals (Beveridge and Murray 1976; Hoyle and Beveridge 1983; Macaskie 1991; Garnham et al. 1992; Gadd and White 1993; Volesky and Holan 1995; Michael 2008; Wang and Chen 2009). But for live cells, metabolic pathways might also contribute to the biosorption process (Gadd and White 1993). The bacterial cell wall structure, although mainly constituted by peptidoglycan, often produces polysaccharides, which play an active role in immobilizing heavy metals, thereby increasing their bioavailability (Kawai et al. 1992; Iyer et al. 2005). Bacteria that naturally produce extracellular polysaccharide demonstrate abilities to absorb metal ions, thereby preventing them from interacting with important cellular macromolecules. Exopolysaccharide produced by *Klebsiella aerogenes* could remove Cd from the growth medium and help those to survive under Cd stress (Scott and Palmer 1990). Moreover, the cell wall of microbial biomass contains structural molecules like proteins, lipids, and carbohydrates. These biological macromolecules are associated with different functional groups such as amino, phosphate, hydroxyl, carboxylate, and sulfate that bind the metals (Rouch et al. 1995; Scott and Palmer 1990). In recent years, modification of biomass, such as overexpression of metalloregulatory protein genes, has been attempted to improve the efficacy or selectivity of microbial biosorbents (Bae et al. 2002, 2003).

24.3.5 Extracellular Precipitation

Microorganisms release a diverse set of specific and nonspecific metal-binding chelators into the extracellular environment, and such release helps in the reduction of the toxic effects of metals. Nonspecific metal-binding microbial metabolites such as organic acids can form complexes with metals and decrease their mobility and toxicity (White and Gadd 1990). Bacteria, algae, and fungi can synthesize macromolecules, which consist of humic and fulvic acids arising from lignocellulose degradation/extracellular polymeric substances (EPS), a mixture of polysaccharides, mucopolysaccharides, and proteins. Such hitherto undefined macromolecules can also bind significant amounts of potentially toxic metals and reduce their toxic effects (Spark et al. 1997). Phosphates, oxalates, and sulfides released by the microbes facilitate extracellular immobilization of available metals. Bioprecipitation of sulfides and phosphate compounds has achieved great importance owing to their low solubility (Gadd 2010). In anaerobic sediments, sulfate-reducing bacteria (SRB) can produce significant amounts of sulfide, which helps in the precipitation of metal ions as metal sulfides. As a consequence, the concentration of available toxic soluble metal ions in the surrounding microenvironment of SRB is decreased. This facilitates SRB to grow in environments with high levels of toxic metals (White et al. 1998).

For example, Cd resistance in *Pseudomonas aeruginosa* occurs through its conversion into CdS (Sinha and Mukherjee 2009). The release of phosphate by hydrolysis has been reported to be an effective arsenal for precipitation of metals on the cell surface as insoluble metal phosphates (Macaskie 1991). Furthermore, release of phosphate due to polyphosphate hydrolysis in the extracellular milieu in certain organisms implicated to their abilities to survive in the environment with higher metal concentration (Gadd 2010). To facilitate the uptake of essential metals at a very low concentration, microorganisms also produce specific extracellular metal-binding compounds. The most studied system is the production of siderophores in the presence of low concentrations of iron in the environment. Siderophores are low-molecular-weight Fe(III) chelating compounds biosynthesized by many microorganisms. They help in solubilizing and complexing insoluble Fe(III) in a form that can be transported into the cell using specific transporters (Neilands 1981). Besides being known as iron-binding compounds, siderophores are also capable of complexing with other metals such as manganese, magnesium, chromium(III), gallium(III), and plutonium(IV) (Birch and Bachofen 1990).

24.3.6 Accumulation Inside the Cell

Bioaccumulation of metals is an energy-dependent transport system. Once the metal enters into the cell via transporters (transport mostly important physiological cations), it might be compartmentalized and/or converted to less toxic forms either by binding or by precipitation in the form of phosphide, sulfide, carbide, or hydroxide (Summers and Silver 1978; Weiss et al. 1978). *Pseudomonas aeruginosa* was found to accumulate Ni in the form of phosphide salts, and 88% of the accumulated metal was mainly partitioned into the membrane and periplasm (Sar et al. 2001). Intracellular accumulation of Cu was also observed in *P. aeruginosa*, where the accumulated Cu was restricted to the periplasm, majorly in the form of copper sulfide (Kazy et al. 1999). Cadmium accumulation has been reported in *Pseudomonas putida* through the production of cysteine-rich soluble proteins metallothioneins (Higham et al. 1984, 1986). Two strains of sulfate-reducing bacteria, *Desulfovibrio desulfuricans* DSM 1926 and *Desulfococcus multivorans* DSM 2059, showed intracellular and periplasmic accumulation of cadmium, respectively (Naz et al. 2005).

24.3.7 Volatilization

Microorganisms often detoxify metal ions through converting them into less soluble and, therefore, less toxic form by volatilization process. This is achieved by oxidation, reduction, methylation, and demethylation of the compounds (Thayer 2004).

Mercury volatilization is a well-known example of this process, which occurs during geochemical cycling of Hg by certain microbes (Mishra and Roy 2008). Hg methylation and demethylation along with oxido-reduction causes volatilization of Hg (Barkay et al. 1989). On the contrary, methylation of arsenic by fungi and other eukaryotes is well known compared to bacterial systems (Gadd and White 1993; Bentley and Chasteen 2002). The pathway of As methylation is a two-step process where in the first step, reduction of arsenate takes place followed by an oxidative inclusion of a methyl group (Challenger 1945; Dombrowski et al. 2005).

24.4 Global Scenario of Metal Pollution

Heavy metal contamination in the environment results mainly due to natural weathering and anthropic disturbances. Anthropogenic sources of metal contamination have been classified into five main groups, viz., (1) metalliferous mining and smelting (Cd, Hg, As, and Pb), (2) industry (Cr, Cu, Zn, Co, Ni, As, Cd, and Hg), (3) atmospheric deposition (Cr, Cu, Cd, As, Pb, Hg, and U), (4) agriculture (Cu, Zn, Pb, Cd, As, Se, and U), and (5) waste disposal (Cr, Cd, Cu, Zn, As, Pb, and Hg) (Ross M. Sheila 1994). Both the type and content of hazardous heavy metals in the environment have been gradually increased in parallel to an advancement of global economy, resulting in the deterioration of the environment. Heavy metals can be biomagnified through the food chain (Han et al. 2002; Su et al. 2014). Figure 24.1 depicts an overview of metal pollution and remediation techniques.

Globally, more than 10 million sites are officially announced to be polluted, of which >50% sites are contaminated with heavy metals and/or metalloids. Heavy metal pollution has a combined impact on global economy (He et al. 2015). An alarming concentration of different heavy metals (Cr, Cu, Zn, Pb, Ni, Cd, Hg, As) has been found in the urban and agricultural soils worldwide (China, Spain, Korea, Slovakia, Iraq, Iran, India, USA, and others) (Su et al. 2014).

Besides soils, a large section of global water resources has also been profoundly affected over the past decades due to human activities, resulting in poor-quality water supply for household and drinking purposes. In many parts of the world, heavy metal (HM) concentrations in drinking water are higher than the recommended values. Metal pollution in drinking water, incorporation into the food chain through biomagnification, and their implications for the human health are one of the major concerns. It is reported that millions of people are affected with chronic metal poisoning and about 1.6 million children die each year due to consumption of metal-contaminated drinking water (Fernández-Luqueño et al. 2013). The magnitude of metal-contaminated groundwater is severe in India, and around 150 million people are at risk (Smith et al. 2000; Su et al. 2014).

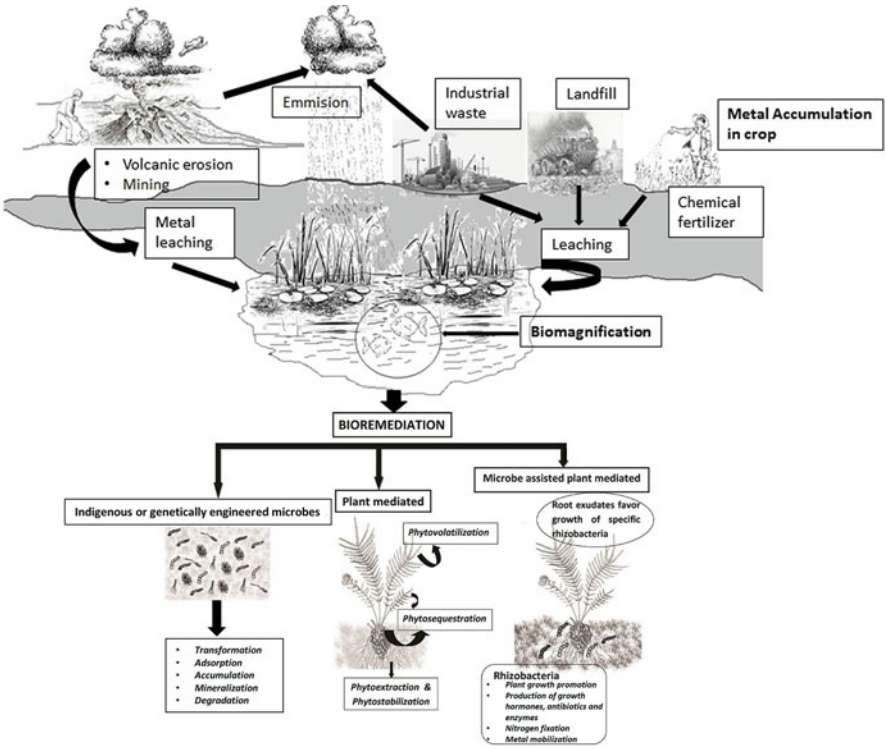

Fig. 24.1 An overview of metal pollution and remediation techniques

24.5 Bioremediation Strategies

Reclamation of metal-contaminated soil is one of the major challenges in the field of environmental engineering. Considering the cost, ineffectiveness, and hazards associated with the existing physiochemical techniques for metal removal from the contaminated environment, new eco-friendly and cost-effective alternative technologies are gaining immense attraction in recent years (Alkorta and Garbisu 2001; Gupta and Joia 2016; Ojuederie and Babalola 2017). Bioremediation of metals involves living organisms, especially bacteria, archaea, fungi, plants, or even an entire microbe-plant system (natural or genetically modified). The common practice adopted for bioremediation involves reduction of the metal solubility either by changing the pH of the system or by changing the redox state of the metal ion. Also common are the adsorption and intracellular accumulation of toxic metals from the contaminated environment (Gupta and Joia 2016; Ojuederie and Babalola 2017).

24.6 Microbe as a Potential Tool

Due to the high cost, low efficiency, and major destruction of soil characteristics and fertility, methods used for soil remediation like thermal treatment, electro-reclamation excavation, landfill, and acid leaching are not suitable for field applications. Other remediation techniques include soil washing, scrubbing with wet screening, and various chemical methods. Chemicals used to extract toxic metals include inorganic and organic acids, bases, salts, and the chelating agents like EDTA and DTPA (Salido et al. 2003; Flora and Pachauri 2010; Jelusic and Lestan 2015). All of these methods generate secondary waste products that necessitate additional waste treatments. Thus, the development of cost-effective, safe, and efficient strategies for toxic metal remediation of contaminated soils is imperative (Wuana and Okieimen 2011). Redox reactions chemically transform toxic metals into non- or less-toxic forms that are more stable, less mobile, or inert such as As, Cr, Hg, and Se when present in natural soils and sediments (Gadd 2010; Rajapaksha et al. 2013; Tandon and Singh 2016). Depending on the type of the contaminants present, diverse approaches can be adopted to improve the process of bioremediation. One such approach is bio-stimulation where organic amendments in metal-contaminated soils facilitate bioremediation. Such an addition brings about alteration in the soil microbial communities, primarily through changing the pH, decreasing the solubility of heavy metals in the soil, and increasing nutrient availability (Hameed 2006; Gupta and Joia 2016). Biomass-obtained crop residue, manure, and solid wastes can be utilized to augment microorganisms for bioremediation by making the environment more amenable (Ojuederie and Babalola 2017). Another approach is bio-augmentation, which involves addition of microbes to the indigenous population to augment the process of biodegradation of a contaminated environment (Lloyd 2002; Gupta and Joia 2016).

In recent years, biosorption techniques, which are based on the metal-binding capacities of biological materials, have attracted immense attention for eliminating toxic metals from the polluted environment. Adsorption of metals by bacterial cell wall components is one of the most promising toxic metal bioremediation techniques. A number of models describing the metal chelation complexes on the bacterial cell surface have thus been defined to account for the degree of metal adsorption (Nakajima and Tsuruta 2004). The process starts with the adsorption of metal ions on the cell surface via interaction with different functional groups followed by transportation inside the cell with subsequent transformation. To enhance the affinity of metal binding on the cell surface, an increased expression of metallothionein and/or metallopeptides has been reported. Metallothionein is a protein family that includes low-molecular-weight cysteine-rich proteins and is involved in binding metals. Metallothioneins are synthesized under metal stress and found in all three domains of life (Singh et al. 2004; Gupta and Joia 2016). Currently, there are varieties of promising microorganisms already studied in detail for their applicability in bioremediation of contaminated environments. Some of these microbes have already shown to be effective in biosorption of heavy metals

(Kim et al. 2008; Gupta and Joia 2016). Biosorption techniques have a number of advantages over the conventional methods. Such advantages include cost-effectiveness, efficiency, minimal sludge, specificity, minimal nutrient requirement, reusability of the biosorbent, and the likelihood of metal recovery (Alkorta and Garbisu 2001; Fan et al. 2007). The essential constituents in such bacterial cells are extracellular polymeric substances (EPS) having ion sequestration capabilities. EPS are natural high-molecular-weight mixed polymers comprising of polysaccharides, proteins, lipids, uronic acids, and a smaller proportion of extracellular DNA (Gupta and Diwan 2017). EPS produced during bacterial biofilm formation play an important role in the biosorption and biomineralization of metal ions (François et al. 2012; Mallick et al. 2015; Bhattacharyya et al. 2017). The biological activities of the EPS can be further chemically modified to expand their biotechnological applications. Such chemical modifications include methylation, phosphorylation, acetylation, sulfonylation, and carboxymethylation (Gupta and Diwan 2017).

Bioaccumulation is another important process in which living organisms remove toxic metals from the environment and accumulate them in the form of particulate matters and/or transform them into further toxic intermediates/insoluble forms at the expense of cellular energy. It includes the adsorption of metals onto the cellular membrane followed by transportation (Jan et al. 2014; Azubuike et al. 2016). For assisting the interaction with metals in the surrounding environment, high surface area of the cell to cellular volume ratio provides certain advantages for microbes. Metal uptake is a complex procedure that depends on various factors like metal chemistry, surface characteristics of the organisms, cellular physiology, and finally the physicochemical parameters such as pH, temperature, and metal concentration. Diverse mechanisms might be adopted by different microorganisms for the same metal ion depending on the surrounding environment (Machado et al. 2010; Lozano and Dussán 2013).

24.7 Phytoremediation

Phytoremediation is a cheap, efficient, and eco-friendly process that is widely used to remove pollutants from soil and aqueous environments involving plants (Rahman and Hasegawa 2011; Vithanage et al. 2012; Jasrotia et al. 2017). Phytoremediation technology operates through different mechanisms like removal (phytoextraction), phytofiltration, phytostimulation, immobilization (phytostabilization), phytovolatilization, or degradation (phytodegradation, rhizodegradation) (Sylvain et al. 2016; Placek et al. 2016; Limmer and Burken 2016). The efficiency of phytoremediation at any polluted site depends largely on the level of metal pollution in the soil, presence of other contaminants in the soil, and the capacity of plants to absorb metals. Phytoextraction of metals involves the following steps: (1) uptake of soluble metal ions from contaminated soil, (2) movement of metal ions through the xylem, and (3) transformation and bioaccumulation of metals into aboveground parts of the plant (Jutsz and Gnida 2015). Hyperaccumulators are capable of taking up

large quantities of toxic metals from contaminated soils in comparison to non-hyperaccumulator plants without suffering from any apparent phytotoxic effect (Jabeen et al. 2009; Rascio and Navari-Izzo 2011). Some plants have been well identified as hyperaccumulators of metals like *Asteraceae*, *Brassicaceae*, *Caryophyllaceae*, *Cyperaceae*, *Euphorbiaceae*, *Fabaceae*, *Lamiaceae*, *Poaceae, Violaceae*, etc. (Muszyńska and Hanus-Fajerska 2016). These hyperaccumulators are capable of taking up large quantities of heavy metals due to their robust root architecture and an efficient root-to-shoot translocation system. Besides, they grow comparatively faster and are highly efficient to sequester large quantities of heavy metals in the shoots (Jabeen et al. 2009; Rascio and Navari-Izzo 2011; Muszyńska and Hanus-Fajerska 2016). Furthermore, decontaminating soil from toxic metals, phytoextraction also produces enough biomass to make it commercially viable. This is however the most preferred technique for bioremediation assisted by root-associated plant growth-promoting rhizobacteria (PGPR) (Vassilev et al. 2004; Ojuederie and Babalola 2017).

Phytofiltration works in the cleanup of aqueous wastes using plants and their associated rhizosphere microflora. On contrary, phytostabilization uses plant roots to absorb contaminants from the soil and sequester them within the rhizosphere (Lone et al. 2008). This technique primarily focuses on toxic metal sequestration within the rhizosphere. The plant species used in phytostabilization are usually equipped with a broad root system and are capable of blocking metal ions from moving toward different plant parts (Islam et al. 2013). Changes in environmental conditions like pH and organic matter can further enhance the phytostabilization ability of a plant. Another technique, phytovolatilization, deals with the removal of soil pollutants in the form of vapor and consequently released into the atmosphere by plants (Ali et al. 2013; Ojuederie and Babalola 2017). Phytodegradation and rhizofiltration are two more efficient techniques to detoxify contaminated environment based on plants' natural enzyme and hyperaccumulation of toxic metals, respectively. In recent years, development of effective green chemistry methods for detoxification of metals has attracted immense attention due to their cost-effectiveness and eco-friendly nature (Gupta and Joia 2016; Ojuederie and Babalola 2017).

24.8 Plant Growth-Promoting Rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) are a diverse group of free-living soil bacteria that can improve growth of plants as well as can assist in removal of toxic metals from the contaminated soil upon successful root colonization. Different bacterial genera are implicated to contribute in biogeochemical cycling of different toxic metals in natural environments. These PGPR live in the rhizosphere of the host plant where they augment plant growth via direct or indirect mechanisms (Bhattacharyya and Jha 2012; Mallick et al. 2014; Mallick and Mukherjee 2015). Direct mechanisms include phosphate solubilization, siderophore production, and 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) synthesis which

Table 24.1 Current research on PGPR for bioremediation

Microorganisms used	Heavy metals	Host plants	Year	Reference
Kocuria flava AB402, Bacillus vietnamensis AB403	As	*Oryza sativa*	2018	Mallick et al. (2018)
Acinetobacter lwoffii	As	*Vigna radiata*	2018	Das and Sarkar (2018)
Bacillus cereus, *Pseudomonas moraviensis*	Cu, Cr, Co, Cd, Ni, Mn, Pb	*Triticum aestivum*	2017	Hassan et al. (2017)
Microbacterium sp. *CE3R2*, *Curtobacterium* sp. *NM1R1*	Zn, Pb, Cu, As	*Brassica nigra*	2017	Román-Ponce et al. (2017)
Bacteroidetes bacterium, Pseudomonas fluorescens	Cd, Cu, Pb, Zn	*Brassica napus*	2017	Dabrowska et al. (2017)
Kocuria sp. *CRB15*	Cu	*Brassica nigra*	2017	Hansda et al. (2017)
Klebsiella pneumoniae	Cd	*Oryza sativa*	2017	Pramanik et al. (2017)
Enterobacter ludwigii, *Klebsiella pneumoniae*	Hg	*Triticum aestivum*	2016	Gontia-Mishra et al. (2016)
Azospirillum	Pb, Cd	*Panicum virgatum*	2016	Arora et al. (2016)
Enterobacter, Leifsonia, Klebsiella, Bacillus	Cd	*Zea mays*	2016	Ahmad et al. (2016)

allow the plants to cope with abiotic stresses and increase plant growth hormone synthesis (Bhattacharyya and Jha 2012; Ahemad and Kibret 2014). On contrary, PGPR act as biocontrol agents and involve in detoxification of heavy metals/pesticides in the indirect mechanisms (Glick and Glick 2012). Many PGPR are capable of surviving at higher metal concentration, and such properties have been implicated to be either intrinsic or induced (Stan et al. 2011). Besides being plant growth-promoting rhizobacteria, such PGPR can remove toxic metals from the contaminated soil either through transformation or immobilization, allowing healthy vegetation in the contaminated environments (Table 24.1). To cope with the metal-contaminated environment, PGPR have evolved several mechanisms by which they could mobilize or immobilize or transform metals rendering them non-bioavailable for biological systems (Nies 1999). The mechanisms as discussed in previous sections include adsorption, accumulation, exclusion, extrusion, transformation, precipitation, methylation/volatilization, and demethylation. These strategies might help to combat the deleterious effect of toxic metals by removing them from the environment in the process of metal-free sustainable crop production.

24.9 Genetically Engineered Organisms

Cleaning polluted environments using indigenous microorganisms have not yet been successful. Like, for instance, in some cases, indigenous soil bacteria cannot remove toxic metals such as Hg from the environment. In those situations, the bacteria that

Table 24.2 List of genetically modified plant-associated microorganisms

Genetically engineered microbe	Modified gene expression	Associated plant	Used to remediate metal (s)	Reference
Pseudomonas putida	Phytochelatin synthase	*Triticum aestivum*	Cd, Hg, Ag	Yong et al. (2014)
Mesorhizobium huakuii	Metallothionein, phytochelatin synthase	*Astragalus sinicus*	Cd, Cu, Zn, As	Ike et al. (2008)
Mesorhizobium huakuii	Metallothionein, phytochelatin synthase	*Astragalus sinicus*	Cd	Ike et al. (2007)
Pseudomonas putida	Expression of metal-binding peptide	*Helianthus annuus*	Cd	Wu et al. (2006)
Mesorhizobium huakuii	Phytochelatin synthase	*Astragalus sinicus*	Cd	Sriprang et al. (2003)

are genetically manipulated to harbor properties of detoxifying the metal contaminants find significant use. These genetic manipulations needed a much deeper knowledge of the metabolic potentials of concerned microorganisms and hence initiated several studies in that direction. With the use of recombinant DNA technology, therefore, several genetically engineered microorganisms (GEMs) are now available that exhibit more efficient remediation of a contaminated environment compared to the indigenous ones. Some examples of GEMs used in bioremediation include enzymes that degrade organic pollutants and transform toxic metals in the environment (Kumar et al. 2013). Genetic engineering has allowed us to engineer bacteria to remove different toxic metals (Cu, Fe, Ni, Cd, As, and Hg) efficiently (Table 24.2). However, the rate and success of degradation largely depend on the catalytic efficiency and induction of the enzymes in cells (D'Souza 2001; Verma and Singh 2005; Azad et al. 2014).

Besides bioremediation, genetic engineering has also been applied to obtain microbes that are designed to act as biosensors. These biosensors are currently being used to monitor pollutants in contaminated sites efficiently and precisely. Despite these advantages posed by the biosensors, they have limited applications. This is mainly due to the variation in the response times, detection limits, sensitivity, stability, and signal relaxation lengths (D'Souza 2001; Verma and Singh 2005; Kumar et al. 2013). Although genetically engineered microbes have made the remediation process more efficient, special attention must be paid while introducing genetically engineered microbes into the environment as it might facilitate horizontal gene transfer between the engineered microbes (with antibiotic markers) and the natural microbes in the environment, leading to the possible development of multidrug resistance varieties.

24.10 Conclusion and Outlook

Recent studies have shown that PGPR that are capable of bioremediation could be the future biotechnological tool for sustainable agricultural practice. However, breakthroughs in this field are still very difficult to achieve without proper knowledge about certain critical factors. Considering the availability of high-throughput technologies, genetic tools for different microbial species, and advancement of biotechnology, questions for future research could be as follows: (1) to understand the ability of genetically modified microorganisms to survive in natural environment and execute bioremediation (Zhuang et al. 2007), (2) the detailed molecular mechanisms of the bioremediation processes and the interaction between biotic components (like plants, etc.) and the microorganisms, and (3) to increase the host range of given microorganism to achieve microbe-assisted phytoremediation under in situ field conditions.

Considerable efforts have been made to design strategies for applying GEMs in the field-based studies. However, researchers are presently looking for an alternative to the antibiotic resistance markers to prevent possible horizontal gene transfer events in natural environment. Moreover, studies are required to fully understand the metabolic potentials of GEMs to be used in bioremediation and an assessment of their effectiveness and possible side effects (Ojuederie and Babalola 2017). With the recent advancement of sophisticated biotechnological and nanotechnological tools, the field of sustainable agricultural practice has attracted immense attention. A new branch of science, nano-agriculture, has emerged as a tool to transform traditional farming practices to precision agriculture including heavy metal remediation (Subramanian and Tarafdar 2011). The expansion of new nanodevices (biosensors, enzyme encapsulation) and nanomaterials (nanotubes, nanowires, and quantum dots) opened up a complete new set of possibilities where heavy metal remediation in agricultural practice has been addressed (Dikshit et al. 2013).

In the last decade, both the environment and agriculture have faced major challenges due to human activities. Random exploitation of environmental resources has reduced its productivity. Under such a scenario, the concept of bioremediation (bacteria, plant, and PGPR) could play a key role in efficient detoxification and management of polluted environments, controlling either metal/pesticide pollution or even nitrogen/phosphorus runoff. An overexposure of heavy metals has led to the bioaccumulation of life-threatening metal conjugates through the food chain, and these are not only hazardous for human consumption but also affect the sustainability of the ecosystem. Such changes can contribute to alteration of the plant-microbe interactions by modifying microbial adaptation followed by an alteration of metal biogeochemistry (Zhuang et al. 2007; Gouda et al. 2018). Application of metal-mobilizing/metal-transforming PGPR can serve as an important factor in sustainable agricultural practice by reducing bioaccumulation of metals in the crops, crop productivity, improving soil fertility, and for maintaining a balanced geochemistry. Considering the hazard and complexity of interaction between toxic metals and PGPR, further studies on selective rhizobacteria would be instrumental in designing

their futuristic application to continue sustainable agricultural practice in the backdrop of human activities.

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