Chapter 19
Plant Growth Promoting Rhizobacteria Improving the Legume–Rhizobia Symbiosis

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Abstract The legume–rhizobia symbiosis is considered the most important nitrogen-fixing interaction from an agricultural point of view. However, biotic and abiotic factors can modify critical parameters of both the legumes and the rhizobia. These changes may lead to differences in the molecular dialogue, consequently reducing the symbiotic effectiveness. Therefore, optimal performance of the N-fixing symbiosis will be guaranteed by selection of both symbiotic partners for adaptation to the target environment. The symbiotic process can be negatively affected by many other rhizosphere interactions, resulting in important ecological, economic, and nutritional losses. The application of agricultural techniques that are friendly with the environment, based on the use of plant growth promoting rhizobacteria (PGPR), can increase the efficiency of the symbiotic process. The use of these beneficial microorganisms could reduce the use of polluting chemicals allowing sustainable production of legumes. Co-inoculations of appropriate rhizobia together with PGPR may profoundly increase the crop yield by different mechanisms. The negative effects of environmental stresses on the legume–rhizobia symbiosis may further be significantly diminished by applying mixtures of rhizobia and PGPR.

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19.1 Introduction

Nitrogen (N) and water are the two major root-acquired resources that limit crop growth worldwide, and the availability of one can affect the utilization of the other. From the 1960s until recently, the main aim of the most of the agricultural industry in developed countries was to optimize output per unit of land area and to achieve this, N fertilizer has been applied at, or close to, "economic optimum levels" on most herbaceous non-legume crops (Firbank 2005). Generally, crop plants are able to take up and convert only 20–40% of the applied N to useful products, and most of the surplus N is lost to the aqueous and atmospheric environment where it can become a serious pollutant and hence requires attention (Jackson et al. 2008). Presently, a large portion (36%) of the global, un-glaciated land area is intensively managed croplands and pasturelands, although effectively all, except the most remote regions, are receiving some type of human intervention (Desjardins et al. 2007). The area of cultivated land increased from about 265 Mha in 1700 to approximately 1,473 Mha presently, while the area under pasture increased from 524 to 3,215 Mha over the same period (Raddatz 2007). Currently, the potential for further expansion of global agricultural lands is limited because most of the good quality arable lands are already in cultivation. In addition, the release of greenhouse gases in the production of synthetic N fertilizer accounts for around 1% (as CO_2, N_2O, CH_4) of global greenhouse gas emissions (Wood and Cowie 2004). Fertilizer demand has historically been influenced by changing and often interrelated factors such as increasing populations and economic growth, agricultural production, prices, and government policies. The production of N fertilizer for 2007 was 130 million tons which is likely to increase further in the coming years (FAO 2008). As a result of these environmental concerns, alternative strategies to the application of N fertilizer are being sought to combat limiting soil N levels in agricultural systems (Andrews et al. 2003). One alternative method is to utilize a nitrogen-fixing legume as, for example, a seed crop, a green manure, or as the main N input in a pasture by growing it in association with grass (Andrews et al. 2007; Jackson et al. 2008).

Legumes, broadly defined by their unusual flower structure and podded fruit, (de Faria et al. 1989), are second only to the Graminiae in their importance to humans. Grain and forage legumes are grown on some 180 Mha, or 12–15% of the Earth's arable surface (Graham and Vance 2003). A key to the success of the legume family, which comprises between 670 and 750 genera with more than 18,000 species (Doyle and Luckow 2003), is the evolution of mutualistic symbioses with nitrogen-fixing bacteria of the family Rhizobiaceae to directly capture atmospheric dinitrogen (N_2) to support plant growth. The incorporation of atmospheric N_2 into organic material resulting from this rhizobia–legume symbiosis is estimated to account for one-third of the total N needed for world agriculture. This unique intracellular association contributes significantly to agricultural yields, with legumes providing 25–35% of the world's protein (Graham and Vance 2003). Why is biological nitrogen fixation (BNF) in legumes so important? In addition to its role as a source of protein in the diet, N from legume fixation is essentially

"free" N for use by the host plant or by associated or subsequent crops. Replacing it with fertilizer, N would cost $7–10 billion annually, whereas even modest use of alfalfa in rotation with corn could save farmers in the US $200–300 million (Peterson and Russelle 1991). Furthermore, fertilizer N is frequently unavailable to subsistence farmers, leaving them dependent on N_2 fixation by legumes or other N_2-fixing organisms (Graham and Vance 2003). Limitations on the amount of BNF in agriculture are predominantly related to management and environment, leading some to argue that any impact of genetically engineered N-fixing non-legume plants are likely to be small (Peoples et al. 2002). Limiting factors for BNF are mainly inadequate moisture, unfavorable temperature regimes, nutrient limitations, and less than optimal nodulation from lack of appropriate inocula. Addressing these issues and expanding legumes into areas where they are not currently grown could have a large impact on global BNF and fertilizer use in the future (Peoples et al. 2002). BNF is often strongly inhibited in arid and semiarid soils due to the poor survival of rhizobia under abiotic stress, which has a negative impact on the sustainability of beneficial microorganisms associated with the plant rhizosphere. Evidence has accumulated that co-inoculation with beneficial organisms having different mechanisms of plant-growth promotion can have additive or synergistic effects on plant growth and crop yield. Beneficial responses due to interaction of plant growth promoting rhizobacteria (PGPR) with rhizobia on legumes have been reported previously (Gray and Smith 2005; Tilak et al. 2006; Estevez et al. 2009). This chapter briefly overviews the use of the beneficial microorganisms, co-inoculations of appropriate rhizobia together with PGPR, and how it can improve legume–rhizobia symbiosis.

19.2 Rhizobia Diversity and Root Colonization

A wide variety of carbon compounds are released from roots to the soil via exudation of low molecular weight, water-soluble compounds; secretion of higher molecular weight compounds involving root metabolic processes; and lysates released from sloughed off root cells and gases (Gregory 2006). Plants exude a variety of organic compounds (e.g., carbohydrates, carboxylic acids, phenolics, amino acids, and flavonoids) (Dardanelli et al. 2008a, 2010) as well as inorganic ions (protons and other ions) into the rhizosphere to change the chemistry and biology of the root microenvironment. All chemical compounds secreted by the plant are collectively named rhizodepositions. Most root products including specific compounds typical of the secondary metabolism of each plant species are available to colonizing microbes. Flavonoids for example excreted by the plant specifically induce the rhizobia to produce Nod factor (NF), a lipo-chito-oligosaccharide nodulation signal. NFs induce several responses in the plant such as, curling of the root hairs and the formation of nodule primordia after the activation of cortical cell division. There are other non-flavonoid related compounds such as xanthones, vanillin, and isovanillin that induce NodD gene expression, but they are required at much higher

concentrations than flavonoids (Cooper 2007), therefore, their importance in natural environments is questionable. Indeed, flavonoid and non-flavonoid *nod* gene inducers, bacterial surface molecules (Broughton et al. 2006; Medeot et al. 2010), and Nod factors are important players in the communication between legume and rhizobia, and other signals play a role also. Before colonization, it is assumed that a continuous dialogue of signals is exchanged between the symbionts to establish colonization.

In legume symbiosis, bacterial invasion can follow different modes of entry into roots and they are host-determined (Gage 2004). Infection thread formation occurs in most of the temperature legumes (e.g., *Medicago* and *Vicia*) and some (sub) tropical ones (e.g. *Glycine*, *Lotus*, *Phaseolus*, and *Vigna*) while crack entry/intercellular infection occurs in (sub) tropical legumes such as *Arachis*, *Neptunia*, and *Sesbania*. Nodules induced by rhizobia are of two general kinds, determinate and indeterminate. These differ in a number of respects, one of the most important being that indeterminate nodules are elongated and have a persistent meristem that continually gives rise to new nodule cells that are subsequently infected by rhizobia residing in the nodule. These newly infected cells, and the bacteria inside them, develop further and form new nodule tissue that actively fixes nitrogen. Determinate nodules, on the other hand, lack a persistent meristem, are usually round, and do not display an obvious developmental gradient as indeterminate nodules do (Gage 2004). Most of the rhizobial species belong to rhizobiaceae in the alpha-proteobacteria. However, recent research has shown that there are other rhizobial species in addition to this in beta-protobacteria, order Burkholderiales (Sawada et al. 2003). Rhizobia, currently consist of 76 species spanning over 13 genera, namely, *Allorhizobium, Azorhizobium, Blastobacter, Bradyrhizobium, Burkholderia, Cupriavidus, Devosia, Ensifer* (formerly *Sinorhizobium*), *Mesorhizobium, Methylobacterium*, *Ralstonia, Rhizobium, and Shinella*. These genera are grouped in six families (Rhizobiaceae, Phyllobacteriaceae, Bradyrhizobiaceae, Methylobacteriaceae, Hyphomicrobiaceae, and Burkholderiaceae).

Agriculture has largely profited from legume–rhizobia symbiosis since its discovery in 1888, the industry of rhizobial inoculants for legume crops being among the oldest in agroindustries and pioneering in the rational use of living bacteria for improving plant health and nutrition (Smith 1992). The most serious problems that affect nodulation and N-fixation are, however, the non-supply of high quality inoculants, infertile-acid soils; stress associated with salinity, and high soil temperatures (Catroux et al. 2001). During industrial development, many strains were selected in laboratory conditions worldwide for high N_2 fixation performance as well as for other desirable characters such as rapid plant infectivity, stress tolerance, and adaptation to a wide range of soil environments and agricultural practices (Lodeiro et al. 2004). Legume inoculation is thus an advisable agricultural practice when there are no specific rhizobia in soil able to nodulate the legumes and when the levels of soil N are low in order to maintain a high level of rhizobia on seeds and in soil, which helps ensure satisfactory nodulation and maximize grain yields (Catroux et al. 2001). The use of rhizobia as inoculants for the main legume crops is reported to increase crop yields and quality, especially in those areas

where N is the principal limiting factor for plant growth (Lodeiro et al. 2004). Despite efforts to improve strains and inoculant formulations, this technology has however not achieved desired results. The interesting part of this technology is that once rhizobia adapt to the soil environment after inoculation, they remain and persist in viable state in the soil for years even after inoculated crop is harvested. These rhizobia in the succeeding season may serve as natural inoculants for the next crop, but after cycles of plant infection, the released population of the best nodule invaders normally increases in size, and therefore, the best plant colonizer genotypes are naturally selected. Thus, a better understanding of the forces that drive rhizobial natural selection and evolution, as well as the factors that distinguish these root-invading mutualistic bacteria from parasitic ones, is the key to sustainable profit from the N_2-fixing potential of legume–rhizobia interactions (Lodeiro et al. 2004).

19.3 Root: A Paradise of Microorganisms in Action

The rhizosphere is a multiple interface between soils, plant roots, microbes, and fauna. As Lorenz Hiltner reported as early as 1904 (Hiltner 1904), the rhizosphere is a place where different biological components strongly interact. These interactions occur not only between soils and plant roots, or plant roots and microbes, but also between plants themselves, and microbes themselves, through numerous signaling molecules and complex pathways. Such complex interactions have major implications for plant nutrition and health. The rhizosphere can be defined as any volume of soil specifically influenced by plant roots and/or in association with roots and hairs and plant-produced materials (Mahaffee and Kloepper 1997). This space includes soil bound by plant roots, often extending a few millimetre (mm) from the root surface and can include the plant root epidermal layer (Mahaffee and Kloepper 1997). Most rhizosphere organisms occur within 50 mm of root surface and populations within 10 mm of root surface may reach 1.2×10^8 cells cm^{-3} or 10^9–10^{12} microbial cells g^{-1} soil. Despite large numbers of bacteria in the rhizosphere, only 7–15% of the total root surface is generally occupied by microbial cells (Pinton et al. 2001).

Bacteria able to colonize plant root systems and promote plant growth are referred to as PGPR (Kloepper et al. 1989). PGPR activity has been reported for strains belonging to a group that includes different diazotrophic bacterial species and strains belonging to genera such as, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus, Burkholderia, Enterobacter, Gluconacetobacter*, *Herbaspirillum, Klebsiella, Paenebacillus, Pseudomonas,* and *Serratia*, among others (Glick 1995; Probanza et al. 1996; Sommers et al. 2004; Spaepen et al. 2009). Rhizobia can also be considered as a soil bacteria with PGPR activity, where root colonization and growth promotion of rice, cereals, and other non-legumes have been reported (Chabot et al. 1996). Plant growth-promoting capacity has been related to different physiological activities: (1) synthesis of phytohormones, such as cytokinins,

gibberellins, and auxins, (2) enhancement of factors affecting mineral nutrition, such as phosphorous solubilization, and (3) protection of plants against phytopathogens (Sommers et al. 2004; Gray and Smith 2005). PGPR can affect plant growth and yield in a number of ways and enhancement of vegetative and reproductive growth is documented in a range of crops like cereals, pulses, ornamentals, vegetables, plantation crops, and some trees. Inoculations with PGPR increase germination percentage, seedling vigour, emergence, plant stand, root and shoot growth, total biomass of the plants, seed weight, and early flowering as well as the yields of grains, fodder, and fruit (Spaepen et al. 2009). However, experimental evidence suggests that bacterially-mediated phytohormone production is the most likely explanation for PGPR activity in the absence of pathogens (Spaepen et al. 2009) while siderophore production by PGPR may be important for plant growth stimulation when other potentially deleterious rhizosphere microorganisms are present in the rhizosphere (Bossier et al. 1988). Figure 19.1 shows how plant roots can communicate with rhizobacteria and establish active rhizospheric interactions.

PGPR control the damage to plants from pathogens by a number of mechanisms including out-competing the pathogen by physical displacement, secretion of siderophores to prevent pathogens in the immediate vicinity from proliferating, synthesis of antibiotics and a variety of small molecules that inhibit pathogen growth, production of enzymes that inhibit the pathogen, and stimulation of the systemic resistance in the plants (van Loon et al. 1998). PGPR may also stimulate the production of biochemical compounds associated with host defense. Enhanced resistance may be due to massive accumulation of phytoalexins, phenolic

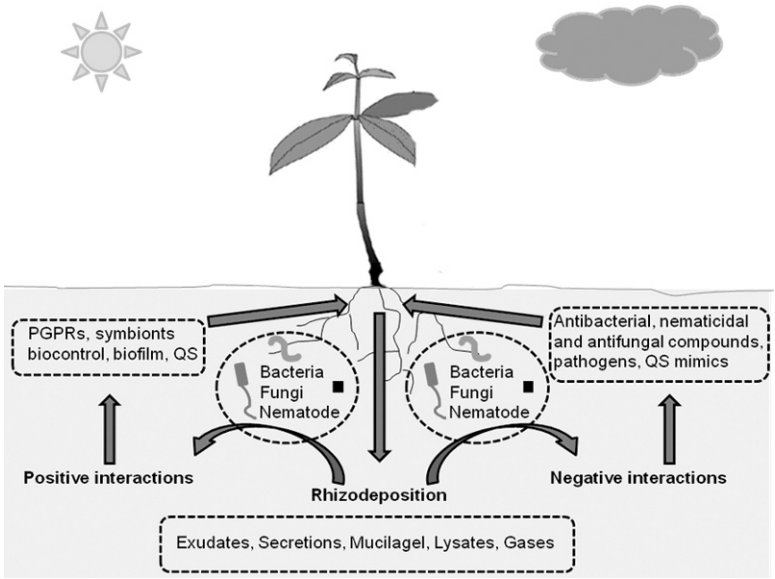

Fig. 19.1 Rhizodeposition and active rhizospherics interactions; *QS* quorum sensing

compounds, increases in the activities of proteins, defense enzymes and transcripts, and enhanced lignifications (van Loon et al. 1998). Biocontrol may also be improved by genetically engineered PGPR to over express one or more of these traits so that strains with several different anti-pathogen traits can act synergistically (Guo et al. 2004). Rhizobacteria-mediated induced systemic resistance (ISR) has been reported to be effective against fungi, bacteria, and viruses, but appears to involve different signaling pathways and mechanisms (van Loon and Bakker 2005). PGPR thus present an alternative to the use of chemicals for plant growth enhancement in many different regions. Extensive research has demonstrated that PGPR could have an important role in agriculture, ornamental plants, and horticulture in improving crop productivity (Larraburu et al. 2007). In addition, these organisms are also useful in forestry and environmental restoration, though research in these areas is minimal (Chanway 1997). PGPR have been shown to cause very real and positive effects when matched correctly to the right plant and the right environmental situation. What is needed for the future is a clear definition of what bacterial traits are useful and necessary for different environmental conditions and plants, so that suitable bacterial strains can either be selected or constructed. Also, it would be very useful to have a better understanding of how different bacterial strains work together for the synergistic promotion of plant growth, novel inoculants delivery systems, and environmental persistence of the PGPR in soil.

19.4 Mechanisms of Plant Growth Promotion by PGPR Affecting Legumes

Many rhizobacteria can affect plant growth and development of legume. More recently, however, attention has focused on the plant growth-promoting capacity of endophytes (Taghavi et al. 2009). A close relationship exists between bacterial strains living in the rhizosphere and those inside the plant (endophytes). Plant growth-promoting capacity has been related with enhancement of factors affecting mineral nutrition. The means by which PGPR enhance the nutrient status of host plants are different, e.g., biological N_2-fixation, increasing the availability of nutrients in the rhizosphere, inducing increases in root surface area, enhancing other beneficial symbioses of the host, and combination of different modes of action (Vessey 2003). It is interesting that even though so many PGPR have the ability to fix N_2, rarely is their mode of action for the stimulation of plant growth credited to BNF. PGPR that have the ability to fix N_2, but for which there is little evidence, or even counter evidence, that their stimulation of growth of a specific host plant is due to nitrogenase activity include *Azoarcus* sp. (Hurek et al. 1994), *Beijerinckia* sp. (Baldani et al. 1997), *Klebsiella pneumoniae* (Riggs et al. 2001), *Pantoea agglomerans* (Riggs et al. 2001), and *Rhizobium* sp. (Antoun et al. 1998). Mechanisms of plant growth promotion by rhizobacteria are briefly discussed in the following section.

19.4.1 PGPR and the Availability of Nutrients in the Rhizosphere

The method by which PGPR facilitate the growth of plants involves solubilization of unavailable forms of nutrients and/or siderophore production which help increase the transport of certain nutrients (notably ferric iron). Several reports have suggested that PGPR stimulate plant growth by facilitating the uptake of minerals N, phosphorus (P), and potassium (K) and microelements by the plant. However, there is some controversy regarding the mechanism (s) that PGPR employ in the uptake of minerals. Many investigators agree that rhizosphere organisms promote uptake of minerals by roots, but there is no generally accepted explanation for the process (Dobbelaere et al. 2003). On one hand, increased mineral uptake by plants has been suggested to be due to a general increase in the volume of the root system, as reflected by an increased root number, thickness, and length, and not to any specific enhancement of the normal ion uptake mechanism (Kapulnik et al. 1987; Biswas et al. 2000). Higher K and Fe uptakes for instance are related to thicker roots (Barber 1985) and higher P uptake is related to the presence of root hairs (Gahoonia and Nielsen 1998).

Phosphorus is second only to nitrogen in mineral nutrients most commonly limiting the growth of terrestrial plants and the process of formation of the N_2-fixing nodule (McDermott 1999). The solubilization of P in the rhizosphere is the most common mode of action implicated in PGPR that increases nutrient availability to host plants (Richardson 2001). Examples of studied associations include *Pseudomonas chlororaphis* and *P. putida* and soybean (Cattelan et al. 1999), *Rhizobium* sp. and *Bradyrhizobium japonicum* and radish (Antoun et al. 1998), and *Rhizobium leguminosarum* bv. *phaseoli* and maize (Chabot et al. 1998). At present, bacilli, rhizobia, and pseudomonads are the most studied phosphate-solubilizers (Peix et al. 2003; Zaidi et al. 2009). An alternative approach for the use of phosphate-solubilizing bacteria as microbial inoculants is the use of mixed cultures or co-inoculation with other microorganisms. Although solubilization of P compounds by microbes is very common under laboratory conditions, results in the field have been highly variable. The inoculation of P-solubilizing bacteria with arbuscular mycorrhizae (AM) and N_2-fixing bacteria has been more successful. Co-inoculation of *Azospirillum, Rhizobium*, and *Azotobacter* with P-solubilizing bacteria showed synergistic effect on plant growth and crop yields. Nevertheless, many rhizospheric, P-solubilizing bacterial species remain unknown and more studies are needed to reveal the high biodiversity of these bacteria. Although the study of rhizospheric bacteria is difficult, due to the high number of bacteria present in soil, characterization and identification of these bacteria are necessary for wide ecological studies of the plant rhizosphere.

Iron is an essential compound for most living organisms. However, despite its abundance on earth and the micromolar concentrations required for cell growth, it is biologically unavailable in most environments. Plants and microbes have evolved active strategies of uptake that are based on a range of chemical processes. Basically, these strategies rely on (1) acidification of soil solution mediated by

the excretion of protons or organic acids, (2) chelation of Fe (III) by ligands including siderophores with very high affinity for Fe^{3+}, and (3) reduction of Fe^{3+}–Fe^{2+} by reductases and reducing compounds. The efficacy of these active iron uptake strategies differs among organisms, leading to complex competitive and synergistic interactions among microbes, plants, and between plants and microbes. The chemical properties of the soil in which they occur have a strong effect on these interactions. In return, the iron uptake strategies impact the soil properties and the iron status. Thus, multiple interactions between soils, plants, and microorganisms are driving a complex iron cycle in the rhizosphere (Lemanceau et al. 2009).

There exists unquestionable potential for managing plant diseases incited by soil borne phytopathogens and increasing crop productivity with the application of certain root-associated microorganisms. Siderophores produced by several fluorescent *Pseudomonas* spp. play a role in the biological control of plant pathogens and in plant growth promotion through competition for iron. Since these plant growth-promoting rhizobacteria produce siderophores with higher Fe^{+3} affinities than the siderophores produced by deleterious rhizosphere microorganisms, the latter microorganisms are out-competed due to iron unavailability (Loper and Henkels 1999). Soils contain siderophores produced by bacteria and fungi; however, the role of siderophores in Fe nutrition of plants is uncertain. The amounts of Fe taken up and transported to shoots from chelates and siderophores are significant considering that 2 micromoles Fe g^{-1} are considered adequate for plant tissues (Epstein 1972). Reid et al. (1984) found that the concentration of siderophores in the rhizosphere may exceed that in the bulk soil by as much as 50-fold. These investigations suggest that roots may encounter concentrations of siderophores in the micromolar range in soils.

Root nodule bacteria produce a number of siderophores, only some of which have been structurally characterized. These include carboxylates such as rhizobactin from *Ensifer meliloti* (Smith et al. 1985); citrate from *B. japonicum* (Guerinot et al. 1990); and catechols from *R. leguminosarum* (Patel et al. 1988), among others. There is little evidence that iron deficient soils affect the numbers of root nodule bacteria. In iron stressed soils, the proportion of siderophore-producing strains appears to increase, though the total population of root nodule bacteria remains unchanged (Carson et al. 2000). Since production and utilization of siderophores could be affected by chemical, physical, and biological factors, the ecological relevance of siderophores depend upon the nature of soil and rhizosphere microenvironments (Buyer and Sikora 1990).

Chebotar et al. (2001) while studying the mechanism by which *P. fluorescens* 2137 co-inoculation with *B. japonicum* A1017 brought about an increase in the nodule number found that the addition of sterile spent medium of *P. fluorescens* 2137 increased the growth of *B. japonicum* A1017 in yeast mannitol broth (YMB). Since *Pseudomonas* sp. are known to be highly proficient at siderophore production, it could be that the active substance may be siderophores. In a similar study, Rajendran et al. (2008), isolated rizobacteria from the surface sterilized root nodules of pigeon pea (*Cajanus cajan*). The *Bacillus* strains NR4 and NR6 were able to produce siderophores which the rhizobial IC3123 was able to cross-utilize.

Under iron starved conditions, IC3123 showed enhanced growth in the presence of *Bacillus* isolates indicating that siderophore mediated interactions may be the underlying mechanism of beneficial effect of the non-rhizobial isolates on nodulation by IC3123.

19.4.2 Role of PGPR in Development of the Host Plant: Phytohormones

PGPR can also influence nutrient uptake, affect root morphology, and more specifically, increase root surface area. More importantly, increases in root length and root surface area are sometimes reported (Spaepen et al. 2009). Different PGPR produce phytohormones that are believed to stimulate plant growth. In most cases, these phytohormones change the assimilate partitioning patterns in plants and affect growth patterns of roots resulting in bigger and more branched roots and/or roots with greater surface area (Spaepen et al. 2009). Many bacteria are capable of producing more than one type of plant hormones (Karadeniz et al. 2006) or produce and degrade the same hormone (Leveau and Lindow 2005), or produce one and degrade the precursor of another (Patten and Glick 2002), or harbor the genes for more than one biosynthetic pathway. For example, *Pantoea agglomerans* pv gypsophilae, has an IAM (indole-3-acetamide) as well as an IPyA (indol 3-pyruvate) biosynthetic pathway for indol-3-acetic acid (IAA) (Manulis et al. 1998). This potential of even single bacterial strains to interfere differently with plant hormone levels remains one of the challenges towards better understanding, predicting, and possibly controlling plant hormone manipulation in complex plant-associated bacterial communities (Faure et al. 2009). Of these hormones, most of the attention has been focused on the role of the phytohormone auxin. The most common and best characterized and at the same time physiologically most active auxin in plants is IAA, which is known to stimulate both rapid (e.g., increases in cell elongation) and long-term (e.g., cell division and differentiation) responses in plants (Cleland 1990; Hagen 1990). The capacity to synthesize IAA is widespread among soil- and plant-associated bacteria. It has been estimated that 80% of bacteria isolated from the rhizosphere can produce IAA (Cheryl and Glick 1996). Several IAA biosynthetic pathways, classified according to their intermediates, have been reported in bacteria and IAA biosynthesis was studied extensively (Spaepen et al. 2007). Tryptophan has been identified as a main precursor for IAA biosynthesis pathways in bacteria. The identification of intermediates led to the identification of five different pathways using tryptophan as a precursor for IAA (Spaepen et al. 2007). In *Bacillus amyloliquefaciens* FZB42, Idris et al. (2007) demonstrated that biosynthesis of IAA affects its ability to promote plant growth. Moreover, this ability is dependent on the presence of tryptophan, which is one of the main compounds present in several plant exudates (Kamilova et al. 2006). The ability to colonize plant roots may depend to some degree on the ability of the bacterium to synthesize IAA. It has been

proposed that bacterial IAA synthesis contributes to enhanced rhizosphere competence by (1) detoxification of tryptophan analogues present on host plant surfaces (Lebuhn et al. 1997) and (2) stimulation of the release of plant exudates (Lambrecht et al. 2000), the downregulation of plant defense (Yamada 1993), or the inhibition of the hypersensitive response of infected plants (Robinette and Matthysse 1990).

Two other plant hormones synthesized by PGPR stimulating plant growth include cytokines, which promote cell divisions, cell enlargement, and tissue expansion in certain plant parts (de Salomone et al. 2001) and gibberellic acid (GA), which cause extension of plant tissues, particularly stem tissue (Santner et al. 2009). Cytokinins are a diverse group of labile compounds that are usually present in small amounts in biological samples and have often been difficult to identify and quantify. Plants and plant-associated microorganisms have been found to contain over 30 growth-promoting compounds of the cytokinin group. One study indicated that as many as 90% of the microorganisms found in the rhizosphere are capable of releasing cytokinins when cultured in vitro (Barea et al. 1976). As cytokinins move from roots to shoots, root exposure to cytokinin could affect plant growth and development. Increases in yield and N, P, and K content of grains obtained after exogenous application of cytokinins in field trials with rice (Zahir et al. 2001) support the hypothesis that cytokinins bacterially supplied to the soil can improve the growth and yield of treated plants. The GA is a complex of molecules of tetracarbocyclic diterpernes and about 100 GAs have been exclusively isolated from plants. The numbering used with GAs is not related to their structure. Those molecules, whose structure has been elucidated, are numbered in the approximate order of their discovery. The most important GA in plants is GA_1, which is primarily responsible for stem elongation. In their early work, Tien et al. (1979) detected gibberellin-like substances in supernatants from *Azospirillum brasilense* cultures at an estimated concentration of 0.05 μg/ml GA_3 equivalent. GA_1 and GA_3 were also identified in cultures of the *A. lipoferum* op33 strain and a quantitative estimation, using the dwarf rice cv. Tan-ginbozu microdrop bioassay, showed that 20–40 pg/ml were produced (Bottini et al. 1989). All data support the concept that the growth promotion in plants induced by *Azospirillum* infection may occur by a combination of both gibberellin production and gibberellin glucoside or glucosyl ester de-conjugation by the bacterium (Piccoli et al. 1997).

There are now considerable experimental evidences that the physiological effects induced by salinity might be modulated by abscisic acid (ABA). Results suggest that ABA application improves the growth and nitrogen fixation parameters of the common bean under saline conditions (Khadri et al. 2007). In addition, changes in enzymes activities of ammonium assimilation and purines catabolism as well as increases of the endogenous ABA content occurred with these treatments, mainly with the NaCl (Khadri et al. 2006). Furthermore, a number of PGPR synthesize the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Shah et al. 1998) which cleaves the plant ethylene precursor ACC, and thereby lower the level of ethylene in a developing or stressed plant. In addition, plants that are treated with ACC deaminase-containing PGPR are dramatically more resistant to the deleterious effects of stress ethylene that is synthesized as a consequence of

stressful conditions such as the presence of phytopathogens (Wang et al. 2000), and drought and high salt (Mayak et al. 2004). In each of these cases, the ACC deaminase-containing PGPR markedly lowered the level of ACC in the stressed plants thereby limiting the amount of stress ethylene synthesis and hence the damage to the plant. These bacteria are beneficial to plant growth as in the natural environment plants are often subjected to ethylene producing stresses. However, it should be emphasized that ACC deaminase-containing PGPR facilitate plant growth to a much greater extent with plants that are ethylene sensitive such as canola, peppers, and tomatoes. It is expected that this activity will be useful in both agricultural and horticultural settings, as well as in environmental clean-up (i.e., phytoremediation) protocols (Weyens et al. 2009). For example, Ma et al. (2003) postulated that *R. leguminosarum* bv. viciae 128 can finely adjust the ethylene production in pea roots, which allows progression of the infection threads inside the cortex and the formation of functional nodules.

19.4.3 PGPR and Stimulation of Legume–Rhizobia Symbioses

Plants are able to establish endosymbiotic interactions with several nitrogen-fixing bacteria. As these microorganisms may be autotrophic or heterotrophic, different strategies for garnering energy (particularly carbohydrates) have evolved in symbiotic rhizospheric-associations with plants as well as in free-living nitrogen-fixing organisms. On average, symbiotic systems have the highest fixation capability not only because energy, in the form of carbohydrates, is provided by the plant, but also other conditions (e.g., export of reduced nitrogen) are optimized for efficient N_2 fixation (Cocking 2003). PGPR have been used in combination with rhizobia and co-inoculation of some *Pseudomonas* and *Bacillus* strains along with effective *Rhizobium* spp. is shown to stimulate chickpea growth, nodulation, and N_2 fixation (Parmar and Dadarwal 1999; Zaidi et al. 2003). Some *Serratia* strains, such as *S. proteamaculans* 1-102 and *S. liquefaciens* 2-68, have beneficial effects on legume plant growth (Chanway et al. 1989; Zhang et al. 1996). The modes of action for PGPR stimulation of legume–rhizobia symbioses implicate different processes such as phytohormone-induced (usually IAA) stimulations of root growth (Srinivasan et al. 1996; Dobbelaere et al. 2003). In this way, the stimulation of nodulation is most commonly an indirect effect; the PGPR stimulate root growth, which provides more sites for infection and nodulation (Vessey 2003). However, Cattelan et al. (1999) found that six of eight isolates positive for ACC (*Bacillus*, *Pseudomonas,* and unknown isolates) increased at least one aspect of early soybean growth, but these rhizobacteria did not affect nodulation positively.

Bacteria of the genus *Azospirillum* are capable of increasing the yield of important crops growing in various soils and climatic regions. The data from field inoculation experiments show statistically significant increases in yield (wheat, sorghum, maize, forage grasses and grains, and forage legumes) in the order of 5–30% in 60–75% of the published reports (Okon and Labandera-Gonzalez 1994;

Castro-Sowinski et al. 2007). Effects of *Azospirillum* inoculation are mainly attributed to improved root development and enhanced water and mineral uptake. Secretion of plant growth promoting substances, mainly IAA, is responsible for this effect (Dobbelaere and Okon 2007; Spaepen et al. 2007). It has been observed that in legumes, such as common bean and peanut, *Azospirillum* promotes root development and mineral uptake but at the same time enhances secretion of flavonoid compounds by the plant, which induce the expression of nodulation (*nod*) genes in *Rhizobium*, resulting in early and faster nodulation, earlier onset of N_2 fixation, and higher crop yield (Burdman et al. 1998; Dardanelli et al. 2008a). Dual inoculation with *Rhizobium* and *Azospirillum* and other PGPR was shown to significantly increase both upper (i.e., those nodules formed in the upper 5 cm of the root system) and total nodule number of several legumes. Co-inoculation of alfalfa, burr medic, vetch, garden and chickpea, white clover, common bean, winged bean, and soybean with *A. brasilense* or *A. lipoferum* (Sarig et al. 1986; Yahalom et al. 1987; Itzigsohn et al. 1993; Burdman et al. 1997), soybean, cowpea, and clover with *Azotobacter vinelandii* (Burns et al. 1981), and common bean with *B. polymyxa* (Petersen et al. 1996), resulted in earlier nodulation and increase in total nodule number as compared to plants inoculated with their respective rhizobial symbiont alone. Furthermore, inoculation with compatible rhizobia influences plant root exudation. Thus, when soybeans were inoculated with *B. japonicum* USDA110, the root exudates contained higher concentrations of daidzein, genistein, and coumestrol in comparison with non-inoculated plants (Cho and Harper 1991). A qualitative change in signal molecules has also been observed in soybean roots when inoculated with PGPR. For example, *Chryseobacterium balustinum* Aur9 changed qualitatively the pattern of flavonoids when compared to control conditions. Thus, in the presence of *C. balustinum* Aur9, soybean roots did not exude quercetin and naringenin (Dardanelli et al. 2010) suggesting that microbial attenuation or alteration of flavonoid may be an important aspect of rhizosphere ecology leading to the establishment of symbiosis (Shaw et al. 2006).

Non-symbiotic nitrogen-fixing bacteria such as *A. chroococcum* and *A. brasilense* and other PGPR like, *P. fluorescens*, *P. putida*, and *Bacillus cereus* when grown together with *Rhizobium* did not antagonize the introduced *Rhizobium* strain but the dual inoculation with either *P. putida*, *P. fluorescens*, or *B. cereus* resulted in a significant increase in plant growth, nodulation, and enzyme activity of pigeon pea over *Rhizobium*-inoculated and uninoculated control plants. The nodule occupancy of the introduced *Rhizobium* strain increased from 50% (with *Rhizobium* alone) to 85% in the presence of *P. putida*. This study suggested that the combination of efficient *Rhizobium* strain and PGPR could serve as an ideal microbial pairing for raising the productivity of pigeonpea in the semiarid tropical regions (Tilak et al. 2006). *Paenibacillus polymyxa* (formerly known as *Bacillus polymyxa*), among rhizobacteria, has however attracted considerable attention because of its great potential in different industrial processes and in sustainable agriculture. Owing to its broad host range, its ability to form endospores, and ability to produce different kinds of antibiotics, *P. polymyxa* is a potential commercially useful biocontrol agent (Lal and Tabachioni 2009). *P. polymyxa* inhabit different niches

such as soils, roots, rhizosphere of various crop plants, forest trees, and marine sediments (Lal and Tabacchioni 2009). The diversity of *Paenibacillus polymyxa* populations associated with the rhizosphere of durum wheat was investigated by Guemouri-Athmani et al (2000). These authors measured nitrogenase activity of some representative isolates of *P. polymyxa* recovered from Algerian soil by acetylene reduction assay (ARA). Results showed that only 14 of the 23 strains tested were able to reduce acetylene. However, it has not been demonstrated that plant growth promotion by *P. polymyxa* is primarily correlated with its nitrogen-fixing ability (Lindberg et al. 1985; Lal and Tabacchioni 2009).

19.5 Relief of Stress and Plant Growth Promotion by PGPR

Agricultural productivity in large terrestrial areas of the world is severely affected by abiotic and biotic stress. In this regard, the damaging effects of salt accumulation in agricultural soils have negatively influenced ancient and modern civilizations (Rengasamy 2006). Typical environmental stresses faced by the legume nodules and their symbiotic partner (rhizobia) may include photosynthate deprivation, osmotic stress, salinity, soil nitrate, temperature, heavy metals, and biocides (Walsh 1995). Like other cultivated crops, the salinity response of legumes varies greatly and depends on factors such as, climatic conditions, soil properties, and stage of plant growth. Among the various stressors, salt stress for example reduces the nodulation of legumes by inhibiting the very early symbiotic events, whereas osmotic stress induces significant changes in water relations, growth, and symbiotic N_2 fixation in stressed plants (Serraj et al. 1999; Dardanelli et al. 2009a). Therefore, under such stressed conditions, a competitive and persistent rhizobial strain is not expected to express its full N_2 fixation ability and consequently the vigour of the host legume is likely to be lost. Water deficiency is another limiting factor in plant productivity and symbiotic nitrogen fixation in many arid regions of the world. The modification of rhizobial cells by water stress has been found to eventually lead to a reduction in infection and nodulation of legumes (Zahran and Sprent 1986). In addition to its depressive effect on nodule initiation, water deficit also results in restriction of nodule development and function (Serraj et al. 1999). The wide range of moisture levels characteristic of ecosystems where legumes have been shown to fix nitrogen suggests that rhizobial strains with different sensitivity to soil moisture can be selected. To overcome drought effect, it has been reported that nodulation and nitrogen fixation in alfalfa can be improved by inoculating plants with competitive and drought tolerant rhizobia (Zahran 1999).

Although many strategies have been adopted to improve the stress tolerance, fewer reports have been published on PGPR as helper to tolerance to abiotic stress such as salt, drought, and heavy metal, among others. When biological activity assays of *Ensifer fredii* SMH12 and *Rhizobium tropici* CIAT899 were carried out on soybean and common bean plants, respectively, salt stress reduced the nodulation rate, growth, and nodule development of both plants. Nevertheless, no

significant differences were observed in the biological activity assays of the Nod factors produced under control conditions versus those produced under saline conditions, for both selected partners (Estevez et al. 2009). In another experiment, the presence of *C. balustinum* Aur9 did not interfere with *R. tropici* CIAT899 root infection and nodule initiation under either control or saline conditions. Likewise, co-inoculation partially overcame the negative effects of salinity on the number and size of nodules and the delay of nodule appearance. Thus, pre-inoculation with strain Aur9 clearly increased the number of nodules by *R. tropici* CIAT899 under saline stress (Estevez et al. 2009). This increase may be related to the changed pattern of root flavonoids in co-inoculated plants and/or to the PGPR production of IAA promoting root hair development and hence provides possible sites for rhizobial entry (Spaepen et al. 2007; Dardanelli et al. 2008b). The co-inoculation with rhizobia and *C. balustinum* Aur9 turned out to be much more effective in improving plant growth than inoculation with the rhizobia alone, especially in soybean plants co-inoculated only with *E. fredii* SMH12 and *C. balustinum* Aur9, as Lucas García et al. (2004) already reported. In this legume, co-inoculation of *B. japonicum* and *S. proteamaculans* 2–68 or 1–102 in the field, increased soybean grain yield by 23 and 29%, respectively, and protein yield by 60 and 50%, respectively (Dashti et al. 1998).

Recent efforts to apply these results to greenhouse and field situations include using mixtures of PGPR strains with symbiotic nitrogen-fixing rhizobia (Figueiredo et al. 2008). The rhizobia are sensitive to drought stress, resulting in a significant decrease of N_2 fixation when faced with low soil-water content. Under drought stress, co-inoculation of bean with *R. tropici* and two strains of *P. polymyxa* resulted in increased plant height, shoot dry weight, and nodule number (Figueiredo et al. 2008). The potential use of rhizobia as growth-promoting bacteria for the remediation of heavy metal contaminated sites is another exciting new area of research. Legumes and rhizobia are often desirable species during, and after, the remediation of heavy metal contaminated land, where legumes have been identified as naturally occurring pioneer species (Carrasco et al. 2005). Recently, non-rhizobial bacteria from genera such as, *Pseudomonas*, *Bacillus*, and *Flavobacterium* have shown promise for their growth-promoting impacts on plants used in the remediation of heavy metal contaminated sites (Weyens et al. 2009). However, an important question that needs to be addressed is how plants in the field can be inoculated more efficiently. To solve this problem, several options such as inoculation of seeds or cuttings and inoculation by spraying techniques in soil or directly onto growing plants have been suggested (Weyens et al. 2009).

19.6 Conclusion

Biological nitrogen fixation represents, annually, up to 100 million tons of N for terrestrial ecosystems, and from 30 to 300 million tons for marine ecosystems.

In addition, 20 million tons result from chemical fixation as a result of atmospheric phenomena (Mosier 2002). Legumes (e.g., faba bean, lupin, soybean, and groundnut) are often considered to be the major nitrogen-fixing systems, as they derive up to 90% of their nitrogen from N_2 fixation (Franche et al. 2009). The cooperative interactions between rhizobia and other plant root colonizing bacteria are of relevance in improvement of nodulation and N_2 fixation in legume plants. The role of rhizosphere processes involving co-inoculations, in particular under stressed environment, could be more effective as such conditions cause extensive losses to agricultural production worldwide. A better understanding of PGPR–rhizobia interaction and their concurrent inoculation is therefore necessary in order to derive full benefits of such associations in legume improvement in a more sustainable and ecologically sound manner.

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