Mohammad Saghir Khan · Almas Zaidi
Javed Musarrat *Editors*

Microbes for Legume Improvement

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Preface

The farmer folks around the world are facing acute problems in providing plants with required nutrients due to inadequate supply of raw materials, poor storage quality, indiscriminate uses and unaffordable hike in the costs of synthetic chemical fertilizers. Beside these factors, the fertility of soil, largely dependent on metabolic activities of microbes, is deteriorating very fast, which further aggravate the agronomic problems. Considering such alarming situations, there is an urgent need to find an alternative so that the chemical based high input modern agricultural practices could be shifted to an economically viable, ecologically sound, and sustainable production system. In this regard, heterogeneously distributed microbial communities play a vital role as organic fertilizers in facilitating uptake of nutrients by crops. Hence, provides a viable and inexpensive alternative to offset dependence on chemical fertilizers applied in modern agriculture on large scale by majority of the progressive farmers around the world for raising the productivity of crops including legumes. Legumes that play an important role in the traditional diets of many regions throughout the world can provide a multitude of benefits to both the soil and other crops grown in combination with them or following them in a rotation. The ability of legumes to fix atmospheric nitrogen is perhaps the most bodacious countenance that distinguishes them from other plants. In addition, legumes can provide a wide range of important soil quality benefits, like, it increases soil organic matter, improve soil structure and porosity, recycle nutrients, decrease soil pH, diversify the rhizosphere microbes and break disease build-up. Application of microbial inoculants very commonly used for legumes, as a component of organic cultivation is therefore, an exciting area for enhancing legume production and has also been suggested as an alternative control measure for mitigation of environmental pollution. Plethora of experiments have been conducted to better understand the impact of naturally abundant microbes in the improvement of legumes but a meagre efforts are made to compile them.

Microbes for legume improvement written by experts in the field provide unique, updated and comprehensive information on how microbial communities could be exploited and practiced for increasing the productivity of legumes in varied production systems in different geographical regions of the world. The book presents the recent developments in the rhizobial taxonomy and discusses

the symbiotic features of rhizobia scrutinizing the frontier of legumes and bacteria promiscuity. Various factors including the exchange of plant and bacterial signaling molecules, such as, flavonoids and nodulation factor (Nod factor), in the early stages of symbiosis that influence symbiotic rhizobial interactions under competitive soil environment, is highlighted. The information relative to proteomic control of legume-rhizobium interaction is explored in a chapter on the role of proteomics in legume-rhizobium symbiosis. The role of ethylene and bacterial ACC-deaminase in legume-rhizobium interaction are also broadly covered in this book. The current developments in the field of soil bacterial biofilms, bacterial functions influencing biofilm formation, effects of exopolysaccharides, quorum sensing, rhizobial proteins, and motility on bacterial biofilms and development, and application of biofilmed biofertilizers in legume improvement are discussed separately. The book further describes as to how the plant growth promoting rhizobacteria either alone or in association with nitrogen fixing bacteria facilitate the growth and nutrient uptake by legumes and how such microbial strategies could be exploited for better productivity of legumes in different agro-ecological regions, are elucidated in greater detail. The interactions/relationships of rhizosphere bacteria with their hosts and performance of wild-type and genetically manipulated beneficial bacterial populations are discussed for their efficient utilization in legume production under sustainable agriculture systems. Phosphorus and its effect on the environment have become hotly contested issues. This book provides a broad and updated view of the strategic and applied research conducted so far to understand as to how phosphate solubilizing bacteria either alone or in synergisms with other symbionts could help to manage phosphorus problems in phosphorus deficient soils, and ultimately enhance nutrient availability, and concomitantly improve the yields of legumes. The mycorrhizosphere interactions for legume improvement, mycorhhizal dependency of legumes, the mechanisms as to how mycorhiza promotes legume growth and consequential impact of mycorrhiza either alone or in combination with other beneficial microbes on legume improvement in conventional or desertified and/or degraded habitats is described. Given the importance of legumes in animal and human consumption and their role in maintaining soil fertility, attention is paid to understand how rhizobia develops resistance to various stressor molecules and how such tolerant naturally gifted microbes could become handy in sustaining the productivity of legumes in the stressed soils. The role of asymbiotic *Azosprillum* either alone or as mixture with other plant growth promoting rhizobacteria (PGPR) in increasing the productivity of legumes are highlighted. The current status of symbiotic nitrogen fixation (SNF) in tropical food grain legumes and strategies adopted for the management of pathogens affecting severely the productivity of legumes, employing plant growth promoting rhizobacteria are well explained. This book collectively provides some novel microbial strategies and proposes alternative solution, which if properly applied could help to boost the overall performance of legumes while reducing the dependence on synthetic agrochemicals. The knowledge and methodologies described in this book offer invaluable research tools, which may serve as an important and updated source material. This edition provides an authoritative overview for individuals interested in legume

research. This book will therefore, be of great interest to the research scientists, postgraduate students, bioscience professionals, decision makers, and farmers who intend to use the naturally gifted wonderful microbes for the improvement of legumes. It would also serve as a valuable resource for agronomists, soil microbiologists, soil scientists, biologists, and biotechnologists involved in nutrient management and legume research.

We are deeply indebted to our well qualified and internationally renowned colleague authors from different countries for providing the important, authoritative and cutting-edge scientific information to make this book a reality. All chapters are well illustrated with appropriately placed tables and figures, and enriched with extensive and most recent references. The help and support provided by research scholars working with us in designing and preparing some of the drawings presented in this book are greatly acknowledged. We are indeed very grateful to our family members for their untiring and sustained support, who, in their own ways inspired us and, subconsciously contributed a tremendous amount to the outcome of this book. We appreciate the great efforts of book publishing team at Springer-Verlag, Austria, in responding to all our queries very promptly and without delay during our ongoing academic/scientific relationship. Finally, this book may have some basic mistakes or printing errors happening accidentally during preparation, for which we regret in anticipation. If pointed out at any stage, we will certainly try to correct and improve them in subsequent print/edition. Suggestions and critical analysis of the contents presented in this book by the readers are most welcome.

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Editors

Mohammad Saghir Khan, PhD, is an Associate Professor at the Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh, India. Dr. Khan received his MSc from Aligarh Muslim University, Aligarh, India and his PhD (Microbiology) from Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, India. He has been teaching Microbiology to post-graduate students for the last 13 years and has research experience of 17 years. In addition to his teaching, Dr. Khan is engaged in guiding students for their doctoral degree in Microbiology. He has published over 60 scientific papers including original research articles, review articles, and book chapters in various national and international publication media. Dr. Khan has also edited three books published by the leading publishers. His interest in the various aspects and applications of Microbiology coupled with his classroom and laboratory experience makes him uniquely qualified to author this book. He is deeply involved in research activities focusing mainly on rhizobiology, microbiology, environmental microbiology especially heavy metals–microbes–legume interaction, bioremediation, pesticide–PGPR–plant interaction, biofertilizers, and rhizo-immunology.

Almas Zaidi received her MSc and PhD (Agricultural Microbiology) from Aligarh Muslim University, Aligarh, India and currently serving as Guest faculty/Assistant Professor at the Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh, India. She has been teaching Microbiology at post graduate level for the last 5 years and has research experience of 13 years. She has published about 40 research papers and reviews in journals of national and international repute. She has also contributed chapters to different books. Dr. Zaidi has edited three books published by the leading publishers. Her main focus of research is to address problems related with rhizo-microbiology, microbiology, environmental microbiology, and biofertilizer technology.

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Chapter 1
Bacteria Involved in Nitrogen-Fixing Legume Symbiosis: Current Taxonomic Perspective

Encarna Velázquez, Paula García-Fraile, Martha-Helena Ramírez-Bahena, Raúl Rivas, and Eustoquio Martínez-Molina

Abstract Bacteria forming nitrogen-fixing symbiosis with legumes were classically named "rhizobia" and currently include more than 50 species distributed in genera *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Azorhizobium*, and *Bradyrhizobium*. These species carry symbiotic genes codifying for nodulation and nitrogen fixation that are located on plasmids or symbiotic islands determining the host range and the ability to fix nitrogen in the nodules. These genes can be transferred from rhizobia to other alpha or beta-Proteobacteria conferring them the ability to nodulate legumes and fix atmospheric nitrogen. In this chapter, an overview of the main different groups of bacteria known up to date to be able of forming symbiosis with legumes is made. The symbiotic features of such bacteria scrutinizing the frontier of legume and bacteria promiscuity are also discussed.

1.1 The "Classical" Species of Rhizobia

Microorganisms able to establish nitrogen-fixing symbiosis with legumes were discovered in the nineteenth century. This symbiosis results in the formation of nodules (on roots or stems) in legumes as the main feature. The nodule is the plant organ where the bacteria, once transformed in bacteroids, carry out the nitrogen fixation process. In 1888, Beijerink obtained the first pure bacterial culture named by him *Bacillus radicicola* from nodule suspension. This isolate able to nodulate *Pisum* and *Vicia* was later renamed as *Rhizobium leguminosarum* (Frank 1889).

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From here onward, the name rhizobia was applied to all bacteria capable of forming nodules on leguminous plants.

The description of the first rhizobial species was mainly based on the legume, which acted as host. This fact led to the definition of the cross-nodulation groups, which was based on the nodulation specificity after infectivity experiments in several legumes (Baldwin and Fred 1929). The description of these species was recorded in Bergey's Manuals that played a fundamental role in rhizobial taxonomy until 1980. In the same year, rhizobial species were officially validated in the International Journal of Systematic Bacteriology (IJSB) and included in the list by Skerman et al. (1980). In the 1974 edition of Bergey's Manual of Determinative Bacteriology, all bacteria able to nodulate legumes were included in a single genus, *Rhizobium* (Frank 1889) within the family *Rhizobiaceae* (Conn 1938). This genus had four fast-growing species: *R. leguminosarum* (Frank 1889), *R. phaseoli, R. trifolii*, and *R. meliloti* (Dangeard 1926) and two slow-growing species: *R. japonicum* (Buchanan 1926) and *R. lupini* (Eckhardt et al. 1931). Only a low number of phenotypic characteristics of these species were recorded in this Manual and the authors indicated that the plant infection data were essential for species classification (Jordan and Allen 1974). In this way, *R. leguminosarum* was considered as endosymbiont for *Vicia*, *Pisum,* and *Lens*; *R. phaseoli* for *Phaseolus*; *R. trifolii* for *Trifolium*; and *R. meliloti* for *Medicago* (Jordan and Allen 1974). The slow-growing species, *R. lupini* (Eckhardt et al. 1931) was found to nodulate *Lupinus* and *R. japonicum* (Buchanan 1926) mainly *Glycine* (Jordan and Allen 1974). This low number of species contrasts with those of genus *Pseudomonas* for which 29 were recorded in the same edition of Bergey's Manual from 1974. It seems evident that the diversity of rhizobia was underestimated probably due to the low number of legumes and soils analyzed and the little attention that rhizobiologists paid to the phenotypic characteristics in the analysis of this group of bacteria.

Since the description of the first species of rhizobia, it has taken nearly a century before there were significant changes within the family *Rhizobiaceae*: *R. japonicum* was reclassified into a new genus, *Bradyrhizobium*. This genus includes the slow-growing rhizobial species (Jordan 1982) that was recorded in the first edition of Bergey's Manual of Systematic Bacteriology (1984). However, although a new species of *Rhizobium, R. loti* (Jarvis et al. 1982) was added to genus *Rhizobium* and the species *R. meliloti* was maintained, the number of rhizobial species was still low due to the reclassification of *R. phaseoli* and *R. trifolii* into *R. leguminosarum* and the elimination of the species *R. lupini* (Jordan 1984). Nevertheless, since the taxonomic changes recorded in Bergey's Manuals are not official, *R. phaseoli* and *R. trifolii* remained as valid species until 2008, when we revised their taxonomic status showing that only *R. leguminosarum* and *R. phaseoli* are valid species being *R. trifolii* a later subjective synonym of *R. leguminosarum* (Ramírez-Bahena et al. 2008). The species *R. lupini* remains still valid.

Considering the taxonomic status of rhizobial species after a century of evolution, we can conclude that it was hampered by the use of symbiotic criteria for species definition, though in the 1980s several other phenotypic and molecular criteria were applied in bacterial taxonomy. At that time, it was already well known

that symbiosis determinants are codified on plasmids in the fast-growing rhizobia (Zurkowski and Lorkiewicz 1979) and their autoconjugative nature made them an inadequate tool for rhizobial classification and identification in spite of their relevance for diversity analysis. Despite this, the symbiotic characteristics were not removed from rhizobial taxonomy for decades and hence, these criteria are still considered as the minimal standards for species description in rhizobia. From our point of view, these criteria are very useful but not for species definition. Since nodulation genes have different phylogenetic origin than core genes, the phylogenetic analysis of these mobile elements is interesting in plant–rhizobial interactions rather than for bacterial taxonomy purposes.

In spite of the taxonomic deficiencies existent in the group of rhizobia, its taxonomic scheme began to change in 1980s. After the publication of the lists by Skerman and collaborators in 1980, most rhizobial species were officially described in IJSB. Only the species *R. loti* (Jarvis et al. 1982) and the genus *Bradyrhizobium* (Jordan 1982) were officially described in this journal before 1980. From this date, only the species *Bradyrhizobium elkanii,* a slow-growing species nodulating soybean, was described outside IJSB (Kuykendall et al. 1992).

In 1984, it was discovered that soybean may also be nodulated by fast-growing rhizobia, and a new species named *R. fredii* was officially recorded (Scholla and Elkan 1984). This not only revealed the nodulation of the same legume by very different species but also introduced the numerical taxonomy in rhizobia, increasing the number of characteristics used for species definition. This was essential for the reclassification of *R. fredii* into a new genus, *Sinorhizobium* (Chen et al. 1988). Since then, the number of phenotypic characteristics studied increased in the description of new taxa such as *R. galegae* isolated from *Galega* nodules (Lindström 1989), *R. huakuii* isolated from *Astragalus* nodules (Chen et al. 1991), and the genus *Azorhizobium* whose type species *A. caulinodans* forms stem nodules on *Sesbania rostrata* (Dreyfus et al. 1988). This study led to the existence of a new way to look at rhizobial infection, since some of them can nodulate not only roots but also stems of legumes.

Although while describing genus *Azorhizobium*, some molecular techniques, such as the rRNA–DNA hybridization, were performed (which was a cutting edge in this bacterial group from 1984 to 1991); the phenotypic and symbiotic characteristics were the main indicators used to determine rhizobial diversity. Thereafter, Woese et al. (1984) using 16S rRNA gene sequences for the classification of bacteria, placed rhizobia within the alpha subdivision of Proteobacteria. From 1991 onward, the minimal standards for validating new species of rhizobia and *Agrobacterium* included the sequencing of the 16S rRNA gene as well the DNA–DNA or rRNA–DNA hybridization RFLP and MLEE analysis and the description of symbiotic characteristics (Graham et al. 1991). Concurrently, description of *R. tropici* was the first report of a rhizobial species based on the 16S rRNA gene sequences (Martínez-Romero et al. 1991). From this date, the 16S rRNA gene sequences were included in all descriptions or reclassifications of the different taxa within family *Rhizobiaceae* and the reclassification of *R. fredii* into genus *Sinorhizobium* was confirmed by the analysis of partial 16S rRNA gene

sequences (Jarvis et al. 1992). In 1993, two different scientific teams published a phylogenetic analysis of family *Rhizobiaceae* based on this gene (Willems and Collins 1993; Yanagi and Yamasato 1993). Although no taxonomic decisions were taken from these results, the closeness of the species of genus *Rhizobium* described until 1993, the phylogenetic relationships of *Rhizobium* with *Agrobacterium*, and the higher phylogenetic divergence of *Bradyrhizobium, Azorhizobium*, and *Phyllobacterium* were confirmed. Also, the existence of two phylogenetic groups within *Rhizobium* was evidenced and in 1997, some species of which were transferred to genus *Mesorhizobium* that includes the rhizobial species showing an intermediate growth rate between *Rhizobium* and *Bradyrhizobium* (Jarvis et al. 1997). The species transferred were *R. loti*, *R. huakuii*, *R. ciceri*, *R. mediterraneum*, and *R. tianshanense*.

In 1998, the analysis of LMW RNA profiles of members of family *Rhizobiaceae* supported the separation of the genus *Mesorhizobium* from *Rhizobium* and *Bradyrhizobium* (Velázquez et al. 1998). These molecules were proposed as genetic markers for rhizobial genera and species differentiation and consequently to find out rhizobial diversity (Jarabo-Lorenzo et al. 2003; del Villar et al. 2008). In the same year (1998), a new bacterium *Allorhizobium undicola* was proposed to include the strains isolated in Senegal from nodules of *Neptunia natans* (de Lajudie et al. 1998).

The most important changes in rhizobial taxonomy took place during 2000 and ahead with a number of species nodulating legumes exponentially increasing, and significant changes in the arrangement of these species in different suprageneric taxa were found. In this way, the reclassification of *Agrobacterium* and *Allorhizobium* into genus *Rhizobium* was proposed by Young et al. (2001) in the official journal of bacterial systematic currently named International Journal of Systematic and Evolutionary Microbiology (IJSEM). After this reclassification, *Rhizobium* is the largest genus of family *Rhizobiaceae*, but many researchers did not accept it and the name *Agrobacterium* is still commonly used by phytopathologists. In our opinion, the reclassification was only justified in the case of the species *Agrobacterium rhizogenes* closely related to the species *R. tropici* on the basis of their 16S rRNA gene (Sawada et al. 1993; Willems and Collins 1993; Yanagi and Yamasato 1993; Young et al. 2001; Velázquez et al. 2005). Nevertheless, as the proposal of Young and collaborators was published in IJSEM, it is currently valid and only if the genera *Agrobacterium* and *Allorhizobium* were recorded in the same journal, the taxonomic status of these genera could be change again.

A nomenclatural change affected to the genus *Sinorhizobium* which has been renamed *Ensifer*. After several requests for an opinion sent to IJSEM (Willems et al. 2003a; Young 2003), the Judicial Commission of the International Committee on Systematic of Prokaryotes (2008) decided that *Ensifer* is the correct name for the genus (Casida 1982), since it was validly described before *Sinorhizobium,* being closely related to this genus on the basis of the 16S rRNA gene.

These changes clearly showed that after 1990, the 16S rRNA gene was the most important diversity marker in rhizobia leading to the distribution of the genera included in the family *Rhizobiaceae* into several families and the creation of a new

order named "Rhizobiales" proposed in Bergey's Manual (Kuykendall et al. 2005). Nevertheless, this name should be changed since it is illegitimate due to the existence of the previously validated order name *Hyphomicrobiales* that include the family *Hyphomicrobiaceae* to which the genus *Azorhizobium* belongs. This order contains symbiotic pathogenic and saprophytic bacteria distributed in several families, including family *Rhizobiaceae*. This family officially include the genera *Rhizobium* and *Ensifer* although in Bergey's Manual also the genera *Agrobacterium* and *Allorhizobium* were still included and the genus *Sinorhizobium* was considered different from *Ensifer*. A new family named *Phyllobacteriaceae* was proposed in Bergey's Manual (Mergaert and Swings 2005) and later validated (Validation list No. 107 2006) that contains the genera *Phyllobacterium* and *Mesorhizobium* together with several nonsymbiotic genera. Genus *Bradyrhizobium* was included in a new family named "Bradyrhizobiaceae" (Garrity et al. 2005), which is also illegitimate, since *Nitrobacteraceae*, a previously described family, includes the genus *Nitrobacter* closely related to *Bradyrhizobium.*

This new arrangement of rhizobia was based on the analysis of a single gene that despite their importance in bacterial classification has limitations in differentiating closely related rhizobial species (Rivas et al. 2004; Valverde et al. 2006; Ramírez-Bahena et al. 2008). In the last decade, several housekeeping genes were identified in different groups of bacteria for species definition (Maiden 2006). In rhizobia, the two firstly analyzed genes were *recA* and *atpD* (Gaunt et al. 2001) and currently they have been sequenced in many rhizobial species showing their usefulness in differentiation of species whose 16S rRNA gene are nearly identical (Valverde et al. 2006; Ramírez-Bahena et al. 2008). The analysis of these genes has allowed the designing of new schemes named multilocus sequence analysis (MLSA) and multilocus sequence typing (MLST) for identification and phylogenetic analysis of bacteria. Applying such techniques, phylogenetic analyses of concrete groups of rhizobia as *Ensifer* (Martens et al. 2007, 2008; van Berkum et al. 2006), *Bradyrhizobium* (Vinuesa et al. 2008; Rivas et al. 2009b) and *Rhizobium* (Ribeiro et al. 2009) were established. The ad hoc committee for reevaluation of the species definition suggested that "species should be identifiable by readily available methods (phenotypic genomic)" and that one promising approach toward this goal is the determination of a minimum of housekeeping genes (Stackebrandt et al. 2002). Zeigler (2003) suggested that analysis of less than five suitable housekeeping genes might be sufficient for a reliable classification. For this reason, in the last descriptions of new rhizobial species, the analysis of at least two housekeeping genes have been included usually to know the closest related species before performing DNA–DNA hybridization experiments.

Although there are no exhaustive taxonomic studies of housekeeping identities in strains from the same species and species from the same genus, the results obtained up to date showed that strains with identities lower than 95% may belong to different species. Therefore, the analysis of several housekeeping genes is currently required for rhizobial species description although no clear indications have been provided in the last reunion of the Subcommittee on the taxonomy of *Agrobacterium* and *Rhizobium* (Lindström and Young 2009). Moreover, they are

very useful for studies on phylogenic biodiversity and biogeography (Stepkowski et al. 2003, 2005; Alexandre et al. 2008; Santillana et al. 2008; Zhang et al. 2008; Alvarez-Martínez et al. 2009; Lu et al. 2009c).

Currently, the 16S–23S intergenic spacer (ITS) has become useful in taxonomic studies of many groups of bacteria including rhizobia (Kwon et al. 2005). It has been shown that identities lower than 95% in the ITS sequences are found among species of rhizobia (Willems et al. 2001; Valverde et al. 2006) and, therefore, they are frequently used in species description (Rivas et al. 2004; Gu et al. 2008; Han et al. 2008b; Ramírez-Bahena et al. 2008; 2009). The difference in the sequences and the presence of inserts within noncodifying regions in the ITS are good tools for diversity analysis (Tan et al. 2001a; Laguerre et al. 2003; Kwon et al. 2005; Romdhane et al. 2006; Iglesias et al. 2007; Safronova et al. 2007; Santillana et al. 2008; Alvarez-Martínez et al. 2009; Menna et al. 2009; Zurdo-Piñeiro et al. 2009).

The analysis of different phylogenetic markers from 16S rRNA gene to ITS and housekeeping genes has revealed one of the most relevant findings in rhizobial taxonomy: There are strains of typical species of rhizobia that are unable to nodulate legumes. The first described species of "rhizobia" unable to nodulate legumes was *Sinorhizobium morelense* (Wang et al. 2002). Nevertheless, as it was isolated from nodules of *Leucaena leucocephala*, the nonnodulating feature could be confusing, and the first species isolated from a completely different source that a nodule was *Bradyrhizobium betae* described by our research group in 2004 (Rivas et al. 2004). This species was isolated from natural tumor-like formations in sugar beet, although it did not develop such malformations and nodulation or nitrogen fixation genes were never found. Many other species have also been isolated from different sources such as *R. daejeonense* (Quan et al. 2005) and *R. selenitireducens* (Hunter et al. 2007), both isolated from a cyanide treatment bioreactor and from a bioreactor that reduced selenate to elemental red selenium, respectively. Other species isolated from plant rhizosphere was *R. alamii* (Berge et al. 2009), while from plant root inner tissues was *R. oryzae* (Peng et al. 2008). In 2007, we isolated *R. cellulosilyticum,* a new species from sawdust of *Populus* with a marked cellulolytic activity (García-Fraile et al. 2007). In the same year, *Mesorhizobium thiogangeticum* was isolated from rhizosphere of legumes (Ghosh and Roy 2006). These results show that diversity of rhizobia is higher than ever expected and that these microorganisms are present in very diverse ecosystems.

Considering the overall developments in rhizobial taxonomy over the years, rhizobia have currently been placed in several families and genera. Of these, genus *Rhizobium* contains 33 species, 24 of which were isolated from legume nodules (Table 1.1); genus *Sinorhizobium* (currently named *Ensifer)* includes nine species isolated from legume nodules; genus *Mesorhizobium* is composed of 18 species isolated from legume nodules; genus *Bradyrhizobium* consists of seven species isolated from legume nodules and genus *Azorhizobium* has two species nodulating legumes (Table 1.1). Moreover, recently described one, isolated from sources other than nodules includes *Shinella kummerowiae* (Lin et al. 2008).The complete list of valid species of rhizobia is constantly updated and recorded in the

Table 1.1 Rhizobia able to nodulate legumes

Species	Host	Reference
Family *Rhizobiaceae*, genus *Rhizobium*		
R. alkalisoli	*Caragana*	(Lu et al. 2009a)
R. cellulosilyticum	*Medicago*	(García-Fraile et al. 2007)
R. etli	*Phaseolus*	(Segovia et al. 1993)
R. fabae	*Vicia*	(Tian et al. 2008)
R. galegae	*Galegae*	(Lindström 1989)
R. gallicum	*Phaseolus*	(Amarger et al. 1997)
R. giardinii	*Phaseolus*	(Amarger et al. 1997)
R. hainanense	*Desmodium*	(Chen et al. 1997)
R. huautlense	*Sesbania*	(Wang et al. 1998)
R. indigoferae	*Indigofera*	(Wei et al. 2002)
R. leguminosarum	*Pisum*	(Ramírez-Bahena et al. 2008)
R. loessense	*Astragalus*	(Wei et al. 2003)
R. lusitanum	*Phaseolus*	(Valverde et al. 2006)
R. mesosinicum	Chinese legumes	(Lin et al. 2009)
R. miluonense	*Lespedeza*	(Gu et al. 2008)
R. mongolense	*Medicago*	(van Berkum et al. 1998)
R. multihospitium	Chinese legumes	(Han et al. 2008b)
R. phaseoli	*Phaseolus*	(Ramírez-Bahena et al. 2008)
R. pisi	*Pisum*	(Ramírez-Bahena et al. 2008)
R. sullae	*Hedysarum*	(Squartini et al. 2002)
R. tibeticum	*Medicago*	(Hou et al. 2009)
R. tropici	*Phaseolus*	(Martínez-Romero et al. 1991)
R. undicola	*Neptunia*	(de Lajudie et al. 1992; Young et al. 2001)
R. yanglingense	*Amphicarpaea*	(Tan et al. 2001b)
Family *Rhizobiaceae*, genus *Ensifer* (formerly *Sinorhizobium*)		
E. arboris	*Acacia*	(Nick et al. 1999; Young 2003)
E. fredii	*Glycine*	(Chen et al. 1988; Scholla and Elkan 1984; Jarvis et al. 1992; Young 2003)
E. kostiense	*Acacia*	(Nick et al. 1999; Young 2003)
E. kummerowiae	*Kummerowia*	(Wei et al. 2002; Young 2003)
E. meliloti	*Medicago*	(de Lajudie et al. 1994; Young 2003)
E. medicae	*Medicago*	(Rome et al. 1996; Young 2003)
E. saheli	*Acacia*	(de Lajudie et al. 1994; Young 2003)
E. terangae	*Acacia*	(de Lajudie et al. 1994; Young 2003)
E. xinjiangensis	*Glycine*	(Chen et al. 1988; Young 2003)
Family *Phyllobacteriaceae*, genus *Mesorhizobium*		
M. albiziae	*Albizia*	(Wang et al. 2007)
M. amorphae	*Amorpha*	(Wang et al. 1999)
M. australicum	*Biserrula*	(Nandasena et al. 2009)
M. caraganae	*Caragana*	(Guan et al. 2008)
M. chacoense	*Prosopis*	(Velázquez et al. 2001)
M. ciceri	*Cicer*	(Nour et al. 1994; Jarvis et al. 1997)
M. gobiense	Chinese legumes	(Han et al. 2008a)
M. huakuii	*Astragalus*	(Chen et al. 1991; Jarvis et al. 1997)
M. loti	*Lotus*	(Jarvis et al. 1997)
M. mediterraneum	*Cicer*	(Nour et al. 1995; Jarvis et al. 1997)
M. metallidurans	*Anthyllis*	(Vidal et al. 2009)
M. opportunistum	*Biserrula*	(Nandasena et al. 2009)

(*continued*)

Table 1.1 (continued)

Species	Host	Reference
M. plurifarium	*Acacia*	(de Lajudie et al. 1998)
M. septentrionale	*Astragalus*	(Gao et al. 2004))
M. shangrilense	*Caragana*	(Lu et al. 2009b)
M. tarimense	Chinese legumes	(Han et al. 2008a)
M. temperatum	*Astragalus*	(Gao et al. 2004)
M. tianshanense	*Sophora*	(Chen et al. 1995; Jarvis et al. 1997)
Family *Nitrobacteraceae*, genus *Bradyrhizobium*		
B. canariense	*Chamaecytisus*	(Vinuesa et al. 2005)
B. elkanii	*Glycine*	(Kuykendall et al. 1992)
B. japonicum	*Glycine*	(Jordan 1982)
B. jicamae	*Pachyrhizus*	(Ramírez-Bahena et al. 2009)
B. liaoningense	*Glycine*	(Xu et al. 1995)
B. pachyrhizi	*Pachyrhizus*	(Ramírez-Bahena et al. 2009)
B. yuanmingense	*Lespedeza*	(Yao et al. 2002)
Family *Hyphomicrobiaceae*, genus *Azorhizobium*		
A. dobereinereae	*Sesbania*	(Souza Moreira et al. 2006)
A. caulinodans	*Sesbania*	(Dreyfus et al. 1988)

list of prokaryotic names with standing in nomenclature by Dr. Euzeby (http://www.bacterio.cict.fr).

1.2 Biovars and Legume Promiscuity in Rhizobia

In the 1970s, it was discovered that symbiosis and pathogenicity genes are harbored in plasmids, in many cases autoconjugative. These kinds of plasmids are found in both genera *Agrobacterium* (Ledeboer et al. 1976) and *Rhizobium* (Zurkowski and Lorkiewicz 1979). The symbiotic genes included those involved in legume nodulation (*nod*) and in nitrogen fixation *(nif*). The *nod* genes are responsible for the synthesis of nod factors (lipochitin-oligosaccharides) that are receptors for the plant flavonoid signal (Downie 1994; Denarié et al. 1996; Broughton et al. 2000). The *nodD* is a regulatory gene of the operon *nodABC* whose genes are determinants of the host range (Winsor 1989; Györgypal et al. 1991; Relic et al. 1994; Roche et al. 1996; Perret et al. 2000). The *nif* genes are involved in nitrogen fixation and are carried by rhizobia but also by free-living nitrogen fixing bacteria (Fischer 1994; Zehr et al. 2003). Symbiotic genes are harbored in plasmids (pSym) in fast- and in some intermediate-growing species of rhizobia, whereas these genes are integrated in the chromosome in the intermediate and slow-growing rhizobia, harbored in symbiotic islands (Barnett et al. 2001; Sullivan et al. 2002; Uchiumi et al. 2004; Flores et al. 2005; Young et al. 2006; Nandasena et al. 2007a, b; Crossman et al. 2008). Symbiotic genes also named "auxiliary" or "accessory" genes, are commonly included in species description of rhizobia and in sometimes MLST analysis is done comparing their phylogeny with that obtained after the "core" gene analysis

(Wernegreen and Riley 1999; Silva et al. 2005; Vinuesa et al. 2005). The auxiliary genes most commonly studied are *nodD*, *nodA*, *nodC*, and *nifH* (Laguerre et al. 2001; Silva et al. 2005; Stepkowski et al. 2007; Laranjo et al. 2008; Steenkamp et al. 2008). Nevertheless, these genes are not useful in taxonomy because of their ability to be transferred in nature (Finan 2002) from plasmids to islands (Nakatsukasa et al. 2008), from bacteria to plants (Broothaerts et al. 2005), and among bacteria (Rogel et al. 2001). Therefore, the analysis of symbiotic genes is overall useful to identify new-rhizobial species forming nodules and to carry out biogeographical studies of legume endosymbionts (Stepkowski et al. 2007; Steenkamp et al. 2008; Wei et al. 2009). Particularly, the nodulation genes are useful to define biovars within rhizobial species (Villegas et al. 2006; Mnasri et al. 2007; Rivas et al. 2007; León-Barrios et al. 2009).

Within rhizobia, the concept of biovar is directly linked to the concept of legume promiscuity. It is known for many years that legume has different promiscuity degree and whereas some of them can be nodulated by several species of rhizobia such as *Macroptilium* (Perret et al. 2000); others are restrictive hosts for nodulation such as *Cicer* (Broughton and Perret 1999). In the same way, rhizobial strains can have broad or narrow host range. For instance, *R. leguminosarum* bv. *trifolii* can only nodulate plants of genus *Trifolium* whereas *Rhizobium* sp. NGR234 nodulates over 100 legumes as well as the nonlegume *Parasponia* (Pueppke and Broughton 1999). Nevertheless, it is necessary to be careful when managing the promiscuity concept, since this feature in a legume should not be based on the number of taxonomic species able to nodulate it but in the different symbiotic genes able to induce the nodulation process (Rivas et al. 2007). Within these genes, *nodC* has been widely analyzed in rhizobial strains and found related with the host range of rhizobia and the promiscuity degree of the hosts (Roche et al. 1996; Perret et al. 2000; Laguerre et al. 2001; Rivas et al. 2007; Iglesias et al. 2008; Zurdo-Piñeiro et al. 2009). In 2006, we described the biovar ciceri within *M. amorphae* and *M. tianshanense* based on the *nodC* gene analysis (Rivas et al. 2007) from which we concluded that *Cicer arietinum* is a very restrictive host, because although it can be nodulated by several species of *Mesorhizobium,* all of them carry nearly identical *nodC* genes (Rivas et al. 2007). By contrast *P. vulgaris* is a very promiscuous legume since it is nodulated by the highest number of taxonomic species, which carry very divergent symbiotic genes (Michiels et al. 1998; Zurdo-Piñeiro et al. 2009).

The first biovars described in rhizobia were those of the species *R. leguminosarum* proposed by Jordan (1984). According to this proposal, the two former species *R. leguminosarum* and *R. phaseoli* were included within *R. leguminosarum* as biovars. This reclassification was made at a time when gene sequencing was not yet performed and therefore, it was based on several phenotypic and molecular data including the transfer of infectivity via plasmids. Recently, we revised the taxonomic status of the three old species and concluded that the species *R. trifolii* is a later subjective synonym of *R. leguminosarum*. However, *R. phaseoli* is a species distinguishable from *R. leguminosarum* and *R. etli*. Moreover, we found that the type strains of *R. leguminosarum* deposited in different culture collections

corresponds to different species and therefore, a new species, named, *R. pisi* was defined to include the former type strains of *R. leguminosarum* kept in DSMZ and NCIMB culture collections (Ramírez-Bahena et al. 2008).

As our work was exclusively based on chromosomal genes, the existence of biovars within *R. leguminosarum* should be decided on the basis of symbiotic genes. Although the reiteration of *nifH* genes was initially proposed to identify the biovar phaseoli (Martínez et al. 1985; Aguilar et al. 1998), the nodulation genes are more adequate for this purpose. Laguerre et al. (2001) pointed out that the biovars like phaseoli, gallicum, and giardinii that include strains from *P. vulgaris*, may be differentiated by the *nodC* gene sequences, although they are phylogenetically related. Later, a new biovar named mediterranense was proposed to include strains of *Sinorhizobium* able to nodulate *Phaseolus* but not *Medicago* (Mnasri et al. 2007). Recently, we have shown that *Phaseolus* can be effectively nodulated by *S. meliloti* strains carrying a *nodC* phylogenetically divergent to those of biovars phaseoli, gallicum, giardinii, and mediterranense (Zurdo-Piñeiro et al. 2009). In addition, strains nodulating *Vicia* and strains nodulating *Trifolium* can also nodulate *Phaseolus,* even though they have *nodC* genes phylogenetically unrelated to those carried by other strains nodulating *Phaseolus* (Alvarez-Martínez et al. 2009; Ramírez-Bahena et al. 2009). Therefore, according to the results of the *nodC* gene analysis, *P. vulgaris* is a very promiscuous host and, therefore, the convenience of keeping the biovar phaseoli should be discussed. Nevertheless as the analysis of the *nodC* gene allows the differentiation of *R. leguminosarum* biovar phaseoli, viciae, and trifolii, these biovars should be maintained in this species.

For other rhizobial species, some biovars have also been proposed as reported in *R. etli* that contains two biovars, phaseoli and mimosae, which differ in the ability of biovar mimosae to nodulate *Leucaena* (Wang et al. 1999). In the case of *R. galegae*, different symbiotic traits allowed the definition of two biovars named officinalis and orientalis (Radeva et al. 2001). In the case of genus *Sinorhizobium,* different biovars have been described in some species. The biovar medicaginis was described in *S. meliloti* on the basis of the *nodA* gene sequences (Villegas et al. 2006) and the *nodC* gene sequences also supported the existence of this biovar (Bailly et al. 2007). The already-mentioned biovar mediterranense has been described in *S. meliloti* and *S. fredii* having closely related *nodC* genes (Mnasri et al. 2007). Finally, a new biovar named lancerottense has been recently described within the species *S. meliloti* to include the strains able to nodulate *Lotus* endemic of Canary Islands (León-Barrios et al. 2009). These strains carry a *nodC* gene phylogenetically unrelated to those carried by other *S. meliloti* biovars and by *Mesorhizobium loti*. After biovar ciceri, a new biovar named biserrulae in *M. ciceri* was described to include rhizobia nodulating *Biserrula pelecinus*. This biovar may be differentiated from biovar ciceri by the *nodA* gene sequences (Nandasena et al. 2007a, b). In the slow-growing species *B. japonicum,* two biovars named glycinearum and genistearum with phylogenetically divergent *nodC* were defined to differentiate strains nodulating soybean or Genisteae legumes, respectively (Vinuesa et al. 2005). Since *nodC* genes of biovar genistearum are closely related to those of *B. canariense* strains, this biovar was also defined in this species

although at present all strains from this species belong to the same biovar (Vinuesa et al. 2005).

All these findings showed the existence of different combinations between chromosomes and symbiotic elements that have been coevolving together with the respective hosts (Aguilar et al. 2004; Moulin et al. 2004; Alvarez-Martínez et al. 2009). The integral knowledge of rhizobia needs the analysis of both the chromosomal and the symbiotic genes that makes possible the study of the geographic distribution patterns of microorganisms able to nodulate legumes (Steenkamp et al. 2008; Stepkowski et al. 2007; Lu et al. 2009a, b).

1.3 The New Rhizobia

As mentioned above, the 16S rRNA gene analysis approach has changed the concept about rhizobia and hence, the systematics of bacteria able to nodulate legumes has been redefined. For more than a century, rhizobia were thought to be the unique bacteria able to originate nodules in legumes and this fact made researchers to discard all those colonies obtained from nodules that had not the aspect of rhizobia on YMA plates. However, in 2001, the report of two atypical bacteria nodulating legumes opened the door of the legume nodulation by "non-rhizobial" bacteria and in successive years other "nonhizobial" genera from alpha and beta Proteobacteria were reported as legume endosymbionts (Table 1.2).

The first "nonrhizobial" bacterium nodulating legumes (e.g., *Crotalaria*) was *Methylobacterium* (Sy et al. 2001) that was later named *M. nodulans* and belongs to the family *Methylobacteriaceae* within the order "Rhizobiales" from alpha-Proteobacteria (Jourand et al. 2004). This species harbored the common nodulation *nodABC* genes and *nifH* gene encoding structural nitrogenase enzyme (Sy et al. 2001; Jourand et al. 2004). In the same year, Moulin et al. (2001) reported the nodulation of *Mimosa* by *Burkholderia,* a genus belonging to the beta-Proteobacteria. This nonrhizobial genus may also nodulate *Aspalathus carnosa* and *Macroptilium atropurpureum* and has nitrogen-fixing genes. The common nodulation genes (*nodABC*) of *Burkholderia* strains are phylogenetically related to those found in "classic" rhizobia supporting the hypothesis of lateral gene transfer in the rhizosphere crossing the boundary between classes alpha and beta Proteobacteria.

Since then several species of *Burkholderia* nodulating legumes have been described: *B. mimosarum* (Vandamme et al. 2002; Chen et al. 2006), *B. phymatum* (Vandamme et al. 2002), *B. nodosa* (Chen et al. 2007), and *B. sabiae* (Chen et al. 2008) nodulating *Mimosa* and *B. tuberum* nodulating *Cyclopia* (Elliott et al. 2007a). These species have been isolated from nodules of different legumes but mainly from *Mimosa* species, for example, *B. mimosarum* carrying *nod* and *nif* genes was isolated from root nodules of *M. pigra* and *M. scabrella* in Taiwan, Brazil, and Venezuela (Chen et al. 2005). *B. nodosa* was isolated from root nodules of *Mimosa bimucronata* and *M. scabrella* in Brazil and produced nitrogen-fixing nodules on *M. pudica, M. diplotricha*, and *M. pigra*. *Mimosa* is also

Table 1.2 Genus and species of new rhizobia nodulating legumes

Species	Host	Reference
Alpha-Proteobacteria		
Family *Rhizobiaceae*, genus *Shinella*		
S. kummerowiae	*Kummerowia*	(Lin et al. 2008)
Family *Nitrobacteraceae*, genus *Blastobacter*		
B. denitrificans	*Aeschynomene*	(van Berkum and Eardly 2002)
Family *Phyllobacteriaceae*, genus *Phyllobacterium*		
P. trifolii	*Trifolium*	(Valverde et al. 2005)
Family *Hyphomicrobiaceae,* genus *Devosia*		
D. neptuniae	*Neptunia natans*	(Rivas et al. 2003)
Family *Brucellaceae,* genus *Ochrobactrum*		
O. lupine	*Lupinus*	(Trujillo et al. 2005)
O. cytisi	*Cytisus*	(Zurdo-Piñeiro et al. 2007)
Family *Methylobacteriaceae*, genus *Methylobacterium*		
M. nodulans	*Crotalaria*	(Sy et al. 2001; Jourand et al. 2004)
Beta-Proteobacteria		
Family *Burkholderiaceae*, genus *Burkholderia*		
B. cepacia	*Dalbergia*	(Rasolomampianina et al. 2005)
B. mimosarum	*Mimosa*	(Chen et al. 2006)
B. nodosa	*Mimosa*	(Chen et al. 2007)
B. phymatum	*Machaerium*	(Vandamme et al. 2002)
B. sabiae	*Mimosa*	(Chen et al. 2008)
B. tuberum	*Aspalathus*	(Vandamme et al. 2002)
C. taiwanensis	*Mimosa spp.*	(Chen et al. 2003a, b; Vandamme and Coenye 2004)

Adapted from Rivas et al. (2009a)

nodulated by *B. phymatum* (Elliott et al. 2007b). Nonetheless, even though *Burkholderia* species are reported to nodulate primarily mimosoid legumes, yet they can also nodulate papilionoid legumes. For example, *Cyclopia* was nodulated by *B. tuberum* (Elliott et al. 2007a) and *Dalbergia louveli* by a strain belonging to the *Burkholderia cepacia* complex (Rasolomampianina et al. 2005). The conclusion of all these works is that beta-Proteobacteria are widespread in legume nodules being in some cases the main *Mimosa* endosymbionts (Barrett and Parker 2005; Chen et al. 2003b). Although this legume is nodulated by rhizobia, some strains of *Burkholderia* are more competitive than *R. tropici* for nodulation of *Mimosa* (Elliott et al. 2009).

Mimosa species are also nodulated by other beta-Proteobacteria initially named *Ralstonia taiwanensis* that nodulated *Mimosa pudica* and *Mimosa diplotricha* (Chen et al. 2003a). This species was erroneously classified and it has been later named *Cupriavidus taiwanensis,* a beta Proteobacteria belonging to the family *Burkholderiaceae* within order *Burkholderiales* (Vandamme and Coenye 2004). *C. taiwanensis* carries ten nodulation genes *nodABCIJHASUQ* and one regulatory gene *nodD* on pRalta. Next to *nod* genes, *C. taiwanensis* carries 19 genes presumably arranged in five operons and covering 25 kb that are involved in nitrogenase synthesis and functioning (Amadou et al. 2008).

Legume nodulation may be performed by beta-Proteobacteria, but this feature is only known in two genera, whereas there are many genera of alpha-Proteobacteria sharing this characteristic. In 2003, we reported the nodulation of *Neptunia natans* by a novel species of genus *Devosia, D. neptuniae*, from family *Hyphomicrobiaceae* and order "Rhizobiales" within alpha-Proteobacteria (Rivas et al. 2003) that carry *nodD* and *nifH* genes closely related to those of *R. tropici* CIAT899^T. The high identity of these genes suggested that they were transferred to *D. neptuniae* from *R. tropici,* an American species nodulating *Leucaena* (Martínez-Romero et al. 1991) and *Neptunia* in America (Zurdo-Piñeiro et al. 2004).

Few years later, two strains from genus *Ochrobactrum* belonging to the family *Brucellaceae* within alpha-Proteobacteria were found in nodules of *Acacia mangium* but no information on their symbiotic genes was reported (Ngom et al. 2004). In year 2005, a new species of this genus carrying symbiotic genes close to those of rhizobia able to nodulate *Lupinus* was reported (Trujillo et al. 2005). The isolated strains harbored megaplasmids of 1,500, 200, and 150 kbp and the *nifH* and *nodD* genes were detected using *nifH* and *nodD* probes. A second new species of *Ochrobactrum*, *O. cytisi*, carrying symbiotic genes and also phylogenetically related to those of rhizobia was isolated from *Cytisus scoparius* nodules in Spain (Zurdo-Piñeiro et al. 2007). In the same year, a new alpha Proteobacteria related to genus *Methylobacterium* was reported to nodulate *Lupinus* in America (Andam and Parker 2007). Although genus *Phyllobacterium* was initially included in the family *Rhizobiaceae*, it was never considered as rhizobia and was proposed to include bacteria isolated from leaf nodules of tropical Rubiaceae and Myrsinaceae (Knösel, 1984). However, a species named *Phyllobacterium trifolii* was isolated from *Trifolium pratense* nodules and was able to nodulate this species as well as *Lupinus albus* (Valverde et al. 2005). Although the type strain of the species *P. trifolii* harbors symbiotic plasmids in which the *nod* and *nif* genes were located, it forms ineffective nodules in the roots of these two hosts. Later on, a new genus (*Shinella)* belonging to the family *Rhizobiaceae* was described (An et al. 2006). The first species of this genus though did not nodulate legumes; the other species *S. kummerowiae* was isolated from nodules of *Kummerowia stipulata* (Lin et al. 2008). This is the first case but probably not the last in which it is difficult to decide whether it is a rhizobial or "non-rhizobial" genus. As mentioned earlier, it is now easy to identify genus and species of rhizobia regardless of their isolation source either having knowledge or not on their symbiotic capabilities and species of "classic" rhizobial genera that have not been isolated from nodules and for which symbiotic abilities are unknown. For example, in *B. betae* the symbiotic genes have, not been found despite a rigorous search (Rivas et al. 2004). This does not imply the absence of such genes in the bacterial genome but without this information it is very difficult to identify the legume host of these bacteria. Until the symbiotic genes are found either by complete genome sequencing or other approaches, those genera will be considered as "non-rhizobia" in which most of strains are unable to nodulate legumes.

In any case, there are numerous reports supporting the presence of alpha and beta Proteobacteria in nodules of several legumes (Liu et al. 2007; Yan et al. 2007;

Benata et al. 2008). Though other studies have reported the presence of gamma Proteobacteria in legume nodules, their symbiotic genes have not yet been detected (Muresu et al. 2008; Ibáñez et al. 2009). Nevertheless, as suggested by Benhizia et al. (2004), they could nodulate legumes, since recently it has been reported that legume symbiosis may occur in the absence of *nod* genes as reported for photosynthetic bradyrhizobia (Giraud et al. 2007).

1.4 Conclusion

Despite the tremendous progress in molecular research including genomics and proteomics, in the recent years, there is still a long way to have a deep insight and thorough understanding of the rhizobia–legume symbiosis, especially when lots of legumes remain unstudied. Therefore, this fascinating area of applied biological sciences requires further attention in order to better understand this intricate relationship between rhizobia and legumes. The forthcoming studies on diversity of rhizobia endosymbionts of many more wild legumes all over the world will draw a definitively more complete and general picture on the peculiarities of this symbiosis in the next few years. In this direction, the advent of some modern massive high throughput sequencing approaches have enabled the complete sequencing of a few bacterial (rhizobial) genomes which is undoubtedly has revolutionized the overall research on microbial diversity, and probably due to these advances it is likely that the legume endosymbiotic bacteria will be much better known and explored in the coming years.

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Chapter 2
Enhancing *Rhizobium*–Legume Symbiosis Using Signaling Factors

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Abstract Rhizobial symbiosis with leguminous plants affects the supply of organic nitrogen. Soil bacteria comprising members of the genera *Rhizobium, Bradyrhizobium*, *Mesorhizobium, Sinorhizobium*, and *Azorhizobium*, commonly referred to as rhizobia, are taxonomically diverse members of the α and β subclasses of the *Proteobacteria*. They possess the ability to induce root nodules on legume plants and provide these plants with fixed nitrogen, enabling them to grow in nitrogen-limited soils. Rhizobia colonize root nodules, fix nitrogen inside, transport usable form of N to plants, and concurrently facilitate the growth and grain yields of legumes. *Rhizobium*–legume symbiosis is a multi-step process requiring the exchange of numerous molecular signals between bacteria and the plant host. Precise fulfilling of all stages of this molecular dialogue is prerequisite to the effective symbiosis, allowing bacteria to invade the host and, conversely, enabling the host to derive benefits from the presence of bacteria. Individual legumes are often nodulated by multiple bacterial strains with varying symbiosis-establishing capabilities. Thus, selection of highly effective strains that successfully compete with less effective ones is required when developing legume inoculants. Various factors that influence symbiotic rhizobial interactions under competitive soil environment, including the exchange of plant and bacterial signaling molecules, such as flavonoids and nodulation factor (Nod factor), in the early stages of symbiosis is highlighted. Beneficial responses of rhizobial inoculants on to legumes, as well as manipulations of symbiotic signaling factors, is likely to increase their potential as biofertilizers for sustainable agriculture to promote growth and nodulation of legume plants.

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2.1 Overview of *Rhizobium*–Legume Symbiosis

2.1.1 Ecological and Agricultural Importance of Symbiotic Nitrogen Fixation

The availability of reduced nitrogenous compounds is a major limiting factor in plant growth and agricultural productivity. The microbiological process that converts atmospheric dinitrogen (N_2) into a plant-accessible species is known as biological nitrogen fixation (BNF). BNF reduces the degree of the requirement for external input of chemical N fertilizers to replenish soil N and improve internal resources (Peoples et al. 1995a; Vance 2001; Herridge et al. 2008). Total global N_2 fixation from BNF has been estimated to 100–290 million tones N/year, with approximately 50–70 million tones N/year in agricultural systems, compared with 83 million tones N fixed industrially in fertilizer production.

Among the wide range of bacteria that have the ability to reduce N_2 to ammonia, the most important are the symbiotic systems of leguminous plants and rhizobial species belonging to β-proteobacteria of the genera *Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium*, and *Sinorhizobium (Ensifer)*, collectively called rhizobia (Perret et al. 2000; Jones et al. 2007; Franche et al. 2009). Recently, several new species of N_2-fixing microsymbionts, such as, *Methylobacterium* (Sy et al. 2001), *Devosia* (Rivas et al. 2002), *Herbaspirillum* (Valverde et al. 2003), *Ochrobactrum* (Zurdo-Piñeiro et al. 2007), *Phyllobacterium* (Valverde et al. 2005), and members of the β-proteobacteria such as *Burkholderia* (Moulin et al. 2001) and *Cupriavidus* (*Ralstonia)* (Chen et al. 2001) have been described. A successful interaction between legume plants and rhizobia leads to the formation of nodules on the roots or shoots. Bacteria in the form of bacteroids reside inside nodules and fix atmospheric N into ammonia (Perret et al. 2000; Gibson et al. 2008). The reduced nitrogenous compounds are transported into the host plant in exchange for organic acids. The symbiotic systems are a major source of nitrogen in most legumes with an average of 80% of N derived from BNF (Vance 2001; Graham and Vance 2003). There are estimates that the rhizobial symbioses with 18,000 legume species (Masson-Boivin et al. 2009), including more than 100 agriculturally important legumes spanning all the geographical regions, contribute nearly half of the annual quantity of BNF in soil ecosystems (Graham and Vance 2003). Rotations of legumes with other non-nitrogen-fixing plants enrich the soil with fixed N and increase the productivity and sustainability of agricultural systems. There is evidence that nitrogen derived from legume sources are less susceptible to losses than chemical fertilizer N, which in long term results in the build-up of a reserve of readily mineralizable organic N. The use of BNF in agriculture provides a renewable source of N to supplement or replace fertilizer N and arrests the decline of soil N fertility (Peoples et al. 1995a, b).

2.1.2 Rhizobial Genomes: Background for BNF and Source of Biodiversity

A common feature of the rhizobial genomes is that genes involved in nodulation and N_2-fixation are clustered on symbiotic plasmid (pSym), or incorporated into the chromosome as symbiotic islands (Palacios and Newton 2005). The architecture of rhizobial genomes may directly underlie the great genetic and physiological variation of rhizobial strains, resulting in a large diversity of populations. Rhizobial genomes are large (e.g., *R. leguminosarum* bv. *viciae*–7.7 Mb; *R. etli*–6.5 Mb; *Sinorhizobium meliloti*–6.7 Mb) and are composed of chromosomal core and plasmids, which comprise up to 50% of total genome (Galibert et al. 2001; Gonzalez et al. 2006; Young et al. 2006). Comparative analyses of rhizobial genomes revealed their mosaic structure: regions showing high degree of conserved synteny are separated by other sequences (Guo et al. 2003; Król et al. 2007). There is evidence that such genomes are dynamic structures, where recombination events are very frequent, permanently creating new versions of individual replicons (Brom et al. 1991; Guo et al. 2003). Moreover, not only the symbiotic plasmid but also considerable fractions of the nonsymbiotic plasmid pool present in rhizobial cells are necessary for establishing an effective *Rhizobium*–legume symbiosis (Brom et al. 1992; Mercado-Blanco and Toro 1996; Galibert et al. 2001). On the one hand, the transfer of symbiotic plasmids between strains in the rhizosphere was evidenced (Broughton et al. 1987), and the lateral transfer of genes was postulated to be a considerable force in rhizobial evolution and diversification (Souza et al. 1992). On the other hand, some data indicate that pSym plasmid transfer frequency is not as high in the field as under laboratory conditions (Wernegreen et al. 1997). Thus, the lateral transfer of genes by plasmid exchange is responsible for an emerging diversity within narrow genetic subdivisions, while main rhizobial genera are quite "reproductively isolated" (Wernegreen and Riley 1999; Bailly et al. 2007).

2.1.3 *Signaling in Rhizobium*–Legume Symbioses

Rhizobia nodulate wide range of legume plants. Some of them, such as *Rhizobium* sp. NGR234, are extremely promiscuous and are able to nodulate many different host plants (over 112 hosts) (Pueppke and Broughton 1999), while others, such as *R. leguminosarum* bv. *trifolii*, have a very narrow host range and nodulates only clover (*Trifolium* spp.) plants. Its close relative, *R. leguminosarum* bv. *viciae,* nodulates pea (*Pisum* spp.), vetch (*Vicia* spp), lentil (*Lens* spp.), and sweet pea (*Lathyrus* spp.) (Perret et al. 2000). The specificity of symbiotic interactions is achieved by exchange of molecular signals. In the early steps of symbiosis, a diverse array of compounds is exuded into the rhizosphere, including flavonoids, isoflavonoids, and non-flavonoid inducers (Fig. 2.1). These compounds are chemoattractants for rhizobia (Caetano-Anollés et al. 1988; Dharmatilake and Bauer 1992), influence

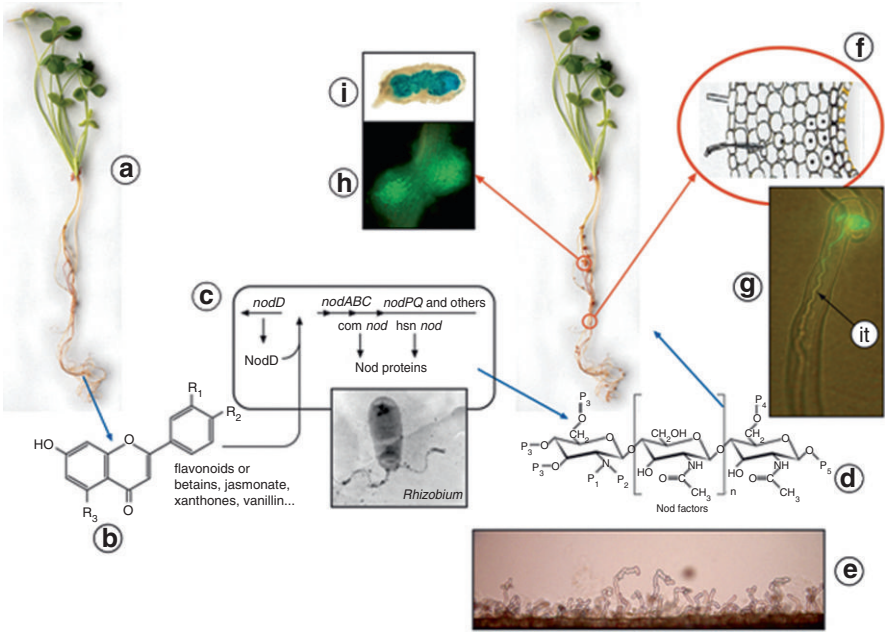

Fig 2.1 The events involved in *Rhizobium*–legume symbiosis. Roots of legume plant (**a**) exude flavonoid compounds (**b**), which are perceived by bacterial NodD regulatory protein (**c**) that activate set of *nod* genes resulting in the production of Nod factor (**d**). Nod factors secreted by rhizobia mediating root hair curling (**e**) and initiation of nodule primordium (**f**). Rhizobia attach to the root hairs and infection threads (it) are formed (**g**). Infection threads penetrate plant tissues, invade nodule primordium, and develops nodule (**h**, **i**). (**h**) Rhizobia tagged with *gfp* inside young nodules (**i**) rhizobia tagged with *gus* in mature nodule

bacterial growth, and induce the expression of nodulation genes (*nod* genes) (Peters et al. 1986; Hungria and Stacey 1997) (for detail see Sect. 2 and 3). As a result of *nod* genes expression, biosynthesis of specific lipochitin oligosaccharides called nodulation factors (Nod factors or LCOs) occurs (Lerouge et al. 1990). Nod factors are structurally diverse and a single rhizobial strain may produce a range of these metabolites (Spaink et al. 1991, 1995).

Nodulation genes have been classified into three categories. First, the common nodulation genes (*nodABC)* that are found in all bacteria including the β-proteobacteria (Moulin et al. 2001) with only one exception, that is, photosynthetic bradyrhizobia (Giraud et al. 2007). These are essential for nodulation and mutations in these genes lead to Nod$^-$ phenotype (Jacobs et al. 1985; Debellé et al. 1986). The encoded NodC is responsible for biosynthesis of β-1,4-linked *N*-acetyl-D-glucosamine trimeric to hexameric backbone, while NodB deacetylates glucosamine at the nonreducing end, following which NodA acylates free amino group of the terminal glucosamine (Spaink 2000; D'Haeze and Holsters 2002). Second, the host-specificity nodulation genes (*nodFE, nodH, nodG, nodPQ*, and several others) whose products modify the *N*-acylglucosamine backbone by adding species-specific

substituents, which are considered the main factors determining the host range of microsymbionts, and influence the rate of nodule formation (Debellé et al. 1986; Horvath et al. 1986; Schwedock and Long 1989). At the reducing-terminal residue, L-fucosyl, 2-*O*-Me-fucosyl, 4-*O*-Ac-fucosyl, acetyl, or sulphate esters are present, and at the nonreducing-terminal residues, *N*-methyl, *O*-acetyl, and *O*-carbamoyl are found, respectively. A fatty acyl chain of varying length and with varying degree of unsaturation is attached to the nonreducing end. These moieties controlled by the host-specificity *nod* gene products make the Nod factors specific for target plant hosts (Dénarié et al. 1996; Spaink 2000; D'Haeze and Holsters 2002). However, variation in Nod factor structure does not fully explain host-range specificity. For example, Nod factors produced by *R. etli* and *Mesorhizobium loti* have the same structure but nodulate distinct host legumes (e.g., *Phaseolus* spp., and *Lotus* spp.) as reported by Cardenas et al. (1995). This indicates that the elements of the molecular dialogue between the legume plant and rhizobia are more complex and that Nod factor is not the sole signal specifying the host range (Perret et al. 2000; Somers et al. 2004).

The third class of *nod* genes is a family of regulatory *nodD* genes (Spaink 2000). NodD proteins belong to the LysR family of transcriptional regulators (Hong et al. 1987; Fisher et al. 1988; Kondorosi et al. 1989). NodD, in complex with a flavonoid, binds conservative sequences upstream of *nod* operons, called *nod*-boxes, acting as transcriptional activator of several *nod, nol*, and *noe* gene promoters (Peck et al. 2006). *Sinorhizobium meliloti* synthesizes four NodD proteins (NodD1, NodD2, NodD3, and SyrM) that interact with different plant flavonoid signals. Some rhizobium species use other sensor-activator systems to control the host range. For instance, *B. japonicum* possesses *NodV–NodW,* a two-component system that is a positive regulator of *nod* genes responding to isoflavone signals. *NodV* and *nodW* are essential for the nodulation of *Macroptilium atropurpureum*, *Vigna radiata,* and *V. unguiculata*, but contribute only marginally to the symbiosis with *Glycine max* (Gőttfert et al. 1990; Sanjuan et al. 1994). Furthermore, several *nod* regulons, which are positively regulated by NodD protein, can be negatively regulated by NolR (Kondorosi et al. 1991; Cren et al. 1995). NolR binds to the promoter regions of *nod* genes and prevents their expression. Expression of *nolR* is negatively regulated by luteolin – a specific *nod* gene inducer in *S. meliloti* (Cren et al. 1995).

For optimal nodulation, proper level of *nod* gene expression is required and special mechanisms down-regulate the expression of *nod* genes in *S. meliloti* and *R. leguminosarum* bv. *viciae* (Somers et al. 2004; Perret et al. 2000; Hogg et al. 2002; Peck et al. 2006). In *R. leguminosarum* bv. *viciae* bacteroids, syntheses of NodA, NodI, NodE, and NodO proteins were reduced at least 14-fold compared with free-living bacteria, whereas the level of NodD protein was reduced only threefold (Schlaman et al. 1991). A decreased amount of NodD was also found in a strain harboring multiple copies of *nodD*. The in situ RNA hybridization of *Pisum sativum* and *Vicia hirsuta* nodules showed that transcription of inducible *nod* genes was switched off by unknown regulatory mechanism before the bacteria differentiated into bacteroids (Schlaman et al. 1991). Moreover, the concentration of Nod

factors in the rhizosphere is modulated by plant root hydrolases (Minic et al. 1998; Ovtsyna et al. 2000). Tetrameric NodD binds to 49 bp *nod*-boxes even in the absence of flavonoids (Feng et al. 2003). However, compatible flavonoids are required to induce the changes in DNA topology at the location of NodD binding in the promoter *nod* gene, thereby allowing RNA polymerase to initiate gene transcription (Chen et al. 2005). In *S. meliloti*, only luteolin is capable of activating in vivo *nod* gene transcription (Peck et al. 2006). Noninducing flavonoids, such as naringenin, eriodictyol, and daidzein, also stimulate an increase in the DNA-binding affinity of NodD1 to *nod* gene promoters but only luteolin is capable of promoting the topological changes necessary for *nod* gene induction. This is consistent with the hypothesis that noninducing flavonoids are acting as competitive inhibitors of inducing flavonoids, preventing NodD1 from activating *nod* gene transcription (Peck et al. 2006).

Flavonoids and *nod*-boxes also regulate other functions, such as: (1) N_2-fixation (Dombrecht et al. 2002), (2) synthesis and/or modification of polysaccharides (Mimmack et al. 1994; Wielbo et al. 2004a), (3) rhizopine catabolism (Rossbach et al. 1994), (4) synthesis of hopanoids (Kannenberg et al. 1995), or (5) synthesis of transcriptional regulators that modulate the synthesis of Tts1 and SyrM2 in *Rhizobium* sp. NGR234 (Kobayashi et al. 2004). The *nodDABC* genes, independently of flavonoids, are required for the establishment of the three-dimensional structure of a biofilm formed by *S. meliloti,* which is enhanced by the flavonoid luteolin – the inducer of *nod* genes (Fujishige et al. 2008). The core Nod factors facilitate bacterial adhesion to the roots until, in the presence of plant flavonoid inducers, a sufficient concentration of the host-specific Nod factors is reached and plant developmental processes are initiated (Fujishige et al. 2008; Faure et al. 2009).

Host-specific Nod factors are perceived by the plant via LysM-type receptor kinases and a complex signal transduction cascade that triggers early plant responses, such as intra and extracellular alkalinization, membrane depolymerization, calcium spiking, deformation of root hairs, initiation of cortical cell division, infection thread growth, and nodule primordia formation (D'Haeze and Holsters 2002; Oldroyd and Downie 2004; Jones et al. 2007). Recently, several genes of the Nod factor-signaling cascade have been identified and cloned from *Medicago truncatula* and *Lotus japonicus* model legumes (Geurts et al. 2005; Oldroyd and Downie 2004, 2006). Nod factors are biologically active at very low concentration (10^{-9}–10^{-12} M). Rhizobia enter the roots at the sites where root hair cell walls are hydrolysed and may produce either hydrolytic enzymes or use plant mechanisms (Perret et al. 2000). They invade the roots through tubular structures called infection threads. It has also been found that Nod factors reduce the salicylic acid level in the roots to help in the suppression of host defence responses and ensure successful infection by rhizobia (Martínez-Abarca et al. 1998).

Purified lipochitooligosaccharides (LCOs) are sufficient to induce root hair curling, reinitiation of cell division, and in some cases, elicitation of nodule-like structures (Dénarié and Cullimore 1993; Stokkermans et al. 1995; Heidstra and Bisseling 1996; Gibson et al. 2008). Furthermore, Nod factors and other chitin

oligosaccharides have been known to have developmental effects on nonleguminous plants, such as carrot (*Daucus carota*) (de Jong et al. 1993), tobacco (*Nicotiana tabacum*) (Schmidt et al. 1993), and Norway spruce *Picea abies* (Dyachok et al. 2000), in the absence of auxin and cytokinin. Global transcriptome analyses of host plants revealed significant changes in the expression of a wide range of genes involved in various aspects of the symbiotic interaction, such as bacterial infection, nodule formation and function, and defense responses (Fedorova et al. 2002; Mitra and Long 2004; Yahyaoui et al. 2004; Barnett and Fisher 2006).

Rhizobia multiply near the tip of growing infection threads, and after reaching the nodule primordium, they are released into the plant cells. Simultaneously, plant-derived peribacteroid membranes (PBM) forming symbiosomes encapsulate them. Inside symbiosomes, they differentiate into bacteroids, which reduces N to NH_4^+ (Brewin 2004; Gage 2004). Numerous rhizobial signaling molecules are required for initiation and elongation of the infection threads and nodule development. They include symbiotically relevant cell-surface polysaccharides such as capsular polysaccharides (CPS), exopolysaccharides (EPS), cyclic β-(1,2)-glucans, and lipopolysaccharides (LPS). Such polymeric compounds play an important role in plants forming indeterminate type of nodules with a persistent meristem such as *Vicia, Medicago, Pisum*, or *Trifolium* (Fraysse et al. 2003; Becker et al. 2005; Skorupska et al. 2006). However, plants that form determinate type of nodules do not have such a requirement (Hotter and Scott 1991). Several possible roles for bacterial polysaccharides in symbiosis are considered: (1) a mechanistic role in protecting bacteria against environmental stresses; (2) acting as signaling molecules triggering plant developmental response; (3) promotion of infection thread initiation and development and suppression of plant defense responses (Fraysse et al. 2003; Skorupska et al. 2006).

Recently, it has been reported that genomes of photosynthetic *Bradyrhizobium* strains (BTAi1 and ORS278) that induce stem and root nodules on aquatic *Aeschynomene* legume plants do not contain the common nodulation genes *nodABC* that are indispensable for Nod factor backbone synthesis, indicating that this group of rhizobia uses mechanism other than Nod factor strategy to enter into symbiosis (Giraud et al. 2007). Purine derivatives, such as cytokinins produced by some strains of *B. japonicum* and *R. leguminosarum*, have been considered as a potentially important signal triggering nodule formation (Giraud et al. 2007; Frugier et al. 2008). On the one hand, even though rhizobially derived cytokinins have not been found essential for Nod-factor-dependent symbiosis, yet they seem to be important for Nod-factor-independent nodulation in *Aeschynomene*. On the other hand, recent data indicate diverse functions of plant cytokinins in symbiosis, such as mediating root susceptibility to rhizobial infection and nodule organogenesis. Cytokinins act downstream of early Nod-factor signaling playing a crucial role in redifferentiation of cortical cells after the induction of Nod-factor-dependent pathways (Lohar et al. 2004; Frugier et al. 2008).

The host plant controls localization, shape, anatomy as well as the infection of the nodules. In general, there are two different kinds of nodules elicited by rhizobia: determinate and indeterminate. The elongated indeterminate nodules have

a persistent meristem that continually gives rise to new nodule cells that are subsequently infected by rhizobia. In this type of nodules, the developmental stages can be observed from the meristem at the nodule tip to the older senescent and saprophytic zones near the root (Vasse et al. 1990; Timmers et al. 2000). Bacteroids within indeterminate nodules undergo a terminal differentiation program and grow outside the host cells (Mergaert et al. 2006), while the bacteria released from the saprophytic zone are viable and grow in the rhizosphere and infect legume roots. Determinate nodules are formed generally by tropical legumes (e.g., *Glycine max, Vicia faba,* and *Lotus japonicus*) and are round due to lack of persistent meristem and do not display developmental zones (Perret et al. 2000; Gage 2004). Bacteroids within determinate nodules can dedifferentiate after release from nodules and grow in soil (Mergaert et al. 2006). These two types of nodules also differ significantly in their C and N metabolism (White et al. 2007).

2.1.4 Nitrogen Fixation

Inside nodules, rhizobia enclosed in PBM are transformed into bacteroids capable of N_2-fixation. This process is catalyzed by oxygen-sensitive molybdenum nitrogenase found in all rhizobia. The nitrogenase catalyzes the following reaction:

$$N_2 + 16\text{MgATP} + 8e^- + 8H^+ \rightarrow 2NH_3 + 16\text{MgADP} + \text{Pi} + H_2$$

In this reaction, 16–18 molecules of ATP are required for the reduction of N_2 molecule to two molecules of NH_3 and $2H^+$ to H_2. Nitrogenase is extremely O_2 sensitive and is rapidly inactivated in aerobic environment. The low O_2 concentration (3–22 nM) in the nodule is achieved by the high concentration of O_2-binding heme protein – leghemoglobin, and the symbiosome membrane diffusion barrier (Kaminski et al. 1998; Patriarca et al. 2002). Energetically, this is a very expensive process, which explains the inhibition of nodulation by the presence of fixed nitrogen (White et al. 2007).

Rhizobia are equipped with several *nif* genes responsible for the N_2 fixation process: structural gene (*nifH*) encoding dinitrogenase reductase, also designated Fe-protein, and *nifD, nifK* genes encoding α and β subunits of dinitrogenase, respectively, that form functional complexes with FeMo cofactor, also named FeMo protein. *nifB, nifE*, and *nifN* genes encode molecular scaffold for the assembly of FeMo cofactor. Prosthetic groups containing 4Fe–4S clusters are covalently bound to MoFe protein bridging the α and β subunits. The 4Fe–4S group is linked also to the Fe protein (Fisher 1994; Newton 2007). The *nifA* gene performs a regulatory role in expression of *nif* genes; NifA functions in low oxygen tension. In rhizobia, besides *nif* genes with significant homology to *Klebsiella pneumoniae nif* genes (Ruvkun and Ausubel 1980), several other *fix* genes involved in nitrogen fixation have been found. Generally, rhizobia show high plasticity not only in the

gene composition of *nif* regulon but also in the mode of *nif* genes regulation (Fisher 1994; Masson-Boivin et al. 2009).

Carbon sources for N_2-fixation are supplied by the host plant in the form of photosynthates (sucrose) that are catabolized to C_4 dicarboxylic acids and transported to the bacteroids to provide energy for N_2-fixation. Ammonium (and alanine) as a product is exported back to the plant through the symbiosome membrane and is further assimilated into glutamine or asparagine in the plant cytosol in indeterminate nodules. In determinate nodules, these amino acids are converted in the uninfected cells, specialized in further nitrogen assimilation into ureides or amides that are transported from the nodules to the shoots (Lodwig and Poole 2003; White et al. 2007).

2.2 Flavonoids Compounds Inducing Nod Factor Production and Enhancing Symbiosis

Plants exude large quantities of sugars (from mono and simple polysaccharides to high molecular weight polysaccharides), acids (aliphatic as well as aromatic acids), amino acids, amines, and many other low molecular weight compounds such as flavonoids, steroids, alkaloids, vitamins, and growth regulators (Gaworzewska and Carlile 1982; Knee et al. 2001; Bertin et al. 2003). All these compounds may affect bacterial soil populations. Some of them, such as sugars, acids, and aminoacids, serve as C and energy sources for microorganisms (Jaeger et al. 1999). Others such as flavonoids are involved in signal exchange between the two symbionts or in anti-pathogen plant defense system (Phillips and Kapulnik 1995). Moreover, some components of plant root exudates have been shown to interfere with quorum sensing-dependent bacterial communication systems (Teplitski et al. 2000). On the basis of these observations, it has been suggested that plants possess a great potential for "soil engineering" and may select for the most suitable bacteria (Simms and Taylor 2002). On the other hand, rhizosphere microorganisms can enhance root exudation of C and energy sources or flavonoids, suggesting the presence of "feedback" in plant–*Rhizobium* interactions related to bacterial nutrition (Lodwig and Poole 2003; Phillips et al. 2004).

Flavonoids are the most important components of plant root exudates for successful *Rhizobium*–legume relationships (Table 2.1). The flavonoid backbone is synthesized by condensation of 4-coumaryl-CoA provided by chalcone synthase (CHS) (Dixon and Paiva 1995). Several modifications of this structure yield different classes of flavonoids: flavanones, flavones, isoflavonoids, coumestans, chalcones, and anthocyanidines (Harborne and Williams 2000). More than 4,000 flavonoids are synthesized in vascular plants and released into the rhizosphere (Perret et al. 2000). The production spectrum of these substances may vary with the physiological state and age of the plant (Schlaman et al. 1998; Harborne and Williams 2001). Great amounts of flavonoids are released near the root hair zone;

Table 2.1 Substitution patterns of plant flavonoids. Deavours et al. (2006)

Flavonoid	Substitution pattern on ring positions				
	3	5	7	3′	4′
Flavones					
7,4′-dihydroxyflavone			OH		OH
Apigenin		OH	OH		
Luteolin				OH	
Isofavones					
Daidzein			OH		OH
Genistein		OH	OH		OH
Flavanones					
Hespertin		OH	OH	OH	OH
Naringenin		OH	OH		OH
Flavonols					
Quercetin	OH	OH	OH	OH	OH

OH hydroxyl

a site of rhizobium infection (Peters and Long 1988; Hartwig et al. 1990; Zuanazzi et al. 1998). Flavonoids play important roles in different stages of nodulation. First, they act as *nod* gene inducers by activation of NodD protein (Recourt et al. 1991; Hungria and Stacey 1997; Brencic and Winans 2005). For example, luteolin and 4, 7′-dihydroxyflavone (Dhf) are the inducers for *S. meliloti* (Caetano-Anollés et al. 1988; Hartwig et al. 1990; Peck et al. 2006), isoflavone genistein induces *nod* gene expression in *B. japonicum* but inhibits *S. meliloti nod* gene expression (Kosslak et al. 1987), and hespertin and naringenin are potent inducers of *R. leguminosarum* bv. *viciae* (Firmin et al. 1986; Begum et al. 2001). They induce Nod factor synthesis in the infection threads (Sharma and Signer 1990) and act in auxin transport regulation and initiation of nodule primordia cell division (Mathesius et al. 1998; Zhang et al. 2009). Recently, different roles of flavonoids in development of determinate and indeterminate nodules have been reported (Wasson et al. 2006; Subramanian et al. 2007; Zhang et al. 2009). For instance, total silencing of flavonoid biosynthesis in *M. truncatula* forming indeterminate nodules leads to a near complete loss of nodulation by *S. meliloti,* whereas flavone-deficiency results in reduced nodulation. Isoflavone-deficient roots are nodulated normally, indicating that isoflavones are not crucial in *M. truncatula* nodulation (Zhang et al. 2009). Addition of 7,4′-dihydroxyflavone but not flavonol kaempferol (an inhibitor of auxin transport) to flavones-deficient roots can completely restore nodulation. On the basis of these observations, Zhang et al. (2009) proposed the sequence of flavonoid involvement during interaction of *M. truncatula* with *S. meliloti*. It has been suggested that the essential role of Dhf is not only as the primary inducer but also in the sustained Nod-factor induction in the infection threads. The sustained Nod-factor induction in turn leads to the accumulation of flavonol kaempferol and accumulation of kaempferol subsequently leading to the localized auxin transport inhibition resulting in cell division, nodule initiation, and development (Zhang et al. 2009). In contrast, silencing of isoflavone biosynthesis in soybean roots forming

determinate nodules lead to reduced nodulation and increased auxin transport suggesting the essential role of isoflavones during soybean nodulation. In this case, the isoflavone might be responsible for the sustained induction of bacterial *nod* genes and Nod-factor biosynthesis inside the roots (Subramanian et al. 2006).

There are several examples of flavonoids, which have been used to increase *nod*-gene transcription and promote legume growth. For example, bean nodulation by *R. leguminosarum* bv. *phaseoli* or *R. tropici* was enhanced by the addition of quercetin and malvidin glucoside (Hungria and Phillips 1993); luteolin added to certain alfalfa cultivars significantly increased nodulation (Kapulnik et al. 1987); pretreatment of *B. japonicum* with genistein increased nodulation, total protein yield, and grain yield of soybean under laboratory (Zhang and Smith 1995) and field conditions (Zhang and Smith 1996); preinduction of *R. leguminosarum* with flavanones hespertin and naringenin, alone or in mixture, stimulated nodulation and plant dry matter accumulation of pea and lentil plants in comparison to uninduced *R. leguminosarum* cells in controlled environment growth chamber conditions (Begum et al. 2001).

Seed exudates, which are mixture of flavonoids, are economically more justified when used as exogenous *nod* genes inducers, although they also contain flavonoid-inhibitors of *nod* gene expression. Evidence exists that compounds sharing structural similarities with daidzein or genistein are the most effective inhibitors of *nod* gene induction in *B. japonicum*. The inhibitors of *nod* genes may act competitively against inducers at a common target site, such as the NodD protein (Kosslak et al. 1990). However, under laboratory conditions, the clover and bean exudates were more potent inducers of *nodA* gene of *R. leguminosarum* than the specific flavonoids alone, indicating the possibility of synergistic effects of *nod*-gene-activating compounds (Maj et al. 2010). Several authors demonstrated that stimulation of genes with combinations of multiple inducers resulted in better *nod* gene induction and might be advantageous for early symbiotic interactions (Cooper 2004, 2007). This would not exclude the specific interaction between NodD protein and individual flavonoid (Peck et al. 2006).

The potential of seed exudates as *nod*-gene inducers could be exploited in the symbiotic activation of inoculants before their use as biofertilizers. As an example, preincubation of *R. leguminosarum* bv. *trifolii* strains with clover seed exudate increased fresh mass of shoots and increased nodule numbers in a strain-specific manner under laboratory conditions (Maj et al. 2010). Preactivation of inoculant strains with flavonoids might increase competitiveness in the soil as well as legume productivity; in the case of *B. japonicum* preactivated with genistein, soybean yields increased by 10–40%, and the seasonal levels of N_2 fixation enhanced by 35% (Zhang and Smith 2002). Similarly, field pea and lentil plants displayed increased nodulation and biomass production when inoculated with *R. leguminosarum* preinduced with hesperetin (Begum et al. 2001). Currently, flavonoids are used commercially to promote *Rhizobium*–legume symbioses and N_2 fixation in agricultural practices (Hungria and Stacey 1997; Mabood et al. 2008). For example, genistein and daidzein, inducers of *B. japonicum nod* genes, are used in commercial inoculants under the name SoyaSignal. Using SoyaSignal technology to early-planted

soybean increased yields by 10% depending on the soybean genotype potential (Leibovitch et al. 2001). These and other associated data thus suggest that the knowledge of the essential role of flavonoids in *Rhizobium*–legume symbioses could be exploited to enhance the nodulation of economically important legume crops by exogenous addition of synthetic or natural flavonoid compounds. However, the practical application of flavonoids or other signaling molecules in a complex soil environment is much more difficult, and hence, the anticipated results may be different in different ecological niches.

2.3 Non-Flavonoid Signaling Molecules Influencing Legume Productivity

Several non-flavonoid plant factors, such as jasmonates, aldonic acids (erythronic acid and tetronic acid), betains, xanthones, and simple phenolic compounds, can induce the expression of *nod* genes. The common features of such inducers are that they act at higher concentrations than flavonoids and can induce *nod* genes and enhance Nod factors production in several legume plants (Cooper 2007). Jasmonic acid (JA) and its ester, methyl jasmonate (MeJA), generally known as jasmonates, are derivatives of linolenic acid and are biosynthesized in plants via the octadecanoic pathway. They are important signal molecules involved in induced disease resistance and mediate many physiological activities, such as environmental stress responses, root growth promotion, or inter-plant communication in plants. Jasmonic acid exogenously applied to the growth medium at high concentration (100 μM) decreases the number of nodules on *S. meliloti* inoculated *Medicago truncatula* roots. At such dose rate, JA decreases the responsiveness of calcium spiking to Nod factor, whereas at low concentrations (10–50 μM), it modifies the calcium signal by decreasing the frequency of spiking. Modulation of calcium signaling might have an important role in the initiation of colonization where number of calcium spikes is critical for triggering the Nod factor-signaling pathway (Sun et al. 2006; Miwa et al. 2006; Gutjahr and Paszkowski 2009).

The direct effect of JA and MeJA on induction of *nod* genes leading to increased Nod factor production has been described for *R. leguminosarum* and *B. japonicum* (Rosas et al. 1998; Mabood et al. 2006). In the case of *B. japonicum*-soybean symbiosis, jasmonates and genistein at 50 μM and 20 μM, respectively, applied alone or together with a Brady*rhizobium* promoted nodulation and N fixation under controlled and field conditions, at both optimal and suboptimal root zone temperatures. In the absence of these compounds, at suboptimal root zone temperature, nodulation, nitrogen fixation, and plant growth were inhibited (Mabood and Smith 2005; Mabood et al. 2008). The synergistic effect of jasmonate and flavonoids (naringenin, genistein) on nodulation, N_2 fixation, and biomass production was also observed for *R. leguminosarum*-bean (*Phaseolus vulgaris* L.) symbiosis (Poustini et al. 2007). These results suggest that both inducers, jasmonates and flavonoids,

utilize different receptors for signal transduction, and a concomitant activation of different regulatory mechanisms enhances the transcription of *nod* genes and Nod factor production (Mabood et al. 2006).

Within the variety of flavonoids, isoflavonoids, and other compounds secreted by lupin (*Lupinus albus)* roots into the rhizosphere, major proportion is composed of aldonic acids, that is erythronic acid (4-C sugar acids) and its analog tetronic acid. These compounds act as *nod* gene inducers of *R. lupini, M. loti,* and *S. meliloti*. Both aldonic acids in mM concentrations stimulated the expression of *nodC* gene in rhizobia. In addition, lupiwighteone, a genistein monoprenyl, added to cultures together with either aldonic acids exerted a synergistic effect on *nod* genes induction of *R. lupini*. Synergistic effect of luteolin and tetronic acid (but not erytronic acid) on *nod* induction was also observed for *S. meliloti*. Concomitantly with *nod* gene induction, the increase in LCO production was observed in the presence of both aldonic acids in *R. lupini* cultures, and of tetronic acid in *M. loti* and *S. meliloti* (Gagnon and Ibrahim 1998).

The next group of non-flavonoid inducers studied to date is betaines (stachydrine and trigonelline), N-methylated derivatives of aspartic acid and ornithine, secreted in large amounts from germinating alfalfa *(Medicago sativa L.)* seeds (Goldmann et al. 1991; Phillips et al. 1992). Stachydrine and trigonelline transcriptionally activated only the gene encoding the NodD2 protein but no apparent activation was reported for NodD1, which was activated by luteolin (Phillips et al. 1992). Betaine inducers, similarly to aldonic acids, function at a 10^3-fold higher concentration than required for *nod* induction with flavonoids. In *S. meliloti*-alfalfa symbiosis, using non-flavonoid molecules that are synthesized via a metabolic pathway distinct from those for flavonoid synthesis could be beneficial in the case of flavonoid soil depletion. Also, the good solubility of stachydrine and trigonelline in water may allow them to diffuse more easily than flavonoids within the soil, increasing the availability of these inducers (Phillips et al. 1992). The *nod* genes of *B. japonicum* can also be induced by xanthones (Yuen et al. 1995).

2.4 Nod Factor-Enhancement of Bacterium–Plant Symbiosis

In early studies, the manipulation of a number of common *nod* genes led to a reduced nodulation of *Vicia faba* inoculated with *R. leguminosarum* harboring a multicopy plasmid carrying *nodABC* genes (Knight et al. 1986). Following this, attempts were undertaken to improve nodulation and N_2-fixation in alfalfa plants by amplifying specific regions of the symbiotic plasmids of *S. meliloti* strains 41 and 1021 (Castillo et al. 1999). Amplified DNA fragments containing *nodD1* regulatory gene, the common nodulation genes (*nodABC*) and *nifN* gene essential for N_2-fixation, were introduced into *S. meliloti* genome by homologous recombination. Derivatives of *S. meliloti* with a moderately increased copy number of symbiotic genes (2.5–3 copies) showed better symbiotic properties and promoted plant growth under controlled conditions. When the number of copies of symbiotic

genes was set to about seven, the nodulation and N_2-fixation decreased (Castillo et al. 1999). These results suggested that the manipulation of structural or regulatory *nod* genes in rhizobia to increase their symbiotic activity is possible but only to some extent.

The key role of the Nod factor in early steps of symbiosis and its mitogenic and morphogenic activity leading to the formation of nodule primordium sparked several attempts to employ it to enhance legume productivity (Spaink et al. 1991). "Hormone-like" effect of purified *B. japonicum* Nod factor (nod Bj-V $C_{18:1}$,MeFuc) was observed in legume (soybean) and nonlegume plants (corn) (Souleimanov et al. 2002). The application of Nod factor at concentrations from 10^{-7} to 10^{-9} M stimulated biomass accumulation and enhanced growth and architecture of roots in host and nonhost plants indicating that the perception of LCO signal is conserved among a variety of species. This was further confirmed by identifying the genes of Nod factor cascade essential for mycorrhiza or an endosymbiosis in many higher plant species (Geurts et al. 2005). Further experiments showed that *B. japonicum* Nod factor stimulated germination of soybean and a variety of economically important plants belonging to diverse families under laboratory and field conditions (Prithiviraj et al. 2003). Stimulation of seed germination and seedling growth of several members of angiosperms suggests that specific receptors might also exist on seed surface or the developing embryonic roots, and Nod factor-induced genes may be present in genomes of numerous leguminous or nonleguminous plants (Prithiviraj et al. 2003). The chitin pentamer did not elicit such responses, which demonstrated that the structure of Nod factor plays a role in specificity of its biological activity. For legumes forming indeterminate nodules, Macchiavelli and Brelles-Mariño (2004) observed a noticeable increase in nodule numbers after treating the seeds of *M. truncatula* with submicromolar concentration of *S. meliloti* LCOs before inoculation. Moreover, clover seeds treated with specific LCOs before planting displayed a significantly enhanced nodulation and clover growth under competitive conditions in the presence of a mixture of chemical signals in the soil. Under these conditions, the symbiotic activity and competitiveness of *R. leguminosarum* bv. *trifolii* test strain were not improved suggesting that mitogenic activity of LCOs was solely responsible for the observed effects (Maj et al. 2009). Similar effect was observed for *B. japonicum* and soybean, which produce determinate nodules. Commercially, the addition of Nod factors into inoculants of *B. japonicum* to promote nodulation and plant growth of soybean has been applied by Nitragin Inc (Mabood et al. 2008).

2.5 Competitiveness in Natural Populations and Its Effect on Legume Crops

From among the several desirable characteristics in rhizobial strains that can be used as biofertilizers, the most essential are: (a) the ability to form nodules and to fix nitrogen in the host plant in a range of environmental conditions, and (b) the

ability to compete for nodulation with indigenous rhizobial population (Brockwell et al. 1995). The soil is an exceedingly complex habitat inhabited by heterogenous microbial communities, which interact with each other *via* different chemical compounds secreted into the soil. Among these microbial populations, rhizobia constitute "common soil inhabitants" in all climatic zones, from arctic to the tropics, and are commonly found in different types of soils (Robertson et al. 1995; Andrade et al. 2002; Fagerli and Svenning 2005). The correlation between the type of soils and the quantity of rhizobia have shown that the number of *R. leguminosarum*, *Sinorhizobium*, or *Bradyrhizobium* in most cases was 10^2–10^5 cells/g soil (Andrade et al. 2002; Martyniuk et al. 2005). The extensive variability in the soil environment may induce the emergence of diversity in rhizobial populations. Moreover, rhizobial diversity may be influenced by soil properties, such as the availability of N, P, Ca, the acidity, chemical stresses, or by agricultural management regimes (Palmer and Young 2000; Andrade et al. 2002; Laguerre et al. 2006). However, most of the rhizobial biodiversity studies were done with strains collected from a single or a few neighboring geographical regions. They revealed differences in allele frequency of selected genes (e.g., 16S rRNA, *nodD*, *nodEF*, *nifDK*) and sequences (16–23S rRNA ITS), which go in parallel with metabolic differences, for example the enzymatic profile or carbon/energy substrate utilization (Louvrier et al. 1996; Mutch and Young 2004; Silva et al. 2007). Further analysis of these traits allowed clustering the strains and finding correlation between genetic content and origin of the strains. Still, there is evidence that large scale biodiversity also exist within populations of rhizobia colonizing single plants (Wielbo et al. 2010).

Unlike soil, root nodules of legumes form a microenvironment not accessible to all rhizobia inhabiting rhizosphere probably because of the following: (1) the relatively high plant host-microsymbiont specificity as the nodules can be induced and consequently colonized only by rhizobia, which recognize and exchange suitable molecular signals with the host; and (2) variation in secreted Nod factors (Perret et al. 2000; Jones et al. 2007). Moreover, such rhizobia are not exposed to plant defense mechanisms, which are activated if bacteria are not recognized as symbionts, for example as a result of changes in lipopolysaccharide or exopolysaccharide structure (Campbell et al. 2002; Wielbo et al. 2004b). Rhizobia recognized as "suitable microsymbionts" are subjected to a selection process during plant tissue invasion and colonization in two ways – they are exposed to intense competition from other strains, and they are under some selective pressure from the plant host.

The effect of the plant host on the structure and composition of rhizobial population is not conclusive, but there are reports that legumes favor particular symbiotic genotypes of rhizobia (Mutch and Young 2004; Rangin et al. 2008). This relationship seems to be more complex, and even slight differences in genotype or developmental stage of plants may have an influence on the constitution of rhizobial populations in root nodules (Depret and Laguerre 2008). The molecular adjustment of the microsymbionts to their host may be observed as prevalence of particular symbiotic genotypes (versions of *nod* genes cluster) in rhizobia isolated from root nodules (Laguerre et al. 2003) or dependence between the susceptibility

of rhizobia for plant-derived flavonoid induction and competitive abilities of microsymbionts (Maj et al. 2010). For this reason, hypotheses about coevolution in *Rhizobium*–legume symbiosis have been proposed (Doyle 1998) and recently modified, emphasizing the effect of plants on the evolution of bacteria (Martínez-Romero 2009).

Independently of the plant host selection pressure on rhizobial populations, a lot of bacterial traits are involved in the process of competition between the individual strains, which run a race to the nodules (Vlassak and Vanderleyden 1997). The "external" environmental conditions, such as soil acidity, salinity, and nutrient availability strongly affect the vegetative growth of rhizobia in soil, thus setting up the initial conditions for rhizobial competition. A lot of data are available concerning the effect of single, defined factors such as acid tolerance (Vinuesa et al. 2003), presence of small cryptic plasmid (Bromfield et al. 1985), production of vitamins (Streit et al. 1996), and rhizopines (Murphy et al. 1987) affecting the competitive properties of rhizobia. The ability for utilization of specific C and energy sources, such as rhamnose (Oresnik et al. 1999) and homoserine (Hynes and O'Connel 1990), as well as the ability to metabolize the most variable set of substrates (including acids and amino acids) (Wielbo et al. 2007) have been proved to be important determinants of competitiveness. Moreover, the direct strain-to-strain antagonistic effect should also be taken into consideration as the effect of bacteriocin production on strain competitiveness was also reported (Robleto et al. 1998; Oresnik et al. 1999).

The competition between rhizobial strains does not vanish following the relocation of the bacteria from the soil into plant and remain present after the root colonization stage, possibly inside the infection threads (Duodu et al. 2009). Because rhizobia are immobile in the infection thread, the rate of bacterial growth inside can determine the rate of infection thread proliferation and subsequent nodule occupancy (Hoang et al. 2008; Duodu et al. 2009). Presence of multiple bacterial strains inside a single infection thread was shown by Stuurman et al. (2000) and Gage (2004). The C and N exchange between bacteroids and plant cells suggests that metabolic traits of rhizobia may also be important during this endophytic part of bacterial life cycle (Prell and Poole 2006; White et al. 2007). From "bacterial point of view," the aim of competition is to reach nodule compartments, which later on serve as a place for growth and massive multiplication. In indeterminate nodules, such a compartment is called saprophytic zone and is an ecological niche where rhizobia take advantage of the interaction with their plant host, escape plant controls, and their morphology, and some of the physiological traits become similar to these characteristic for a saprophytic (nonsymbiotic) stage (Timmers et al. 2000; Wielbo et al. 2009). In summary, the better the competitive abilities of a strain relative to the autochthonous strains of a local population, the more are the chances for colonization and multiplication in the nodules. And consequently, for the "return to the soil" after plant's vegetative period, albeit in higher number, which may lead to a dominance in the population. The success of a strain is also dependent on the diversity and total viable cell number of the local population. In rhizobia-rich soils, an introduced strain may be quickly dissipated into the

autochthonic population, and its "half-life" may not exceed 1–2 years (Jensen and Sorensen 1987). On the other hand, when the soil is depleted for rhizobia, the persistence of the introduced strain may exceed a few years, and the strain's good competitive abilities may enable a progressive elimination of rivals (Svenning et al. 2001).

One of the strategies that have been postulated a century ago and that has been a common practice for years to enhance legume nodulation and N_2 fixation is the introduction of rhizobial inoculants into the cultivated soil (Martínez-Romero 2003). Inoculation has enhanced plant growth and yield in the cases where even the specific rhizobia were absent or inefficient (Streeter 1994; Brockwell et al. 1995; Giller and Cadisch 1995). On the other hand, strain effectiveness and competitiveness are traits not linked to each other, and bacteria introduced as biofertilizer for target host plants might be outcompeted by autochthonous rhizobia abundant in the fields with little end success (Vlassak and Vanderleyden 1997; Burgos et al. 1999). Therefore, there is a need to investigate the strain competitiveness for nodulation as part of the process of converting a "potentially useful strain" into a "commercial inoculant strain". As discussed earlier, numerous individual traits affecting the competitiveness were identified and some recommendations for improving strain competitiveness have been made (Maier and Triplett 1996), and even genetically engineered strains with competitive abilities targeted in a specific manner were constructed (van Dillewijn et al. 2001). Moreover, promising mathematical models describing nodulation competitiveness were formulated (Beattie et al. 1989). On the other hand, original optimistic attempt to identify and clone the "nodulation competitiveness genes" had to be rejected, with the idea of "spontaneous genetic changes," which render the strains competitive (Beattie and Handelsman 1993) gaining prominence. The advances in understanding about individual metabolic traits responsible for competitiveness (Murphy et al. 1987; Hynes and O'Connel 1990; Streit et al. 1996; Robleto et al. 1998; Oresnik et al. 1999) has replaced the previous simple or very inaccurate theories, and shed some light on the network of factors involved. In this context, the role of point mutations underlying diversity and thus increasing the adaptive potentials of microbial species was confirmed (de Weert et al. 2004), resulting in inclusion of this phenomenon in the list of factors affecting competitiveness. It is possible that such small-scale evolution and/or recently discovered factors may explain some unsolved problems, for example, why strains selected for a particular trait (e.g., acid tolerant) and competitive under laboratory conditions are outnumbered in the "appropriate" (i.e., low pH) soils by indigenous strains (Gemel and Roughley 1993). The practical consequence of such issue requires a continuous search for identifying effective and competitive strains. Nowadays, more efforts are made in countries with weak agricultural practice (Africa or Southerneastern Asia) or with poor fertility of soils (Australia) (Lupwayi et al. 1997; Slattery and Pearce 2002), while in North America and European Union, such work is still conducted despite the availability of numerous patented and industrially-made rhizobial inoculants. They are often coupled with the investigation of problems relating to agricultural use of the inoculants: bacterial carriers, terms of storage, time and method of application,

etc. (Brockwell and Bottomley 1995; Herridge et al. 2002), which may strongly affect the viability and metabolic status of bacteria, and thus exert considerable effect on inoculant competitiveness and legume improvement.

2.6 Conclusions

Research to date has shown that the productivity of *Rhizobium*–legume symbiosis can be enhanced by manipulating bacterial and plant signals, selecting well-adapted bacteria, so that they could be introduced into soil or by modifying plant and bacterial activities. However, the complexity of competitiveness of rhizobia and plethora of factors influencing this phenomenon, which allow them to multiply and out-compete autochthonous rhizobia, still remains to be elucidated. The advanced global analyses of cellular state through approaches such as genomics, transcriptomics, and metabolomics have however made it possible to investigate whole microbial communities, which in turn, has enhanced further the knowledge of diversity on the genetic and metabolic levels. These are likely to result in better formulation of the inoculants applied as biofertilizers in legume production across different ecological regions. All strategies that positively influence legume biomass and global fixed N are important for sustainable agriculture especially when soils lack specific rhizobia or when their number is low. Nowadays, despite a myriad of "traditional" rhizobial inoculants available in the market, new-formulated biofertilizers supplemented with flavonoids or Nod factors needs to be designed and developed and further tested for the promotion of legumes productivity.

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Chapter 3
Key Molecules Involved in Beneficial Infection Process in Rhizobia–Legume Symbiosis

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Abstract The symbiotic relationships between nitrogen-fixing rhizobia and their legume hosts are the result of an intricate signaling network between the host and symbiont. The success of the symbiotic process depends on the competitiveness, specificity, infectivity, and effectiveness of rhizobia and follows a series of events, which are the result of the expression of different molecules from the bacteria, the host plant, or both partners. In this chapter, we review a serial of key molecules involved in the establishment of an efficient rhizobia–legume symbiosis and their role in the different steps of this process.

3.1 Introduction

Legumes establish symbiosis with diverse type of microorganisms able to infect plant cells through different mechanisms. The invasion of host tissues may result in the establishment of a disease process or not. Rhizobia able to originate nodules have the ability to infect plants, but not to cause disease and are, therefore, beneficial for the plant. This type of infection process requires a full coordination

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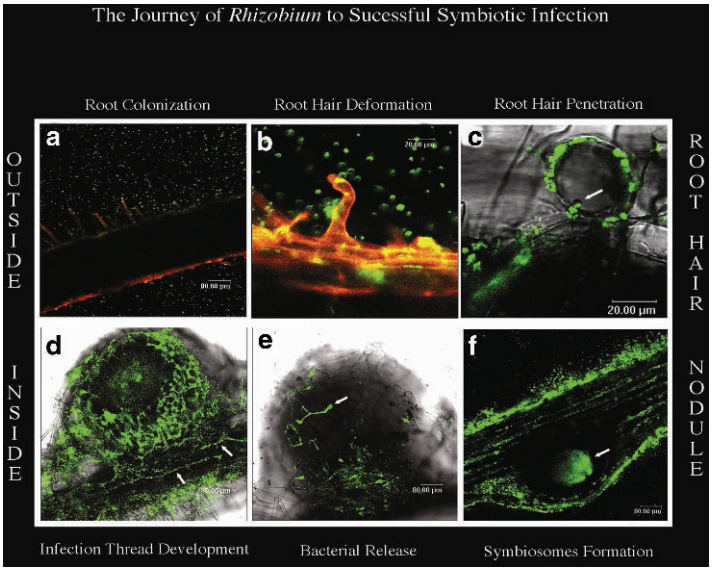

Fig. 3.1 The journey of *Rhizobium* to successful symbiotic infection. Cascade of events outside and inside the plant root for rhizobia–legume symbiosis establishment

between the macro and the microsymbionts. The rhizobia–legume symbiosis (Fig. 3.1) depends on the competitiveness, specificity, infectivity, and effectiveness of rhizobia and follows a series of steps, which are the result of the expression of different molecules by the bacterium, the host plant, or both (Table 3.1). Therefore, the symbiotic relationships formed between the nitrogen-fixing rhizobia and their corresponding legume hosts are the result of an intricate signaling network between the host and symbiont. Quorum sensing has been reported to be a key player in the symbiotic process (Daniels et al. 2002; Loh and Stacey 2003; Marketon et al. 2003) that leads to the concentration of bacteria in and around the plant roots and nodules. The rise in rhizobial cell density, as determined by quorum sensing, is therefore an important component of the signaling process (González and Marketon 2003). The quorum sensing has been studied in several rhizobia like *R. leguminosarum* bv. viciae (Lithgow et al. 2000; Blosser-Middleton and Gray 2001; Wilkinson et al. 2002; Wisniewski-Dyé et al. 2002; Danino et al. 2003; Cantero et al. 2006; McAnulla et al. 2007; Edwards et al. 2009), *Rhizobium etli* (Daniels et al. 2006; Braeken et al. 2008), *Sinorhizobium meliloti* (Pellock et al. 2002); *Mesorhizobium huakuii* (Wang et al. 2004), *Meshorhizobium loti* (Yan et al. 2007), or *Mesorhizobium tianshanense* (Zheng et al. 2006; Cao et al. 2009). Recently in the most promiscuous rhizobia, *Rhizobium* sp. strain NGR234, a remarkable number of secretion systems that allow its rapid adaptation to changing environmental stimuli in soil, rhizosphere, and plant have been detected. In this strain, at least six loci related to the quenching of quorum-sensing signals have been recently identified (Schmeisser et al. 2009). The quorum sensing in rhizobia has been recently

Table 3.1 Summary of main molecules involved in the rhizobia–legume symbiosis, their function and involvement in different stages of the process

Molecule	Released by	Function in rhizobia–legume symbiosis	Part of process in which it is involved
Flavonoids (flavones, isoflavones, flavanones, flavonols, anthocyanins)	Plant	Attraction of rhizobia by the plant	Initial symbiotic process stages
Lipochitin-oligosaccharides (Nod factors)	Bacteria	Activation of LysM receptor kinases	Initial symbiotic process stages
LysM receptor kinases	Plant	Recognition of bacteria by the host	Initial symbiotic process stages
Lectins	Plant	Attachment of bacteria to plant root	Early steps of plant infection
Cellulose	Bacteria	Enhancement of contact of bacteria to the root surface	Early steps of plant infection
Exopolysaccharides (Lipopolysaccharides, capsular EPS, acidic EPS, neutral beta 1-2 glucans)	Bacteria	Protection against stress, attachment to surfaces and nutrient gathering	Early steps of plant infection. Later stages of nodulation process (penetration of infection threads, nitrogen-fixing phenotype)
Polygalacturonases	Plant/ bacteria	Softening of root-hair wall	Infection thread formation
Cellulases	Bacteria	Erosion of root-hair wall	Penetration of bacteria into the plant root
Nodulines (early, late)	Plant	Maintenance of nodule function	Infection/invasion process Beginning of nitrogen-fixation activity
Nitrogenase	Bacteria	Reduction of nitrogen to ammonium	Atmospheric nitrogen fixation
Hidrogenases	Bacteria	Hydrogen recycling	Atmospheric nitrogen fixation

reviewed (Wisniewski-Dyé and Downie 2002; González and Marketon 2003; Sánchez-Contreras et al. 2007; Soto et al. 2009). It involves the use of acylated homoserine lactones (AHLs) as signal molecules which are a general mechanism found in Gram negative bacteria (González and Marketon 2003). This chapter highlights the role of specific molecules involved in the plant–rhizobia signal exchange during the nitrogen-fixing nodule formation in legumes.

3.2 Flavonoids and Nod Factors

During the early stages of infection, two types of molecules are involved, the flavonoids synthesized by plant and the nodulation factors produced by the micro-symbiont. Flavonoids are the principal signals released by the host and perceived by

rhizobia in soil and are derived from the flavonoid family (2-phenyl-1,4-benzopyrone derivatives) of secondary plant metabolites (Gibson et al. 2008). Specific flavonoids secreted by legume roots serve as chemoattractants for the rhizobial symbiont (Gulash et al. 1984) and act as signal molecules in the early stages of rhizobia–legume interactions (Broughton and Perret 1999; Wei et al. 2008) inducing the nodulation (*nod*) genes of rhizobia (Cooper 2007; Zhang et al. 2009). Nodulation genes are responsible for the synthesis of nod factors (lipochitin-oligosaccharides) that are receptors for the plant flavonoid signal (Schultze and Kondorosi 1998). The *nodD* gene inducers include different types of flavonoids such as flavones (Yeh et al. 2002; Zhang et al. 2009), isoflavones (Subramanian et al. 2006; Lang et al. 2008), flavanones (Recourt et al. 1991), flavonols (Maxwell et al. 1989; Hungria et al. 1991; Zhang et al. 2009), and anthocyanins (Hungria et al. 1991). Nevertheless, flavones and flavonols play different critical roles during nodulation of *Medicago truncatula* by *Sinorhizobium meliloti* (Zhang et al. 2009). Some flavonoids are anti-inducers of *nod* gene expression by competition, and inhibition may be overcome by increasing the inducer concentration (Djordjevic et al. 1987a, b). It has been reported that naringenin induces the expression of *nod* genes in *R. leguminosarum–Pisum sativum* interaction, whereas quercetin is an inhibitor of nodulation (Novák et al. 2002). The isoflavone daidzein can induce *nod* gene expression in *B. japonicum* (nodulating soybean) inhibiting by contrast the expression in *R. leguminosarum* strains nodulating clover (*Trifolium pratense)* or peas (*P. sativum*), thus contributing to host specificity (Andersen and Markham 2006). In the case of *S. meliloti-Medicago sativa* symbiosis, in the presence of luteolin, NodD1 exhibited increased binding to *nod* gene promoters compared to binding in the absence of luteolin. The flavonoids naringenin, eriodictyol, and daidzein, did not stimulate *nod* gene expression in *S. meliloti*, but stimulated an increase in the DNA binding affinity of NodD1 to *nod* gene promoters. In vivo competition assays demonstrate that these noninducing flavonoids act as competitive inhibitors of luteolin, suggesting that both inducing and noninducing flavonoids are able to directly bind to NodD1 and mediate conformational changes at *nod* gene promoters but only luteolin is capable of promoting the downstream changes necessary for *nod* gene induction (Peck et al. 2006).

Although *nod* genes expression may be induced by exogenous isoflavones, it has been showed that endogenous isoflavones are essential for the establishment of symbiosis between soybean (*Glycine max*) and *B. japonicum*. Expression of isoflavone synthase (IFS), a key enzyme in the biosynthesis of isoflavones, is specifically induced by *B. japonicum*. When IFS was silenced using RNA interference in soybean hairy root composite plants, these plants severely reduced nodulation. Surprisingly, pretreatment of *B. japonicum* or exogenous application to the root system of either of the major soybean isoflavones, daidzein, or genistein, failed to restore normal nodulation (Subramanian et al. 2006). Genistein from soybean has been recently reported to have much broader function than mere induction of *nod* genes since about 100 genes were induced in *B. japonicum* (Lang et al. 2008). Within a great variety of flavonoids including apigenin, daidzein, genistein, hesperetin, kaempferol, luteolin, naringenin, and rutin, hesperetin and naringenin

were found to be the most effective plant-to-bacteria signal molecules in the case of *R. leguminosarum* symbiosis with *P. sativum* and *Lens culinaris* (Begum et al. 2001). In the case of *R. galegae–Galega orientalis* symbiosis, an unidentified chalcone is the better inducer of *nodD1* gene, whereas apigenin or luteolin revealed a moderate induction potential (Suominen et al. 2003). Daidzein was found to induce 18 of the 19 nod-boxes harbored by the broad host-range strain *Rhizobium* sp. NGR 234 (Kobayashi et al. 2004).

In the last years, several flavonoid compounds have been identified in different legumes involved in plant–rhizobia interactions, for example, genistein, daidzein, and chrysin are involved in peanut–bradyrhizobia interaction (Taurian et al. 2008) or apigenin in *Methylobacterium nodulans–Crotalaria podocarpa* symbiosis (Renier et al. 2008).

Flavonoids bind bacterial NodD proteins, which are members of the LysR family of transcriptional regulators, and activate these proteins to induce the transcription of rhizobial genes (Perret et al. 2000; Barnett and Fisher 2006). The expression of these genes produces the Nod factors which were identified firstly in *S. meliloti* as modified lipo-chitooligosaccharide molecules and were considered as a primary morphogenetic signal for nodulation because it triggers on a compatible host the earliest stages of nodule development, including root-hair deformation and curling, as well as cortical cell divisions (Lerouge et al. 1990; Spaink et al. 1991). Nod factors consist of a backbone of β-1,4-*N*-acetyl-D-glucosamine residues, which can differ in number not only among bacterial species but also within the repertoire of a single species (Perret et al. 2000). Nod factors are *N*-acylated at the nonreducing terminal residue with acyl chains that may also vary among rhizobial species (Perret et al. 2000). These Nod factors are recognized by a specific LysM domain containing receptor kinases (Limpens et al. 2003). Besides the LysM receptor kinases, several other components of this Nod factor-induced signaling cascade have been identified. These components include the putative cation channel DMI1 (Ané et al. 2004), the leucine-rich-repeat-containing receptor kinase DMI2 (Limpens et al. 2005), and the calcium calmodulin-dependent kinase DMI3 (Lévy et al. 2004). The Nod factor receptors trigger a signal-transduction cascade that is essential for the induction of all early symbiotic events, including root-hair deformation, pre-infection thread formation, and the induction of cell division in the root cortex that marks the formation of the nodule primordium (Jones et al. 2007). However, the addition of purified compatible Nod factors to plant roots is not sufficient to cause the formation of tightly curled root hairs (shepherd's crooks), a complete differentiation of the infection thread and mature nodules indicating that it is not the only required effector produced by these symbionts to enter plant tissues and colonize plant cells (Gage 2002; Jones et al. 2007; Gibson et al. 2008).

Although Nod factor signaling is a nearly universal means of establishing the rhizobia nitrogen-fixing symbiosis with compatible legumes, exceptions are emerging. The recent genome sequencing of some photosynthetic *Bradyrhizobium* strains that form nitrogen-fixing nodules on the roots and stems of an aquatic host, *Aeschynomene sensitiva*, revealed that the common *nodABC* genes are absent in these species (Giraud et al. 2007). Thus, the host initiates nodule development in a

NF-independent manner and instead may respond to the secretion of bacterial purine derivatives with cytokinin-like activity, highlighting the importance that the host hormone balance plays in nodule formation (Gibson et al. 2008).

3.3 Lectins and Polysaccharides

After recognition of the bacteria by the host, a successful infection requires the bacteria to actively colonize the plant. During this process, several events are involved such as motility of rhizobia and their pronounced chemotactic responses toward specific compounds exuded by the compatible host legumes (Miller et al. 2007). Rhizobial root colonization is a dynamic, multiphase process including several nonspecific and host-specific events in which plant lectins and bacterial polysaccharides are involved (Dazzo et al. 1984).

The attachment to hair roots includes a first nonhost-specific interaction of rhicadhesin on rhizobial individual cells and the root-hair tip (Smit et al. 1992), followed by a more host-specific aggregation of immobilized cells at the root-hair tip mediated by excreted, multivalent host lectin (de Hoff et al. 2009). Lectins are defined as proteins that reversibly and nonenzymatically bind specific carbohydrates. They have been recently redefined by de Hoff et al. (2009) and are classified into different families according to their carbohydrate recognition domains (van Damme et al. 2004). Legume lectins constitute one of these families and bind different sugar residues, for example, concanavalin A, a lectin from *Canavalia ensiformis* binds glucose/mannose residues, the *Glycine max* agglutinin binds *N*-acetyl-D-galactosamine/galactose, and *Ulex europaeus* lectin binds L-fucose (de Hoff et al. 2009). The legume lectins is the most extensively studied group (Van Damme et al. 2004) and generally consist of two or four 25–30 kDa subunits that may present identical chains as for phytohemagglutinin (PHA; *Phaseolus vulgaris* L. lectin) or distinct, as occurs in the case of *P. sativum* L. lectin (Lioi et al. 2006).

In the 1970 decade, seed lectins were proposed as mediators of specificity (Hamblin and Kent 1973; Bohlool and Schmidt 1974), since they play a role in rhizobial binding to the plant roots (Kijne et al. 1997; van Rhijn et al. 2001; Hirsch 1999). The successful bacterial attachment to root hairs through plant lectins facilitates infection thread formation, a necessary requisite for effective root nodule development (de Hoff et al. 2009). Several experiments with transgenic plants supported that lectins facilitate rather than direct the symbiosis (van Rhijn et al. 2001; Sreevidya et al. 2005). According to these experiments, the rhizobia preferentially get attached to root-hair tips, a location where legume lectins are typically localized and the authors hypothesized that recognition of lectin and enhanced attachment by rhizobia led to structural modifications of the cell wall, similar to a model proposed by Kijne et al. (1997).

A second phase in the attachment process implies a significantly increased force of adhesion of attached rhizobial cells concurrent with the formation of extracellular microfibrils that enhance the degree of contact of bacteria to the

root-hair surface (Dazzo et al. 1984). The extracellular microfibrils made in vitro by *R. leguminosarum* bv. trifolii have been isolated and shown by chemical analysis to consist of microcrystalline cellulose (Napoli et al. 1975). However, the nature of the microfibrils associated with rhizobia firmly attached to the legume root epidermis is more difficult to define. The combined use of scanning electron microscopy, enzyme cytochemistry, and computer-assisted image analysis has provided direct in situ evidence of the cellulosic nature of the extracellular microfibrils extending from *R. leguminosarum* bv. trifolii cells colonizing white clover root epidermis (Mateos et al. 1995).

Besides lipochitooligosaccharides (nod factors) and cellulose, rhizobia produce surface polysaccharides that have nonspecific functions such as protection against environmental stress, attachment to surfaces or nutrient gathering and are also crucial for establishment of successful symbiosis with legumes in which the genes responsible for production of different types of cell-surface polysaccharides played a major role in the elongation of infection threads (see reviews by Fraysse et al. 2003; Skorupska et al. 2006). Involvement of polysaccharides in bacterial adherence to the tip of root hairs and the subsequent development of the infective process seems to be critical, because besides their role in competitiveness of strains it has been demonstrated that mutants defective in their biosynthesis are characterized by low infectivity, a low capacity for nodulation, and in some cases, changes in the host range (Gibson et al. 2008).

Rhizobial exopolysaccharides (EPS) are species-specific heteropolysaccharide polymers composed of common sugars that are substituted with noncarbohydrate residues. Synthesis of repeating units of EPS, their modification, polymerization, and export to the cell surface is controlled by clusters of genes, named *exo*/*exs*, *exp*, or *pss* that are localized in rhizobial megaplasmids or chromosome. Production of EPS is influenced by a complex network of environmental factors such as phosphate, nitrogen, or sulfur (Skorupska et al. 2006). They are involved in the establishment of rhizobia–legume symbiosis and numerous evidences indicate also that they play important roles in protection against the host defense (D'Haeze and Holsters 2004).

Polysaccharides produced by rhizobia may be of different types, such as lipopolysaccharides (LPS), capsular polysaccharides (CPS or K-antigens), acidic EPS, and neutral beta-1,2-glucans. CPSs form an adherent cohesive layer on the cell, whereas EPSs and cyclic beta-glucans are loosely attached to the surface of the outer bacterial membrane. Cyclic beta-glucans are thought to be predominantly present in the periplasmic space, although they are also secreted under certain circumstances. LPSs are anchored in the outer membrane and are constituted by lipid A, a core oligosaccharide, and an O-antigen polysaccharide (Fraysse et al. 2003; Skorupska et al. 2006).

The acidic EPSs are high-molecular mass complex heteropolymers with repeating units ranging from seven to nine hexose residues. The glycosidic linkage is variable, which can be alpha, beta linear, or branched with side chains. Also, the EPSs mostly contain noncarbohydrate substituents such as succinate, pyruvate, or acetate and their acidic nature is due to the presence of uronic acids, pyruvate

ketals, and succinates. Although the role of EPS in rhizobial legume symbiosis is not well known, they could be involved in protecting bacteria against environmental stresses, in early steps of plant infection, such as attachment of bacteria to the roots, in the infection thread formation and release of bacteria from infection threads, bacteroid development, suppression of plant defense responses, and protection against plant antimicrobial compounds (Fraysse et al. 2003). EPSs appear to be essential for the early infection process and may be involved in nodule ontogenia (Gray et al. 1991), which suggest a key role for the establishment of nitrogen-fixing symbiosis on legume developing indeterminate nodules such as *S. meliloti*-alfalfa, *R. leguminosarum* bv. viciae/*Vicia sativa*, and bv. trifoli/*Trifolium*, ssp. and strain NGR234/*Leucaena* (Djordjevic et al. 1987a, b). This is not the case for associations leading to determinate nodules such as *S. fredii-Glycine max* and *R. etli*/*Phaseolus* ssp. (Diebold and Noel 1989; Kim et al. 1989). In these cases, other polysaccharides such as LPS could complement the EPS deficiency in the determinate nodule formation. Numerous works demonstrating different rhizobial EPS requirements between the two basic nodule ontogenies have been reviewed by Fraysse et al. (2003).

The cyclic beta-(1,2)-glucans were mostly studied in the extracellular medium of rhizobial cultures and their high level as cell-associated saccharides was ignored (Fraysse et al. 2003). They are predominantly localized in the periplasmic compartment (Breedveld and Miller 1994) and consist of a neutral homopolymer of about 20 beta-(1,2)-linked glucose residues – often substituted by phosphoglycerol, phosphocholin, or succinyls – and probably play a passive role in the bacterial cell adaptation to hypo-osmotic conditions in its surroundings (Chen et al. 1985). Nevertheless, the possible involvement in some aspects of the symbiotic interactions has been shown in several works (Breedveld and Miller 1994, 1998; Fraysse et al. 2003; Skorupska et al. 2006).

CPSs surround the bacterium and constitute a hydrated matrix, which confers bacterial resistance to bacteriophages and to the dry conditions often encountered in the rhizosphere environments (Fraysse et al. 2003). These polysaccharides contain a high proportion of 3-deoxy-D-manno-2-octulosonic acid (Kdo) and are structurally analogous to one subgroup of K antigens found in *Escherichia coli* (Reuhs et al. 1993). Basically, all known KPSs have been described from *Sinorhizobium* species (Reuhs et al. 1993; Forsberg and Reuhs 1997; Forsberg and Carlson 1998). They differ by size range and contain a repetitive motif of a hexose linked with a Kdo or related 1-carboxy-2-keto-3-deoxy sugars such as pseudaminic and neuraminic acid. According to Reuhs and collaborators (Reuhs et al. 1993, 1995), the K-antigens are strain-specific antigens, whereas the EPS are conserved within a species, the production of one or more KPS could be dependent on the growth conditions of cultured cells and are only located directly around the bacterial membrane and not secreted outside. It is possible that KPS mediates the contact between legume and rhizobia (Fraysse et al. 2003; Skorupska et al. 2006).

Lipopolysaccharides exhibit specific roles in the later stages of the nodulation process such as penetration of the infection thread into the cortical cells or the setting up of the nitrogen-fixing phenotype (Fraysse et al. 2003). LPSs are

polysaccharides that are attached to the membrane by a lipidic part inserted into the bacterial phospholipid monolayer, the saccharidic part being oriented to the exterior. The general structure of this compound consists of an anchor named lipid A associated with a core polysaccharide, which can bear an O-antigen domain. Rough LPS are formed by the first two domains, while the smooth LPS are formed by the three domains. LPS are acidic polysaccharides found in the aqueous phase when extracted by the hot water/phenol method. However, they can be partitioned between the two phases, as, for example, the LPS from *B. japonicum* 61A123 (Carrion et al. 1990). Dazzo and coworkers demonstrated that *R. trifolii* LPS plays an important role by modulating infection thread development in white clover root hairs (Dazzo et al. 1991). Although LPS is a constitutive component of the bacterial membrane, it could be found in very low concentrations in growth media. Consequently, a putative role from a distance or in the early steps of symbiosis could be attributed to rhizobial LPS. The importance of LPS in the rhizobia–legume symbiosis has been widely explored (Fraysse et al. 2003; Becker et al. 2005). Despite the numerous studies suggesting the involvement of rhizobial EPS in the establishment of symbiosis, no conclusive evidence on the specific role of such molecules in this process is available. Nevertheless, the sequencing of complete genomes of different rhizobia is likely to facilitate the identification of genes involved in the synthesis of EPS and the understanding of their function during the establishment of nitrogen-fixing legume symbiosis.

3.4 Cellulases and Polygalacturonases (Pectinases)

A key event of the infection process required for development of the *Rhizobium*–legume root-nodule symbiosis is the passage of the bacteria across the root-hair wall. The process of wall degradation must be delicately balanced in order for the slow, localized penetration of the bacterial symbiont to occur without overdestruction of the root hair and subsequent abortion of the infection process. Several hypotheses have been proposed to explain how this event occurs. For example, Nutman et al. (1973) proposed that rhizobia redirect growth of the root-hair wall from the tip to the localized site of infection, causing invagination rather than penetration of the root-hair wall. Ljunggren and Fahraeus (1961) proposed that homologous *Rhizobium* strains specifically induce the host plant to produce polygalacturonases, which soften the root-hair wall at the site of infection, thus allowing the bacteria to penetrate between microfibrils to the cell membrane and initiate an infection thread. The third model (Hubbell 1981) proposes that wall-degrading enzymes produce a localized degradation that completely traverses the root-hair wall, allowing direct penetration by the bacteria. Electron microscopic studies support the hypothesis that hydrolytic enzymes are involved in various steps in the infection process, including the entry of rhizobia into root hairs, the crossing of root cortical cell walls by rhizobia in the advancing infection thread, and the release of rhizobia from infection threads into the nodule cell cytoplasm. The

strongest evidence for involvement of wall hydrolysis in the *R. leguminosarum* bv. trifolii-white clover infection process was obtained by Callaham and Torrey (1981), who reported a localized degradation of the root-hair wall coinciding with the deposition of a new wall layer, above the site of degradation, which is continuous with the root-hair wall. From this point two hypotheses have been developed, the possibility that the wall-degrading enzymes involved in the process are associated with the bacteria or locally induced in the plant by components of the bacteria.

Favorable data for "plant hypothesis" have been reported for *S. meliloti-Medicago* symbiosis (Muñoz et al. 1998). Specific genes encoding polygalacturonase (MsPG3) are expressed in all cells of nodule primordia and in the cells of the invasion zone in *M. truncatula*. This finding suggests the involvement of MsPG3 gene in cell wall rearrangement, penetration of the bacteria through the root-hair wall, or infection thread formation and release of bacteria in plant cells. Preferential accumulation of an MsPG3-GFP fusion protein in the tip of the growing root hair at different developmental stages was found, confirming the delivery of MsPG3 to the newly synthesized cell wall (Rodríguez-Llorente et al. 2003). Later studies showed that the symbiotic *MsPG3* and *MtPER* genes from *M. truncatula* could have, as ancestors, pollen-expressed genes involved in polar tip growth processes during pollen tube elongation. Moreover, they could have been recruited after gene duplication in the symbiotic interaction to facilitate polar tip growth during infection thread formation (Rodríguez-Llorente et al. 2004).

Extending the role of hydrolytic enzymes in the active penetration of plant cell walls by some pathogenic microorganisms, McCoy (1932) was the first to investigate the possible involvement of hydrolytic enzymes in the infection of legumes by rhizobia. She found no evidence for these enzymes from rhizobia, though sensitive procedures to detect minute amounts of cell wall-degrading enzymes were not yet available. Later, several studies detected pectinolytic (Hubbell et al. 1978), cellulolytic (Morales et al. 1984), and hemicellulolytic (Martínez-Molina et al. 1979) enzyme activities from pure cultures of rhizobia. In general, the activities of these rhizobial enzymes are very low and at the limit of sensitivity of conventional reducing sugar assays. Using improved, reliable assays of increased sensitivity (Mateos et al. 1992), we established that cellulases are produced by all of the official type strains of rhizobia (Jiménez-Zurdo et al. 1996b; Robledo et al. 2008). In contrast, polygalacturonase is less commonly found in rhizobia (Jiménez-Zurdo et al. 1996b). The model wild-type strain of clover-nodulating rhizobia, *R. leguminosarum* bv. trifolii ANU843, produces atleast two cell-bound cellulase isozymes, CelC1 and CelC2 (Mateos et al. 1992). By use of pSym-plasmid-cured and *nod*-recombinant derivatives of ANU843, the cellulase CelC1 gene locus was localized to the symbiotic plasmid (pSym), outside the *nod* region, whereas the cellulase CelC2 gene locus was not located on the pSym (Jiménez-Zurdo et al. 1996a). Using a combination of phase contrast/polarized light microscopy and enzymology, we found that only cellulase CelC2 can completely erode the root-hair wall at a highly localized site on the isotropic, noncrystalline apex of the root-hair tip ("Hole on the tip", or "Hot" phenotype), and can more extensively degrade clover root-hair walls

when grown in the presence rather than the absence of chitolipooligosaccharide Nod factors from clover rhizobia (Mateos et al. 2001). Other studies have shown that Nod factors from clover rhizobia induce a localized disruption in the normal crystallization of the host cell wall architecture in growing root hairs (Dazzo et al. 1996). These and other associated data suggest a complementary role of rhizobial cellulases and Nod factors in promoting root-hair infectibility at strategic sites during primary-host infection.

Rhizobial CelC2 is a 1,4-β-D-endoglucanase (EC 3.2.1.4) with high substrate specificity for noncrystalline (amorphous) cellulose. It has an approximate molecular mass of 33.2 KDa, an optimal pH of 5, a maximal rate of carboxymethylcellulose hydrolysis at 40°C, and an apparent Km of 84.4 mg/ml for CMC as the substrate (Robledo et al. 2008). These biochemical characteristics restrict (and hence tightly controls) the symbiotically relevant activity of CelC2 cellulase during primary host infection. The cell-bound location (rather than largely excreted), the high Km value, and the relatively low activity of CelC2 cellulase are all characteristics that would restrain its degradative action on roots, thereby minimizing indiscriminate host cell lyses and death. These characteristics provide further opportunity to restrict its short-range action based on physical positioning of the bacterium at the host wall interface. The specificity of CelC2 cellulase for noncrystalline cellulose significantly restricts its in vivo site of erosive action to the highly localized root-hair infection site that lacks crystalline wall architecture. The pH 5 optimum for CelC2 cellulase is consistent with the slightly acidic pH at the external surface of white clover root hairs. Finally, the host plant specificity exhibited by the Hot biological activity of CelC2 cellulase, which includes the compatible white clover legume but excludes the heterologous, nonhost legume alfalfa, is consistent with the host specificity of infection thread formation in legume root hairs (Robledo et al. 2008).

Purified CelC2 can completely erode the highly localized noncrystalline tip of the host root hair, forming a complete hole whose geometry and location match the entry point of primary host infection into white clover. CelC2 knockout mutants are unable to breach the host wall at the root-hair tip, form infection threads within the host root hair and induce effective nodules, indicating that this bacterial enzyme is absolutely required for development of the nitrogen-fixing *R. leguminosarum* bv. trifolii-white clover symbiosis. Transfer of the cloned wild-type gene into the CelC2 knockout mutant restored the symbiotic phenotypes, providing further compelling evidence for the requirement of this enzyme in successful development of the canonical *Rhizobium*–white clover symbiosis (Robledo et al. 2008). Molecular genetic analysis shows that *celC*-encoded protein is homologous to other rhizobial endoglucanases (*R. leguminosarum*, *R. etli,* and *S. medicae*). Interestingly, these *celC* genes are located near putative cellulose synthase genes confined to a region of the chromosome (*celABC*) involved in bacterial cellulose biosynthesis. This finding raises the possibility of new infection strategies based on cellulases associated with cellulose biosynthesis with a view to avoiding the elicitation of plant defenses.

3.5 Early Nodulins and Leghemoglobins

Nodulins are expressed proteins in the root nodules although some of them have been found in other parts of the plant being highly up-regulated during symbiosis. The early nodulins are generally expressed during the infection and invasion process whereas the late nodulins are expressed at the beginning of measurable nitrogen fixation activity and are involved in nodule function and maintenance. Several early nodulins are involved in signaling during the early stages of *rhizobia*–legume symbiosis, such as those codified by the plant genes *enod40, enod2, and enod12* (Bladergroen and Spaink 1998; Bahyrycz and Konopinska 2007; Laporte et al. 2007; Hashimoto et al. 2008). The expression of the early nodulin genes is elicited by the Nod factors produced by rhizobia (Horvath et al. 1993; Crespi et al. 1994; Stacey et al. 2006; Mathesius 2009). Studies on the role of nodulin genes encoding proteins in plant development and nodule organogenesis have been conducted in several legumes such as *Glycine* (Kouchi and Hata 1993; Matvienko et al. 1994; Girard et al. 2003), *Pisum* (Matvienko et al. 1994), *Medicago* (Asad et al. 1994; Sousa et al. 2001; Campalans et al. 2004; Wan et al. 2007), *Vicia* (Vijn et al. 1995), *Phaseolus* (Papadopoulou et al. 1996), *Lotus* (Flemetakis et al. 2000, Niwa et al. 2001; Kumagai et al. 2006; Gronlund et al. 2005), *Trifolium* (Varkonyi-Gasic and White 2002; Crockard et al. 2002), or *Lupinus* (Podkowinski et al. 2009). The nodulin codified by *enod40* gene are not exclusive of legumes (Kouchi et al. 1999; Vleghels et al. 2003) but is one of the earliest nodulins to be expressed upon *Rhizobium* inoculation whose role as "riboregulator" of the *enod40* genes during plant development was proposed by Crespi et al. (1994). Later, Charon et al. (1997) showed that the early nodulin gene *enod40*, which encodes a small peptide comprising 12 or 13 amino acids, induces dedifferentiation and division of root cortical cells in *Medicago*. Therefore, it was proposed that *enod40* gene is involved in the initiation of root nodule organogenesis in legumes. A year later, the involvement of *enod40* gene in the nodule development on stems of *S. rostrata* inoculated with *A. caulinodans* was also reported (Corich et al. 1998). Recently, it has been reported that RNAi knock-down of *enod*40s leads to significant suppression of nodule formation in the model legume *Lotus japonicus* (Kumagai et al. 2006). Based on the data from Rohrig et al. (2002), soybean *enod40* encodes two peptides that bind to sucrose synthase and may be involved in the control of sucrose use in nitrogen-fixing nodules.

There are two types of late nodulins, metabolic nodulins and symbiosome membrane nodulins. Metabolic nodulins include leghemoglobin, uricase, which is a key enzyme of uriede biosynthesis, glutamine synthetase, which catalyzes the first step in ammonium assimilation, and sucrose synthase, which catalyzes the cleavage of sucrose to begin the pathway to produce carbon metabolites for bacteroid energy production. In addition, several enzymes have been detected in root nodules of legumes that differ in their physical, kinetic, and immunological properties from the corresponding root enzymes: phosphoenolpyruvate carboxykinase, choline kinase, xanthine dehydrogenase, purine nucleosidase, and malate dehydrogenase. These

enzymes may not be true nodulins but rather posttranslational modifications of the root enzymes. Symbiosome membrane nodulins originate from the plasma membrane with modifications due to coalescence with golgi vesicles. The symbiosome membrane serves as the interface between eukaryotic and prokaryotic symbionts and thus is expected to possess transporters for nutrient exchange. Examples of soybean symbiosome membrane nodulins include nodulin 24 and 26. Nodulin-24 is synthesized as a lumenal protein in the endoplasmic reticulum and posttranslationally attached to the membranes *en route* to the symbiosome membrane (Cheon et al. 1994). Nodulin 26 is an aquaporin channel with a modest water transport rate (Guenther et al. 2003). Phosphorylation of nodulin 26 on Ser-262, which is catalyzed by a symbiosome membrane-associated calcium-dependent protein kinase, stimulates its intrinsic water transport rate. Phosphorylation is sustained at steady-state levels until entry into senescence.

Hemoglobins (Hbs) are hemoproteins that reversibly bind O_2 being the transport of this gas in different organisms their main function (Appleby 1992; Ross et al. 2002, Kundu et al. 2003, Garrocho-Villegas et al. 2007, Hoy and Hargrove 2008). Three types of Hbs have been identified in plants: symbiotic (sHb), nonsymbiotic (nsHb), and truncated (2/2) Hbs (tHbs) (Ross et al. 2002). The first identification of Hbs in plant was reported in legume nodules by Kubo (1939), who concluded that the physiological role of the hemoprotein in nodules is to stimulate the assimilation and transport of O_2. Kubo's plant Hb was named as leghemoglobin (Lhb) by Virtanen and Laine (1946), and it is also known as plant symbiotic Hb. Further analyses showed that Kubo's hemoprotein is a plant Hb with similar (i.e., structural) properties to animal Hbs.

For many years, Hbs were identified in nitrogen-fixing legumes and were called leghemoglobins (Lhb), because they were first discovered in legumes. The role of Lhb in nitrogen-fixing nodules was elucidated by Wittenberg and coworkers (Wittenberg et al. 1974). The function of Lhb in nodules is to facilitate the diffusion of O_2 to bacteroids at an internal concentration too low to inhibit or destroy their O_2-sensitive nitrogenase. This concept of high O_2 flux at low free O_2 in the bacteroid vicinity is now generally accepted. Although sHb were initially found in legumes, sHbs have been also found in the root nodules of nonlegumes such as *Parasponia andersonii* in symbiosis with *Rhizobium*, and dicotyledoneous plants such as *Casuarina glauca* in symbiosis with the actinobacteria *Frankia* (Appleby et al. 1998). Tjepkema (1983) detected high concentrations of Hb-like proteins in nodule extracts of actinorhizal plants (which are nodulated by *Frankia*), such as *C. cunninghamiana* and *Myrica gale*, and low concentrations in nodules of *Comptonia peregrina*, *Alnus rubra,* and *Eleagnus angustifolia*. The spectral properties of actinorhizal Hbs are similar to those of Lhbs and nonplant Hbs.

The origin of plant Hbs was proposed to be due to a unique act of horizontal gene transfer (HGT) from a phytophagous insect to a primitive legume via viral vector (Jeffreys 1982). Nevertheless, leghemoglobins are encoded by genes that are interrupted by three introns (Jensen et al. 1981) and *hb* gene from the insect *Chironomus* contains no introns (see Appleby et al. 1998). Therefore, this horizontal hypothesis was discarded in favor of the vertical hypothesis supported by the identification of

an Hb in the nitrogen-fixing root nodules of the nonlegume dicotiledoneous plant *Parasponia* (Appleby et al. 1980). *Parasponia* Hb is similar to those of other legumes (Gibson et al. 1989) suggesting that the function in *Parasponia* nodules is similar to that of Lhb in legume nodules, that is, to facilitate the diffusion of O_2 to bacteriods. The detection of sHbs in *Parasponia*, *Trema*, and actinorhizal plants suggested that plant Hbs are widespread in land plants and that plant *hb* genes vertically evolved from a common ancestor. Also, the detection of Hb transcripts in *Parasponia* and *Trema* roots showed that *hb* genes are expressed and Hbs probably function in nonsymbiotic plant organs.

Although Hbs are abundant in nodules playing a crucial role in nitrogen fixation (Ott et al. 2005), nsHb have been found in many nonlegume plants such as barley, rice, maize, wheat (Taylor et al. 1994), actinorrhizal plants *Causarina* (Jacobsen-Lyon et al. 1995), and leguminous plants such as soybean, clover, alfalfa, and pea (Andersson et al. 1996). This confirmed their existence in monocot and dicot plants including legumes and strengthened the theory that Hbs have an ancient origin and are ubiquitous in the plant kingdom through vertical evolution.

NsHbs differ from sHbs and mammalian Hb and myoglobins (Mb) in that they are generally "hexacoordinate" in both the ferric and ferrous states due to a histidine in the distal pocket that reversibly binds the sixth coordination site of the hemo iron (Arredondo-Peter et al. 1997). Two classes (classes 1 and 2) of nsHbs have been distinguished using phylogenetic analysis and shown to differ in their patterns of expression (Trevaskis et al. 1997). Despite hexacoordination, class 1 nsHbs have high oxygen affinities and low oxygen dissociation rate constants (Arredondo-Peter et al. 1997; Hoy et al. 2007) due to stabilization between the distal histidine and the bound ligand akin to that in Mb (Arredondo-Peter et al. 1997; Das et al. 1999). Class 2 nsHbs have lower oxygen affinities and greater similarity to sHbs than nsHbs, consistent with the observation that most sHbs evolved from class 2 nsHbs (Trevaskis et al. 1997). In addition to Lhbs and nsHbs, Hb sequences that are similar to those of microbial truncated (2/2) Hbs (Pesce et al. 2000 and Wittenberg et al. 2002) detected in primitive and evolved plants. However, the function of 2/2-like Hbs in plant organs is not yet known, although kinetic properties of a recombinant *Arabidopsis* 2/2-like Hb suggest that these proteins may function as O_2-carriers (Watts et al. 2001).

3.6 Nitrogenase and Hydrogenase

Within symbiosomes, rhizobia are differentiated morphologically and biochemically to bacteroids, which can derepress nitrogenase, the enzyme complex that reduces atmospheric N to ammonium. The reduction of atmospheric dinitrogen to ammonium is the primary function of the symbiosis. Nitrogenase catalyzes the MgATP-dependent reduction of N_2 to ammonia:

$$N_2 + 8e^- + 16\,\mathrm{MgATP} + 8H^+ \rightarrow 2NH_3 + H_2 + 16\,\mathrm{MgADP} + P_i.$$

The most studied nitrogenase contains two metallocomponents, dinitrogenase [molybdenum-iron (MoFe) protein] and dinitrogenase reductase (Fe protein). Nitrogenase requires an electron donor and a minimum of 16 mol of ATP per mole of N_2, the energy needed for the complete nitrogen-fixation process is around 40 mol of ATP per mole of N_2, or in terms of carbon: 6 g of carbon for every gram of N reduced (Vance and Heichel 1991). Electrons are generated in vivo either oxidatively or photosynthetically, depending on the organism. These electrons are transferred to flavodoxin or ferredoxin, a (4Fe–4S)-containing electron carrier that transfers an electron to the Fe protein of nitrogenase, beginning a series of oxido-reduction cycles. Two molecules of MgATP bind to the reduced Fe protein and are hydrolyzed to drive an electron from the Fe protein to the MoFe protein. The actual reduction of N_2 occurs in the MoFe protein in a multistep reaction. Electron transfer must occur six times per each fixed N_2 molecule. Therefore, a total of 12 ATPs are required to fix one N_2 molecule, but as nitrogenase also reduces protons to H_2 consuming two electrons, the total cost of N_2 reduction is eight electrons transferred and 16 MgATPs (Cheng 2008).

In nitrogen-fixing bacteria, nitrogenase is encoded by a set of operons that includes regulatory genes (such as *nifLA*), structural genes (such as *nifHDK*), and other supplementary genes. The free-living diazotrophic bacterium, *Klebsiella pneumoniae*, has been the most extensively analyzed and provides a model for studies of nitrogenase regulation, synthesis, and assembly. A 24-kb DNA region contains the entire *K. pneumoniae nif* cluster, which includes 20 genes (Dos Santos et al. 2004).

Although there are several different types of nitrogenases, rhizobia possess only the molybdenum-containing type (Howard and Rees 2006). The Mo nitrogenases are composed of two proteins: a MoFe protein and a Fe protein. The MoFe protein is a 220–240 kDa tetramer, a $(\alpha\beta)_2$ complex, of the *nif*D (α-subunit) and *nif*K (β-subunit) gene products each of which contains complex metalloclusters. Each tetramer of two $\alpha\beta$ pairs contains two P-clusters [Fe_8S_7] and two FeMo cofactors. The FeMo cofactor, located within the α-subunit, consists of a $MoFe_3$–S_3 cluster bridged to a Fe_4–S_3 cluster by three sulfur ligands with a homocitrate co-ordinated to the molybdenum (Howard and Rees 2006). The Fe protein is $\sim$60 kDa dimer of the *nif*H gene with a single 4Fe–4S cluster located between the subunits. A MgATP-binding site is located on each subunit (Howard and Rees 2006). During catalysis, electrons are delivered one at a time from the Fe protein to the MoFe protein in a reaction coupled to the hydrolysis of 2 MgATP for each electron transferred (Rees and Howard 2000). The P-clusters are thought to mediate electron transfer from the Fe protein to the FeMo-cofactor of the MoFe protein, the site for substrate binding and reduction.

Hydrogen evolution is an inherent step of the catalytic mechanism of nitrogenase (Simpson and Burris 1984) being the nitrogen-fixation process one of the most relevant biogenic hydrogen sources (see review by Palacios et al. 2005). For the *Rhizobium*–legume symbiosis, over 1 million tonnes of hydrogen/year was evolved from root nodules into the air (Evans et al. 1987). A number of strains of the slow-growing rhizobia possess hydrogenases that recycle the hydrogen thereby

recapturing some of the lost energy (Albrecht et al. 1979; Baginsky et al. 2002). Until now the hydrogenase system has been characterized in a limited number of rhizobia such as *Sinorhizobium meliloti* (Ruiz-Argüeso et al. 1979), *Rhizobium leguminosarum* (Hidalgo et al. 1990; Rey et al. 1993), *Bradyrhizobium* (van Soom et al. 1993; Baginsky et al. 2005), *Azorhizobium caulinodans* (Baginsky et al. 2004). Although the hydrogen-uptake system of *Rhizobium leguminosarum* bv. viciae UPM791 has been analyzed in detail (Ruiz-Argüeso et al. 2000), only few strains have this system (Fernández et al. 2005). However, sequencing of some regions of Hup cluster showed that they are conserved in these strains (Fernández et al. 2005). In *Rhizobium leguminosarum* bv. viciae UPM791, the hydrogen-uptake system is based on a membrane-bound, heterodimeric [NiFe] hydrogenase (Hidalgo et al. 1990; Brito et al. 2002) similar to those described in *Bradyrhizobium japonicum* and other aerobic bacteria. In these bacteria, electron flow from hydrogen to oxygen results in the generation of ATP, although for *R. leguminosarum* the degree of coupling is variable for the different strains (Ruiz-Argüeso et al. 2000). *R. leguminosarum* hydrogenase contains two subunits: a large subunit (HupL) of approximately 60 kDa carrying the heterometallic FeNi active center and a small subunit (HupS) of approximately 30 kDa harboring three FeS clusters. As in other bacteria, the synthesis of this enzyme is a complex process that occurs in the cytoplasm through the concerted action of over 15 proteins (*hup* and *hyp* gene products). Functions ascribed to the proteins involved in hydrogenase synthesis and activity includes electron transport (HupC), processing of large subunit (HupD), nickel provision (HypAB), and synthesis of NiFe cofactor (HypFCDE). In *R. leguminosarum* bv. viciae, hydrogenase genes (*hupSLCDEFGHIJKhypABFCDEX*) are clustered in a 20-kb DNA region of the symbiotic plasmid (Leyva et al. 1990). This plasmid also contains genes for nodulation and nitrogen fixation. The location of hydrogenase genes in the symbiotic plasmid is a general trait for hydrogenase positive strains of *R. leguminosarum* (Leyva et al. 1987), suggesting an adaptation of hydrogen recycling to the symbiotic life style in this bacterial species. The expression of *R. leguminosarum* hydrogenase activity is affected by the environment surrounding the bacteroid, and permissive (*Pisum*, *Vicia)* and nonpermissive *(Lens*) hosts for hydrogenase activity in bacteroids have been described (López et al. 1983). Recent studies suggested the existence of a plant-dependent mechanism that affects hydrogenase activity during the symbiosis by limiting nickel availability to the bacteroid (Brito et al. 2008). Also, the hydrogenase activity is limited by the availability of nickel in agricultural soils (Ureta et al. 2005).

3.7 Conclusion

Appart from the molecules reviewed in this chapter, there are several other molecules involved in nodule formation induced in legumes by rhizobia, since this is a very complex process not completely understood yet. Further investigation will

probably lead to the discovery of many other molecules essential for an effective nitrogen-fixing symbiosis establishment between rhizobia and legumes. The knowledge of these molecules will undoubtedly contribute to the better understanding of this fascinating ecological event, and therefore to the comprehensive management of the symbiosis for an enhanced legume production and environment protection in a sustainable agriculture context.

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Chapter 4
Recent Advances in *Rhizobium*–Legume Interactions: A Proteomic Approach

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Abstract Nitrogen-fixing symbioses between legumes and rhizobia over the years have played a major role in sustainable agricultural ecosystems. Owing to specific interactions with rhizobia, the leguminous plants form specialized nitrogen-fixing organ called as nodule, wherein rhizobia dwell and bring out the conversion of atmospheric nitrogen (N) to its usable form. This symbiosis in turn may abate the demand for external application of nitrogenous fertilizers while growing legumes under natural soil environment. Contemporary genomic research has provided a better understanding of the *Rhizobium*–legume interaction at molecular level. Several genomic approaches have been employed to define and demonstrate the involvement of rhizobial genomes in the symbiotic events. The genomes of two rhizobial species namely *Mesorhizobium loti*, the symbiont of several *Lotus* species, and *Sinorhizobium meliloti*, the symbiont of alfalfa, have now been completely sequenced, which have revealed interesting information about the genome evolution and structure, plant–microbes communication, and physiological diversity among the microsymbionts of legumes. While for legumes, numerous expressed sequence tags representing tens of thousands of different genes involved in root nodule formation and nitrogen fixation from three major legume species, *Glycine max*, *Medicago truncatula,* and *Lotus japonicus* have been deposited in the public domain. Currently, biological research is directed to understand gene expression and function involved in rhizobia–legume interaction. In this context, proteomics with continually evolving set of novel techniques to study all facets of protein structure and function is being considered as a promising and effective tool in

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the postgenomic era to explore further the intricacies of symbiotic process. It is likely that the proteomics approach may reveal the newer possibilities for better understanding the complex interactions of rhizobia and legumes, and also the mechanisms as to how rhizobia survive under stressed environment. The major breakthroughs from the contemporary proteome-level investigations into legume–rhizobia interactions are discussed.

4.1 Introduction

The capability of leguminous plants to form a symbiotic association with plant growth promoting rhizobacteria (PGPR) belonging to the order rhizobiales in the family rhizobiaceae with the potential of transforming atmospheric nitrogen into usable form (Newton 2000) has exerted a profound impact on legume productivity (Zaidi et al. 2003; Zaidi and Khan 2007; Franche et al. 2009). Legumes are grown globally on approximately 250 million ha, which in symbiotic association with heterogeneously distributed soil bacteria belonging to the genera *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Phylorhizobium*, *Azorhizobium,* and *Sinorhizobium*, provides about 90 million metric tons of N per year (Kinzig and Socolow 1994). Members of these genera are collectively called rhizobia (Vance 2001), which includes a taxonomically and physiologically diverse group of the α and β subclasses of the proteobacteria. Legumes adequately nodulated by rhizobial species are reported to fix up to 300 kg of N per ha annually, which is equivalent to 625 kg of urea fertilizer. Indeed, the rhizobia empower legumes to generate protein-rich foods that are extremely important both for humans and animals and thus play a major ecological and economic role. Rhizobial strains, which serve as suitable inoculants, are able to (1) colonize the soil and tolerate environmental stresses (2) compete with indigenous rhizobia or other naturalist microflora (3) form effective nodules (4) fix atmospheric nitrogen, and (5) have no deleterious effects on nontarget hosts (Brockwell et al. 1995; Howieson 1999). Strains of rhizobia, however, differ widely in their survivability as well as nodulating and nitrogen-fixing efficiency in different agro-ecosystems leading to a variation in legume productivity.

The interaction between rhizobia and its specific legume host, generally referred to as symbiosis, has been well documented (Long 1989; Pawlowski et al. 1996; Fauvart and Michiels 2008; Chang et al. 2009; Catherine et al. 2009; Herder and Parniske 2009). Also, many bacterial and some plant genes affecting nodulation have been characterized (Javier et al. 2007; Laranjo et al. 2008; Wei et al. 2009; Lu et al. 2009). The formation of nitrogen-fixing nodules (Oldroyd and Downie 2008) on legumes requires coordinated expression of several bacterial and plant genes. Initial stages of nodule formation require expression of specific nodulation (nod) genes by rhizobia leading to the synthesis of a group of signal molecules that induce nodule morphogenesis. Inside nodule, the nitrogen-fixing form of rhizobia, referred to as bacteroids, are surrounded by a host-derived membrane called the

peribacteroid membrane (PBM), which controls molecular exchanges between the bacteroid and the legumes (Panter et al. 2000). The elicitor or *Rhizobium* nod factor responsible for nodule initiation is a lipochitin oligosaccharide (LCO) and plays a pivotal role in the induction of symbiotic developmental responses in legumes, leading to the formation of nodules onto the root systems of growing legumes (Spaink 1996; Rolfe et al. 2003).

To understand the plant–microbe interactions, several model organisms have been chosen, which provide either genomic or expressed sequence tag (EST), used to identify gene transcripts, and are instrumental in gene discovery and gene sequence determination, a prerequisite for large-scale protein identification (Cordwell et al. 1995; Kaneko et al. 2000; Galibert et al. 2001; Kaneko et al. 2002; MacLean et al. 2007). Genome size is influenced by environmental factors, and the soil-dwelling species such as rhizobia tend to have larger genomes (Bentley and Parkhill 2004). For example, *Bradyrhizobium japonicum* has the largest chromosome size (approximately 9.2 Mb), and the variation in chromosome sizes among the rhizobial species may in part be due to the presence of extra-chromosomal (plasmid) DNA. Thus, the complexity and heterogeneity of microbial populations within soil requires a large inventory of genes in order to maximize survival of free-living cells (Bentley and Parkhill 2004; Young et al. 2006), while the ability to establish a symbiotic relationship with a host plant imposes an additional genetic requirement upon rhizobia. The genomes of *Rhizobium leguminosarum* bv viciae (Young et al. 2006), *Rhizobium etli* (González et al. 2006), and two photosynthetic *Bradyrhizobium* strains (Giraud et al. 2007) have recently been completed, which has increased the number of available complete rhizobial genome sequences to seven, including sequences obtained for *B. japonicum* USDA 110 (Kaneko et al. 2002), *Mesorhizobium loti* (Kaneko et al. 2000), and *Sinorhizobium meliloti* (Barnett et al. 2001; Capela et al. 2001; Finan et al. 2001; Galibert et al. 2001). Following the success of *Arabidopsis*, the genome sequencing of two legume species, *Lotus japonicus* (Japanese trefoil) and *Medicago truncatula* (barrel medic) was launched (Mathesius et al. 2001; Young et al. 2005) and a substantial amount of information about their gene structures as well as physical and genetic maps has been made public. For instance, 176 Mb (89 Mb finished, 9 Mb at phase 2, and 78 Mb at phase 1) and 189 Mb (122 Mb finished, 37 Mb at phase 2, and 30 Mb at phase 1) nonredundant sequences of the *L. japonicus* and the *M. truncatula* genomes, respectively, has been released. These correspond to approximately 40% of the entire genomes of both *L. japonicus* and *M. truncatula* with estimation of more than 60% coverage of the euchromatic regions, and cover 69% and 58% of public ESTs of *L. japonicus* and *M. truncatula*, respectively (Sato et al. 2007). Now, since the genome sequence of both the symbiotic partners, that is, rhizobia and some legumes have been completed, how can we understand the biological mechanisms of interaction between the two symbionts? To explain such interaction further, Djordevic and his group from Australia described the use of proteomics to study flavonoid induced proteins in *R. leguminosarum* (Guerreiro et al. 1997). Later on, they identified differentially displayed proteins expressed during the symbiotic interaction between *S. meliloti* 1021 and the legume *Melilotus alba,* and characterized

Table 4.1 Proteomic studies in the field of legume–*Rhizobium* interactions

Rhizobia	Proteins characterized by 2 DE	Identified proteins	References
Rhizobium sp. NGR234/ *R. fredii*/*Sinorhizobium meliloti*	16	0	Krause and Broughton (1992)
R. leguminosarum bv. *trifolii* strain ANU843	1700	12	Guerreiro et al. (1997)
Bradyrhizobium japonicum	12	1	Winzer et al. (1999)
B. japonicum	17	17	Panter et al. (2000)
S. meliloti	600	100	Natera et al. (2000)
R. leguminosarum	16	10	Morris and Djordjevic (2001)
R. leguminosarum	4	12	Guerreiro et al. (1997)
R. leguminosarum	22	5	Guerreiro et al. (1998)
S. meliloti	52	23	Guerreiro et al. (1999)
S. meliloti	189	52	Chen et al. (2000a)
S. meliloti	60	11	Chen et al. (2000b)
S. meliloti	51	7	Bestel-Corre et al. (2002)
S. meliloti	41	11	Bestel-Corre et al. (2002)
Rhizobium etli CE3	5	0	Encarnación et al. (2003)
Sinorhizobium medicae	50	5	Reeve et al. (2004)
S. meliloti strain 1021	67	67	Djordjevic et al. (2003)
R. etli strain EBRI 26	49	0	Shamseldin et al (2006)
S. meliloti strain 2011	10	1	Shamseldin et al (2006)
Rhizobium sp. VMA301	16	0	Mandal et al (2009)
B. japonicum	100	68	Hempel et al. (2009)

Modified from Bestel-Corre et al. (2004)

novel proteins produced during symbiosis by comparing proteome of free-living bacteria and bacteroids of *S. meliloti* (Siria et al. 2000). Since then, proteomic studies have focused on mutualistic symbioses of legumes with nitrogen-fixing rhizobia (Table 4.1) in order to identify proteins that are specifically induced by microbes (Vij 2003; Mathesius 2009). These studies have led to new insights into the detection of microbial signal molecules by plants, the balancing of defence responses, nutrient exchange, and the alteration of plant development by microbes.

4.2 Rhizobia–Legume Interactions: An Overview

Symbiosis in general, is an interaction between two species, where the association results in a mutually beneficial relationship. All legume crops in general are capable of establishing nitrogen-fixing root nodule symbiosis with rhizobia. Nitrogen-fixation efficiency within a single legume species, however, vary more than tenfold, providing a tremendous opportunity for engineering legumes for

nitrogen fixation. Similarly, only a very few rhizobial species can induce root nodules and infect them, but fix nitrogen only poorly or not at all. Such rhizobial species predominates in the rhizosphere and pose a serious threat to legume improvement in conventional soils (Kiers et al. 2007; López-García et al. 2009). In this context, the data collected so far for signal exchange, infection, and early nodulation, however, suggests that even though 99.99% of the rhizobial population can interact with roots, but cannot necessarily produce an effective infection (Pérez-Giménez et al. 2009). The symbiosis between legumes and rhizobia is hence, a result of complicated interconnection that leads to the formation of nodules on root systems or stems of legumes (Prell and Poole 2006). Competition for nodulation is, however, a major problem, where bacterial adhesins (required for root colonization), can play a significant role in symbiosis (Elías et al. 2009). Generally, the overall symbiotic process includes (1) plant infection (2) nodulation and nodule maturation (3) senescence (4) release of rhizobia and (5) persistence of rhizobial populations in soil. Of these, the early interaction period comprises less than 10% of the whole symbiotic cycle, while the steps from nodule senescence to rhizobial persistence in soil occupy more than 60% of it (Pérez-Giménez et al. 2009).

The process of nodule formation involves a cascade of events that starts with movement of rhizobia toward the chemical signals released by legume hosts (Redmond et al. 1986), which ultimately results in the physical contact between the two symbiotic partners. During this preinfection stage, rhizobia, however, essentially be able to colonize and attach firmly to the root surfaces, out compete the neighboring organisms, respond to nodulation *(nod)* gene-inducing flavonoid compounds and possibly other plant factors (present in seed and root exudates) and release lipo-oligosaccharide signals (Nod-factors) (Cullimore et al. 2001) that are important for induction of nodulation. Studies have shown that flavonoid compounds cause the initiation of legume–rhizobial interactions by attracting rhizobia to host roots leading this to curl and affect many of the symbiotic events like (1) stimulated growth, (2) modified composition of bacterial cell wall components, (3) induced expression of *nod* genes leading to production of Nod factors, induce expression of the TTSS, and (4) induced expression of plant cell wall degrading enzymes, required for successful root infections by rhizobia (Cooper 2004). A number of specific plant proteins, referred to as nodulins, are also expressed during infection, nodule maturation, and maintenance to support the nitrogen-fixation process (Sánchez et al. 1991). The Nod factors in association with polysaccharides (Skorupska et al. 2006) and effector proteins (Dai et al. 2008; Kambara et al. 2009; Elías et al. 2009) allow rhizobia to attach to root hairs and to penetrate within the root through a tubular structure called the infection thread (Cermola et al. 2000), where the cell wall gets disrupted and rhizobial cells come into direct contact with the host-cell plasma membrane (Brewin 2004). The plant cell membrane then outgrows and bacteria are taken up into the plant cell lumen by endocytosis. Once the rhizobia are endocytosed within a host-membrane-bound compartment called symbiosome (Roth et al. 1988), a horizontally acquired organelle, the bacteria differentiate into a new endosymbiotic form, the nitrogen-fixing bacteroids.

Rhizobia, in general, produce both indeterminate (e.g., *M. truncatula* or *Pisum sativum*) and determinate (e.g., *L. japonicus* or *Glycine max*) types of nodules. Indeterminate nodules are characterized by different zones (1) the distal meristem, where bacteria are internalized, (2) an inter zone with amyloplast accumulation and differentiation of bacteroids, (3) a fixation zone that includes plant cells: in this zone, the bacteria differentiate to become nitrogen-fixing bacteroids, leghemoglobin of the plant cytoplasm protect nitrogenase from oxygen toxicity yet it facilitate bacteroid respiration (Ott et al. 2005), and (4) a senescence zone in the basal region that contains degrading bacteroids and collapsing plant cells (Pawlowski and Bisseling 1996). In comparison, determinate nodules are typically round shaped and are derived from the cessation of meristem activity after nodule initiation and growth of the nodule mainly by cell expansion. Such bacteroids cause the enzymatic reduction of atmospheric nitrogen to ammonia and make this N accessible to host plants and allow plants to grow even in the absence of an external nitrogen source (Jones et al. 2007). In return, the bacteria are supplied with carbohydrates mainly succinate and malate (Prell and Poole 2006) in a protected environment. The host plants, however, regulate the number of nodules formed, the maturation of nodules, and the nitrogen fixation of the nodules. The amino acid cycling has also been reported in the *Rhizobium*–legume symbiosis (Lodwig et al. 2003). However, bacterial differentiation and nitrogen fixation depends on the microaerobic environment and other support factors provided by the plants. In addition, the polysaccharide composition of the rhizobial cell wall (EPS, LPS, and cyclic glucans) are also reported to be important for successful infection, invasion, and nodule development, bacterial release from infection threads, bacteroid development, suppression of plant defence response and protection against plant antimicrobial compounds (Gibson et al. 2008; Jones et al. 2008; Robledo et al. 2008). And hence, there is a strong suggestion that production of a variety of symbiotically active polysaccharides may allow rhizobial strains to adapt to changing environmental conditions and interact efficiently with legumes. Recently, tyrosinase (EC 1.14.18.1), a monophenol oxidase responsible for the synthesis of the black pigment known as melanin, and a plasmid-encoded product in many rhizobial species including *R. etli* has been found to be involved during early symbiosis where it provides resistance against reactive oxygen species (ROS) and phenolic compounds generated as part of the plant protective responses (Silvia et al. 2007).

Of late, substantial progress has been made in the identification of genes involved in plant–microbe symbioses and decoding their functions (Franken and Requena 2001). However, despite substantial progress in biological sciences, the mechanisms regulating legume root nodule development are still inadequately explained, and very few regulatory genes have been cloned and characterized. For instance, ethylene response factor (EFD) required for the formation of functional nitrogen-fixing nodules and upregulated during nodulation in *M. truncatula* has recently been characterized (Vernie et al. 2008). With the completion of the genome sequence of certain rhizobial species, proteomics techniques may now further be employed to understand the expression of the gene products in *Rhizobium*–legume interactions in natural conditions.

4.3 What is Proteomics?

The word "proteome" is a blend of "protein" and "genome" and was coined by Marc Wilkins in 1994 in the symposium: "2D Electrophoresis: from protein maps to genomes" in Siena, Italy (Wilkins 2009), which represents the complete set of proteins that are determined by the genome. Analogous to genomics, proteomics is the study of proteins and describes mainly the structure and functions of the entire complement of proteins, including the modifications made to a particular set of proteins, produced by an organism, or system at a given time (Wilkins et al. 1996; Anderson and Anderson 1998; Kav et al. 2007). Proteome analysis has been used to compare the simultaneous accumulation of hundreds of plant proteins in response to a variety of bacterial signaling molecules. The term "proteomics" was first coined by Klose (1975) to make an analogy with genomics, the study of the genes, and after genomics, proteomics is often considered the next step in the study of biological systems because knowledge of where and when proteins are expressed is essential for understanding biological events. Proteomics can broadly be classified into "classical proteomics" for protein identification and "functional proteomics" for the detailed characterization of protein structure and function as well as protein–protein interactions (Yarmush and Jayaraman 2002). Proteomics in general involves the use of two-dimensional electrophoresis (2DE), which allows the separation of denatured protein polypeptides according to their isoelectric points and molecular weights combined with high-throughput mass spectrometry (MS) identification methods. Moreover, the peptide mass fingerprinting or de novo sequencing and bioinformatics tools (Fig. 4.1) are used to identify and characterize the proteins, their activities and interactions (Jungblut and Wittmann-Liebold 1995; Pasquali et al. 1996; MacBeath 2002; LaBaer and Ramachandran 2005). However, proteomics is much more complicated than genomics mostly because while an organism's genome is more or less constant, the proteome differs from cell to cell and from time to time. Interestingly, the study of proteins introduces another level of complexity at the level of the posttranslational modification (PTM) and the biological relevance of such modifications. These changes in PTM during the growth and development of organisms (including plants) or in response to stress (including disease) cannot be deduced from studies investigating genome sequences and/or transcript abundance but can only be deciphered through proteomics (Dubey and Grover 2001; Gygi et al. 1999; Park 2004; Thurston et al. 2005). Even though proteome analysis possesses high resolution power and sensitivity, there may be limitations in the analysis of total cellular protein. The inability to detect some proteins may indicate that they (1) are present in relatively low amounts (2) are not soluble (3) comigrate with more abundant proteins or (4) are of very low molecular masses and are not resolved in the second dimension. Despite these problems, proteome analysis may provide a sensitive new tool to examine plant–microbe interactions (Guerreiro et al. 1997) under natural conditions.

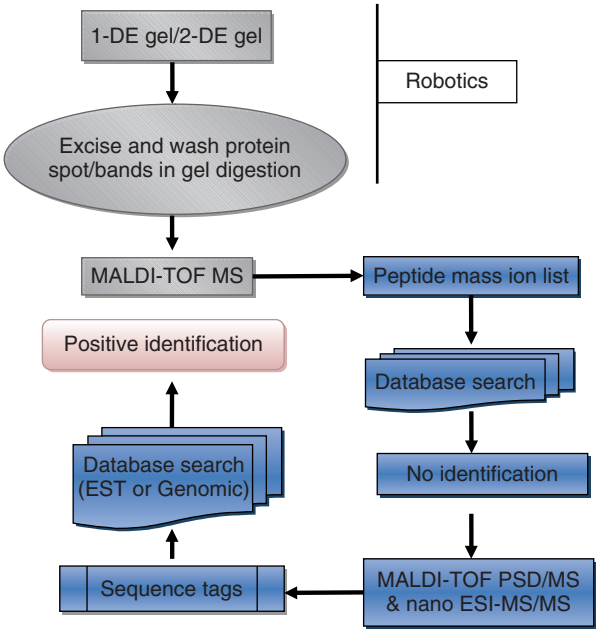

Fig. 4.1 A typical flow chart for the analysis of proteomes by MS. Proteins are separated by 1-DE or 2-DE visualized and selected for identification. The protein spots are excised and used to determine the mono-isotopic peptide ion masses by MALDI-time-of-flight (TOF) MS

4.3.1 How Proteomics Can Be Useful in Rhizobium*–Legume Symbiosis Studies?*

Currently, the proteome analysis of plant–microbe interactions is not well established, though the majority of researches have been directed toward understanding the proteome of microbial partner, probably due to ease of culturing as such organisms are single celled and the availability of complete genome sequence (Djordjevic et al. 2003). However, the research is required to discover new proteins involved in symbiotic relationship and hence to understand the proteomic basis of legume–*Rhizobium* interactions, the PTMs, identification of specific isoforms of proteins involved in certain metabolic pathways, and the construction of biochemical pathways in which the newly identified proteins can act. Proteomics of the rhizobia–legume symbiosis was started a decade ago (Krause and Broughton 1992), and there was renewed interest in the technique several years after, with studies focusing either on nodule proteins (Winzer et al. 1999; Panter et al. 2000; Natera et al. 2000; Morris and Djordjevic 2001) or on the isolated rhizobia (Guerreiro et al. 1997, 1998, 1999; Chen et al. 2000a, b). The nodule-forming rhizobia are currently underrepresented in the available databases compared to numerous agronomically

important bacteria. Most of the genes identified so far have been found associated with infection, polysaccharide production, or nitrogen fixation (van Rhijn and Vanderleyden 1995; Gray and Rolfe 1990; Fischer 1994). With increasing interest in the complex regulatory changes that occur during *Rhizobium*–legume interactions under different environments, together with the ever-increasing availability of mutants, it is desirable to characterize the *Rhizobium* gene expression via proteome analysis (Kav et al. 2007). After the completion of the genome sequence of *S. meliloti* and the determination of a 410-kb DNA region of *B. japonicum* chromosome, harboring potential symbiosis-specific genes, the focus is shifted to employ proteomics to identify the proteins induced during symbiosis (Giel et al. 2007) or to analyze protein–protein interaction in the nitrogen-fixing bacterium (Shimoda et al. 2008). During symbiotic studies, proteomic analysis is likely to provide a broad spectrum of the proteins produced by both partners, that is, rhizobia and legumes. In this context, the recent discovery of several plant receptor kinases responsible for the early detection and signal transduction of Nod factor perception (Endre et al. 2002; Stracke et al. 2002) and autoregulation of nodule numbers (Krusell et al. 2002; Nishimura et al. 2002; Searle et al. 2002), suggests that many early plant–microbe signaling events are regulated by phosphorylation events and key receptor kinases. Among legumes, proteomic analysis has mainly focused on *M. truncatula*, for which a proteome reference map has been established (Mathesius et al. 2001). Nevertheless, the proteomics approaches have been used to study protein patterns in several rhizobial species including *R. leguminosarum*, *R. etli,* and *S. meliloti* (Guerreiro et al. 1997, 1999; Encarnación et al. 2003). The first two-dimensional protein map of *R. leguminosarum* bv. *trifolii* strain ANU843, grown in defined medium in the presence and absence of flavonoid, resolved over 1,700 constitutive proteins representing about 30% of the estimated genomic output. While the global expression pattern of proteins was largely unaltered by the treatment, yet four inducible proteins were detected, which together with other 20 constitutively expressed proteins were sequenced to develop internal standards for the construction of a two-dimensional *Rhizobium* protein database. Of the identified proteins, NodE was present throughout the growth of the cells but decreased quantitatively during stationary phase cells, whereas NodB was not detected in the later stages of growth. Two of the induced proteins did not match with any known nodulation gene product, with one of these being present in mid-late log and stationary phase cells and possessing four consecutive His residues at the N terminus. Also, the reference maps for the *S. meliloti* during the early and late exponential growth phases, as well as protein patterns for bacteroids, were established before the genome sequence of *S. meliloti* became available (Guerreiro et al. 1999). The *S. meliloti* genome sequence paved the way for more sophisticated profiling of protein patterns. Djordjevic et al. (2003) used a combination of two-dimensional (2D) gel electrophoresis and peptide mass fingerprinting to investigate the protein patterns of nodule bacteria and of cultured bacteria in response to various stress conditions. About 1,180 protein products derived from 810 genes (13.1% of the predicted genes), demonstrated that the proteomic analysis is a powerful approach for global analysis of protein profiles. A large number of studies that include proteomic and

transcriptomic approaches have been initiated with the wide spread acceptance of these methods in the investigation of rhizobia–legume symbioses.

An obvious difference in the proteome of both symbiotic bacteria and cultured bacteria has been observed and putative nodule-specific and nodule suppressed proteins were identified. The data were further analyzed using metabolic pathway prediction programs and used to assess the biochemical and genetic changes. While considering the central carbon and nitrogen metabolism, the data revealed a greater similarity between the proteomic and biochemical findings and suggested that a highly specialized nutrient exchange occur between the nodule bacteria and the host plants. Proteins embossed in nodule bacteria are associated with vitamin synthesis and stress-related processes, like heat shock, chaperoning, detoxification of ROS, regulation of stress, and osmo-regulation. These findings clearly demonstrate the level of the shift in metabolism that occurs when *S. meliloti* invade legumes and form an effective symbiosis (Djordjevic 2004).

During the development of symbiosis, infection of legume root hairs by rhizobial species is considered the first of several complex events leading to nodule formation. To identify proteins involved in this process, proteomic studies have been conducted. For instance, Wan et al. (2005) have used proteomics to identify proteins in the soybean (*G. max*) root hairs after infection by *B. japonicum*. Root-hair protein preparations were obtained after 0, 3, 6, and 12 h exposure to *B. japonicum*. Mainly, the proteins previously identified as lipoxygenases, agglutinin, actin, peroxidase, and phenylalanine ammonia lyase, and some novel proteins such as phospholipase D and phosphoglucomutase have been reported. In a follow up study, a proteome-level analysis of the bacteroid of *B. japonicum* has been performed and proteins involved in nitrogen and carbon metabolism were identified along with numerous proteins related to protein synthesis, scaffolding, and degradation. Similar to the *M. alba–S. meliloti* interaction, the proteomes of *B. japonicum* inhabiting root nodules were significantly different from that of the culture-grown free-living bacterium (Sarma and Emerich 2006). Furthermore, while comparing the proteome-profiles of *B. japonicum* grown under in vitro condition and those obtained from bacteroid, proteins related to fatty acid and nucleic acid syntheses were considerably more abundant in cultured cells, whereas proteins related to nitrogen metabolism were present in higher amounts in bacteria living in nodules (Sarma and Emerich 2006) suggesting that the bacteroid state differs from the free-living state (Finan et al. 1991; Ampe et al. 2003; Barnett et al. 2004; Becker et al. 2004; Hoa et al. 2004) basically due to (1) changes in energy resources to support nitrogen fixation (2) changes in the protein degradation machinery and (3) enhanced expression of chaperonins to extend the life span of nascent proteins. Additionally, a small protein proteome of *M. truncatula* nodules establishes the presence of ribosomal proteins S6 and L24, a histone-like protein, and a peroxidase precursor (Zhang et al. 2006). In a similar study, the protein profiles of root hairs of *Vigna unguiculata* inoculated with *Rhizobium* sp. and a hair-deformation minus mutant of *Rhizobium* sp. were analyzed using 2-DE (Krause and Broughton 1992). Twelve symbiosis-specific proteins were observed and seemed to be associated with root-hair deformation and nodule development. These findings suggest that the

proteome analysis could serve as an attractive source for the study of root-hair infection by rhizobia and, in a more general sense, the functional genomics of a single, plant cell type. The results obtained also indicate that proteomic studies with legumes, lacking a complete genome sequence, are practical. Furthermore, the proteomic analysis of nodule cytosol proteins of soybean cv. Williams 82 led to conclude that of the 69 identified proteins, three were involved in glycolysis, which were further characterized to support their roles in the sucrose synthase pathway to provide malate for the bacteroids (Oehrle et al. 2008).The host-derived symbiosome membrane (SM) in intracellular symbioses represents both a structural and functional boundary between the two symbionts and hence, is strategically located to control the molecular interactions between the symbionts. Symbiosome membrane proteins from soybean nodules inoculated with wild-type and mutant *B. japonicum* were investigated using 2-DE, which revealed several quantitative differences between wild-type- and mutant-inoculated soybean nodules, including an observed significant downregulation of nodule-specific proteins in mutant-inoculated nodule (Winzer et al. 1999). While proteins of the PBM of soybean root nodules using combination of 2-DE and N-terminal sequencing was identified by Panter et al. (2000). This study identified homologues of hsp60 and hsp70 and presented evidence of the presence of a molecular machinery in the symbiosome for importing cytoplasmic proteins during co- or posttranslational modification. Proteins involved in the early stages of nodulation between the subterranean clover cultivar Woogenellup and two strains of *R. leguminosarum* bv. *trifolii* were identified using a comparative proteome approach (Morris and Djordjevic 2001). Proteins involved in nodule formation, early hormonal response upon infection and cell-wall strengthening and loosening were identified. Several symbiosis-related root proteins of *M. truncatula* were identified associated with the nitrogen-fixing bacterium *S. meliloti* using 2-DE, MALDI-TOF, and tandem MS (Bestel-Corre et al. 2002). In other studies, Natera et al. (2000) examined the proteome of *M. alba* root and nodules and the bacteroid of *S. meliloti*. In this study, *S. meliloti* proteins involved in carbon and nitrogen metabolism as well as protein synthesis were identified. In order to further characterize the changes that occur in *S. meliloti*, while it occupies the root nodule of *M. truncatula* and *M. alba*, a comparison of the proteomes of the nodule bacteria with that of *S. meliloti* cultured in the laboratory was performed using 2-DE and MS. This study identified nodule-specific proteins, nodule-suppressed proteins, stress-related proteins, transporters, and vitamin synthesis-related proteins, which suggested the presence of a highly specialized nutrient exchange system between the bacterium and the host (Djordjevic 2004). A proteome analysis of *M. alba* nodules revealed that over 250 proteins were differentially expressed in nodules compared to uninfected roots, and over 350 proteins changed in nodules compared to free-living rhizobia (Natera et al. 2000). Bestel-Corre et al. (2002) used a time-course analysis of root protein profiles to study *M. truncatula* inoculated with *S. meliloti*. A recent proteome study of early *M. truncatula* root responses to *S. meliloti* was carried out using 2D Difference in Gel Electrophoresis (DIGE). Within 24 h of inoculation of roots with rhizobia, 174 of approximately 3,700 proteins showed differential expression (van Noorden et al. 2007). Overall, 140

proteins were identified by MALDI-TOF/TOF. These proteins included a large number of enzymes involved in energy, carbohydrate, amino acid, and flavonoid metabolism, which could reflect the metabolic adaptations in the roots as part of the developmental changes in preparation for nodule initiation. A more detailed study of the *S. meliloti* proteome under free-living and symbiotic conditions identified 810 gene products involved in at least 53 metabolic pathways (Djordjevic et al. 2003; Djordjevic 2004). Of these, several proteins appeared to be nodule specific, which included enzymes required for nitrogen fixation, heme synthesis as well as heat-shock and stress-related proteins, and proteins involved in detoxification processes. In addition, a number of transport proteins, in particular ABC transporters, showed specific changes between free-living (cultured) and nodule-inhabiting rhizobia, highlighting the specialized changes in nutrient transfer that develop in a functioning nodule (Djordjevic 2004). These investigations concluded that a substantial change occurred both within the bacterium grown under different circumstances and the temporal changes within the inoculated plant. In other study, the proteins of the PBM of soybean nodule bacteroids and their possible involvement in protein processing and the biogenesis and function of the PBM is reported (Panter et al. 2000).

4.3.2 Survival of Rhizobia in Stressed Environment: A Proteomic Approach

Microbial communities, in general, have evolved a wide range of strategies that accord them to confront with varying environmental challenges. The dynamism among microbes to overcome such adverse situations is critical for their survival and growth, which depends on rapid and efficient control of genetic expression and metabolic responses (Nystrom 1998). Of the heterogeneous microbial populations, a few can survive in strictly undesirable environments while others can be affected adversely under stressed conditions. When bacteria encounters adverse environments like heat, heavy metals, salt and nutrient limitations, and other factors, the level of expression increases substantially. For example, bacteria initiate a program of gene expression in response to osmotic stress by high salt concentrations, which are manifested as a set of proteins produced in increased amounts in response to the stress. Like other bacteria, soluble proteins from the salt-tolerant *R. etli* strain EBRI 26 separated by two-dimensional gel electrophoresis and visualized by Commassie staining demonstrated that the expression of at least six proteins of varying molecular weight were increased following 4% NaCl compared to *R. etli* grown in medium without salt. These proteins analyzed by MALDI-TOF after digestion with trypsin revealed a very good peptide mass fingerprint data, which could not be identified since the genome sequence of *R. etli* is not yet published. In another experiment, the soluble proteins from salt-induced or nonsalt-induced cultures from *R. etli* strain EBRI 26 when labeled separately with different fluorescent cyano-dyes

prior to two-dimensional gel electrophoresis affirmed that 49 proteins were differentially expressed after the addition of sodium chloride. Of these, 14 proteins were over expressed and 35 were downregulated. Similar experiments using *S. meliloti* strain 2011 identified four overexpressed and six downregulated proteins. Among the overexpressed protein was a carboxy-nospermidin decarboxylase, which plays an important role in the biosynthesis of spermidin (polyamine) while enzyme catalase was among the downregulated proteins. These proteins may play a role in salt tolerance (Shamseldin et al. 2006).

Another important unfavorable factor that adversely affects the growth of bacteria is the high acid environment. However, rhizobial species have also evolved mechanisms to cope with such deleterious environmental factor, where proteomics play a key role in identifying the proteins responsible for tolerance to high acidity. For example, the proteome analysis of *B. japonicum* USDA110 revealed 568 and 628 protein spots of cells grown at pH 4.7 and pH 6.8, respectively, (Puranamaneewiwat et al. 2006). Of these, only 84 protein spots with at least threefold differential expressions were further identified by MALDI-TOF MS. The annotated proteins were assigned to four different classes (1) proteins produced only at pH 4.7 (15 proteins such as D-alanine aminotransferase, 2-haloalkanoic acid dehalogenase, and periplasmic mannitol-binding protein) (2) proteins produced under both conditions but strongly induced at pH 4.7 (27 protein spots such as triosephosphate isomerase, UTP-glucose-1-phosphate uridylyl transferase, and glyceraldehyde 3-phosphate dehydrogenase) (3) proteins downregulated during growth at pH 4.7 (25 proteins such as GroEL, acyl-CoA dehydrogenase, and ATP synthase beta chain) and (4) proteins specific to growth at pH 6.8 (17 proteins such as ATP dependent protease ATP-binding subunit, N-utilization substance protein A, and 2-isopropylmalate synthase). The data of the differential protein expression can be a basis for mechanism elucidation of the acid response in *B. japonicum* USDA110. In a similar study, to elucidate the mechanisms of pH response in an acid-tolerant *Sinorhizobium medicae*, Reeve et al. (2004) have identified acid-activated gene transcription and now complement this approach by using a proteomic analysis to identify the changes that occur following exposure to acidity. Protein profiles of persistently or transiently acid-stressed *S. medicae* cells were compared to those grown in pH neutral, buffered media. A total of 50 pH-regulated proteins were identified; N-terminal sequences for 15 of these were obtained using the Edman degradation. Transient acid exposure downregulated GlnA and GlnK and upregulated a hypothetical protein while consistent acid exposure downregulated ClpP, an ABC transporter, a hypothetical protein, a lipoprotein, the Trp-like repressor WrbA1 and upregulated DegP, fructose bisphosphate aldolase, GroES, malate dehydrogenase, and two hypothetical proteins. These findings implicate proteolytic, chaperone, and transport processes as key components of pH response in *S. medicae*.

Like the bacterial partners, nitrogen-fixing legumes are also sensitive to stressed factors. For example, water limitation has been reported to reduce nitrogen fixation substantially (Guerin et al. 1990; Zahran 1999) possibly due to downregulation of key enzymes involved in symbiosis (Gordon et al. 1997; Arrese-Igor et al. 1999),

oxygen limitation (Diaz del Castillo et al. 1994), nitrogen feedback (Serraj et al. 2001; King and Purcell 2005) and a shortage in nodule carbon flux (Arrese-Igor et al. 1999). It is now well established that plants have evolved many adaptations to counteract dehydration by dehydration-responsive changes in expression of proteins, which may lead to cellular adaptation against water deficit conditions (Bray 2004; Blum 2005). For example, proteomics approach to identify dehydration-responsive extracellular matrix (ECM) proteins in several commercial varieties of a food legume like chickpea, is reported (Bhushan et al. 2007). The comparative proteomics analysis led to the identification of 134 differentially expressed proteins that include predicted and novel dehydration-responsive proteins. It has been demonstrated that over a hundred ECM proteins are presumably involved in a variety of cellular functions like cell wall modification, signal transduction, metabolism, and cell defence and rescue. Moreover, under water stressed condition, synthesis of trehalose (α-D-glucopyranosyl (1-1) α-D-glucopyranoside), an uncommon sugar in the plant kingdom (Mellor 1992) is increased either directly via bacterial osmo-regulatory mechanisms or indirectly via oxygen partial pressure and accumulates in the root nodule but can be exported from nodules . Under similar conditions, trehalose synthesis is triggered in cultured rhizobia (Hoelzle and Streeter 1990) and if the trehalose concentration is high enough, that is, if synthesis is high and hydrolysis low, then some trehalose may escape the confines of the nodule (Streeter 1980) and may be able to act as osmo-protectant in other parts of the plant.

Proteomics also help to evaluate the impact of sewage sludges polluted with heavy metals or polycyclic aromatic hydrocarbons, on legume–*Rhizobium* interaction. For example, although control sludge showed positive effects toward *M. truncatula* plants noninoculated or inoculated with *S. meliloti*, the polluted sludges exhibited clear negative effects on plant growth and root symbioses. A clear correlation was established between some symbiosis-related proteins and the levels of nodulation, revealing a potential use of this technology for environmental studies (Bestel-Corre et al. 2002). Sewage sludge-related proteins were also identified in nodulated *M. truncatula* roots (Bestel-Corre et al. 2002), and in cultured *S. meliloti* cells (Bestel-Corre et al. 2002), thus giving some supplementary information when these data were compared to physiological data. Likewise, *Rhizobium* sp. VMA301 was isolated from the root nodules of *Vigna mungo*, grown in arsenic contaminated field. The LC50 value of arsenite for VMA301 was found to be 1.8 mM. A total of 16 differentially expressed proteins were identified using RP-HPLC and MALDI TOF mass spectrometry from arsenite-induced whole cell lysate soluble proteins. Of these, nine proteins were upregulated and seven proteins were downregulated in comparison to the cells grown without arsenite. These differential protein expressions mitigated the toxic effect of arsenite and stimulate the detoxification process (Mandal et al. 2009). Such studies thus suggested that the proteins expressed by rhizobial species as revealed by proteomic studies under stressed environment may help rhizobia to establish an effective symbiosis even under derelict or stressed soils leading thereby to improve the performance of legumes under polluted soils.

4.4 Conclusion

The interaction between legumes and rhizobia though has been widely studied but looks still in an actively diversifying evolutionary phase. The genetic approaches adopted to understand the mechanistic basis of legume–*Rhizobium* interaction has provided significant insight into nodule development and function. However, the genes able to knock down the symbiotic events like nodulation, nitrogen fixation, and other symbiotic processes may not necessarily be the ones that were identified as essential for symbiosis through genetic screens. Moreover, numerous evidences suggest that genome architecture and even content are influenced greatly by the multiphasic lifestyle adopted by nodule bacteria. To further understand how legume–rhizobia partnership continues leading to successful nodulation and nitrogen fixation, proteomics in recent times has provided valuable insight into the symbiotic interaction of the rhizobia and their respective host legumes despite certain limitations associated with this technique. Proteomic analysis is hence, likely to extend our knowledge of the fascinating partnership that exists between legumes and rhizobia. Furthermore, the development of other functional genomic approaches, such as studies focusing upon the metabolomics of stem- and root nodule bacteria (Barsch et al. 2004; Colebatch et al. 2004) may help to extend our understanding of the interaction occurring between legumes and their corresponding rhizobial partners. However, the combination of proteome-based techniques along with information generated from genomic sequencing is likely to lead to a better understanding of various events occurring during *Rhizobium*–legume interactions, which may ultimately lead to model both legumes and rhizobia for enhancing legume productivity in both conventional and stressed soils across different geographical regions of the world.

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Chapter 5
Role of Ethylene and Bacterial ACC Deaminase in Nodulation of Legumes

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Abstract Rhizobia–legume symbiosis is a complex process involving a set of plant and bacterial genes leading to formation and development of root nodules. Plant hormone, ethylene plays an important role in regulating nodule developmental processes and signaling networks in response to a wide range of biotic and abiotic stresses. Ethylene is known as a negative regulator of nodulation. Several studies have shown that inoculation of nitrogen-fixing bacteria collectively called rhizobia leads to a temporal stimulation of ethylene production that suppresses nodule formation. Application of exogenous ethylene gas or its precursors and/or ethylene-releasing compounds also reduces nodulation on legumes. Nonetheless, inhibitors of ethylene synthesis or its physiological action have been found to promote nodulation in legumes. Plant growth-promoting rhizosphere bacteria containing 1-aminocyclopropane-1-carboxylate (ACC) deaminase can increase nodulation in legumes by degrading ACC (an immediate precursor of ethylene) and thus, by lowering ethylene concentrations in the plant. In this chapter, the role of ethylene and bacterial ACC deaminase in nodulation of legumes is reviewed critically.

5.1 Introduction

The relationship between rhizobia and legume plants has been studied for over 100 years as a classic example of mutualistic association between the two organisms. This association is commonly known as symbiotic nitrogen fixation, which occurs

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as a result of series of interactions between a microsymbiont such as *Rhizobium* and its host legume plant, leading to the formation of nodules in legumes. Nodulation, considered as the primary feature of the symbiotic association is strictly controlled by the internal autoregulation mechanism of host plants (Figueiredo et al. 2008; Lohar et al. 2009). Further, the host plant regulates nodule number in response to biotic and abiotic factors (Arshad and Frankenberger 2002; Cooper 2007).

Plant growth regulators play very imperative role in the processes of nodule formation and development (Frankenberger and Arshad 1995). Ethylene, a gaseous plant hormone regulates many physiological processes of plants, ranging from germination of seeds to senescence of various organs and in many responses to environmental stresses (Csukasi et al. 2009; Santner and Estelle 2009). It also acts as an autoregulator to control nodule formation and development during symbiosis as reported by Ligero et al. (1991) and Lohar et al. (2009). It is effective in evoking physiological responses in plants even when present in extremely low concentrations. Even though ethylene is crucial for many physiological processes, yet it inhibits nodulation in numerous plant species when produced by the plants in excessive amounts (Saleem et al. 2007). Ethylene is generated in plant tissues from the precursor, ACC which is converted to ethylene by the enzyme ACC oxidase. Production of ethylene in plant tissues is directly related to stress conditions. During growth, plants are commonly exposed to various environmental stresses (Bari and Jones 2009) and resultantly more ethylene is produced (Yoo et al. 2009). During the process of nodulation, infection of roots with microsymbiont imposes biotic stress and results in increased ACC in the infected roots and consequently ethylene level in plant tissues. High concentration of ACC and ethylene in root tissues serve as negative regulators of nodulation in legume plants (Yuhashi et al. 2000).

Any factor or stimulus that changes ethylene levels of a plant either by altering its synthesis endogenously or in the close vicinity of the roots can also affect nodulation process. Previously, chemical inhibitors of ethylene synthesis (aminoethoxyvinylglycine, AVG and cobalt, Co^{2+}) and action (silver, Ag^{+}) have been used to lower the production of ethylene and promote growth and nodulation of various legumes (Ligero et al. 1991; Guinel and LaRue, 1992). Ethylene production can also be suppressed by converting ACC, partially or completely, into other products instead of ethylene. Under stress conditions, high levels of ACC accumulate in the plant tissues and are excreted into the rhizosphere, where presumably it is reabsorbed by growing root tips such as occurs with many organic acids or converted into ethylene by rhizosphere microflora. At the same time, some plant growth-promoting bacteria are able to lower the plant's ethylene concentration by taking up ACC and destroying the precursor by using it as a N source. These bacteria carry gene for an enzyme ACC deaminase that hydrolyzes ACC into ammonia and α-ketobutyric acid and, thus, promote root growth and nodulation of certain plant species by suppressing ethylene biosynthesis (Pandey et al. 2005; Shaharoona et al. 2006). Furthermore, proliferation of primary or lateral roots through lowering of ethylene as a result of bacterial ACC deaminase activity provides more infection sites and contact to rhizobia for nodule formation. In this

chapter, we discuss the role of ethylene, being negative regulator, in nodulation and how the bacterial ACC deaminase could promote nodulation in legumes via modulation of ethylene biosynthesis.

5.2 Ethylene Vs. Nodulation

Several factors such as soil physicochemical and host-microsymbiont compatibility conditions, and the presence of known and unknown biomolecules could affect nodulation on the roots of legumes (Tak et al. 2004; Collavino et al. 2005; Kinkema et al. 2006). The role of ethylene in the formation and development of nodules is very critical (Ding and Oldroyd 2009). Ethylene could be involved in several phases of symbiosis, including initial response to bacterial *Nod* factors, nodule development, senescence, and abscission (Lynch and Brown 2006; Csukasi et al. 2009; Patrick et al. 2009). However, nodulation response to ethylene is variable and is dependent on plant species as well as concentration of the ethylene released in root tissues during nodulation process.

5.2.1 Ethylene as a Negative Regulator of Nodulation

Several authors have reported that ethylene affects nodulation negatively (Oldroyd and Downie 2008; Musarrat et al. 2009). Ethylene controls the epidermal responses during the nodulation process and thus negatively regulates multiple epidermal responses in order to inhibit rhizobial infection (Nukui et al. 2004; Sugawara et al. 2006). Although not all legumes respond similarly, addition of exogenous ethylene to most of the nodulating plants reduces the frequency of nodule primordium formation (Nukui et al. 2000; Oldroyd et al. 2001). In the presence of ethylene, the number of infected root hairs did not change; however, many infection threads were aborted and the epidermis or outer cortex and nodule primordia did not form (Lee and LaRue 1992b). This leads to reduction in infection as well number of nodules in legumes. It has been observed that ethylene production significantly increases in roots infected by *Rhizobium* or *Bradyrhizobium* and decreases the number of nodules that form on the infected plants (Gonzalez-Rizzo et al. 2006; Middleton et al. 2007). Endogenous ethylene interferes with nodulation in legumes (Prayitno et al. 2006). Furthermore, ethylene applied directly as a gas or indirectly as its precursor inhibits nodulation on the roots of legumes (Peters and Crist-Esters, 2001). In contrast, inhibitors of ethylene synthesis or its physiological activity enhances nodulation (Tirichine et al. 2006). Use of ACC (the precursor of ethylene) and AVG (an inhibitor of the ethylene response) in wild-type plants indicates that ethylene inhibits rhizobial-induced epidermal responses such as root-hair deformation, calcium spiking, the expression of early nodulin genes such as *RIP1* and *ENOD11* and the frequency of infection threads (Ding and Oldroyd 2009). Like

chemical inhibitors, bacteria containing ACC deaminase that are able to lower ethylene synthesis by degrading its precursor ACC have also been reported to promote nodulation in leguminous plants (Shaharoona et al. 2006).

To understand at molecular level how decreased levels of ethylene enhance nodulation, several models have been proposed depicting the relationships between signal transduction, ethylene sensing, and nodule development (Gresshoff et al. 2003; Stearns and Glick 2003; Sun et al. 2006). For example, Nukui et al. (2004) in a study transformed *L. japonicas* B-129 with a mutated ethylene receptor gene Cm-ERS1/H70A. A point mutation was introduced into the melon ethylene receptor Cm-ERS1 by abolishing its ethylene-binding ability. The *L. japonicus* transgenic plants exhibited low sensitivity to ethylene and produced substantially higher numbers of infection threads and nodule primordia on their roots than did either wild-type or azygous plants without the transgene. Moreover, the amount of transcripts of NIN, a gene governing formation of infection threads, increased in the inoculated transgenic plants as compared with the wild-type plants. These results imply that endogenous ethylene in *L. japonicus* roots inhibits the formation of nodule primordia, as well as other infection processes. These studies clearly demonstrate that ethylene acts as a negative regulator of nodulation, and reduction in ethylene concentration has stimulatory effect on formation and development of nodules in legumes.

5.2.2 Accelerated Production of Ethylene During Nodulation Process

It has been generally observed that inoculated nodule-forming legumes produce ethylene at accelerated rates than noninoculated/nonnodulated legumes (Ligero et al. 1999). The higher production of ethylene during nodulation is most likely a plant response to the nodulating bacteria (Zaat et al. 1989). Several authors have reported that ethylene release was stimulated after inoculation in alfalfa (Caba et al. 1998), Vicia (van Workum et al. 1995), and soybean (Suganuma et al. 1995). Suganuma et al. (1995) also reported that production of ethylene by soybean roots was facilitated by inoculation with *B. japonicum* and stimulation was maximum for 3 days after bacterization. The rate of ethylene synthesis thereafter fell to the extent as observed for the roots of uninoculated plants. Caba et al. (1999) compared ethylene evolution activity in roots of soybean cv. Bragg (wild type) vs. the supernodulating mutants "*nts* 382 and *nts* 1007" after inoculation and treatment with ACC or ethephon. They observed that ethylene release was greater in inoculated Bragg than its mutants in the absence of ACC or ethephon. The skl mutant is an ethylene-insensitive legume mutant showing a hypernodulation phenotype when inoculated with its symbiont *Sinorhizobium meliloti* (Prayitno et al. 2006). The skl mutant was used to study the ethylene-mediated protein changes during nodule development in *Medicago truncatula*. The root proteome of the skl mutant was

compared to its wild type in response to the ethylene precursor ACC. Then, the proteome of skl roots were compared to its wild type after *Sinorhizobium* inoculation to identify differentially displayed proteins during nodule development at 1 and 3 days postinoculation. Six proteins (pprg-2, Kunitz proteinase inhibitor, and ACC oxidase isoforms) were downregulated in skl roots, while three protein spots were upregulated (trypsin inhibitor, albumin 2, and CPRD49). ACC induced stress-related proteins in wild-type roots. For example, pprg-2, ACC oxidase, proteinase inhibitor, ascorbate peroxidase, and heat-shock proteins were stimulated in response to ACC. However, the expression of stress-related proteins such as pprg-2, Kunitz proteinase inhibitor, and ACC oxidase was downregulated in inoculated skl roots. It was hypothesized that during early nodule development, the plant induces ethylene-mediated stress responses to limit nodule numbers. When a mutant defective in ethylene signaling, such as skl, is inoculated with rhizobia, the plant stress response is reduced, resulting in increased nodule numbers (Prayitno et al. 2006).

Nitrate (NO_3^-) and light have also been documented to increase biosynthesis of ethylene by roots and affect nodulation. In a study, Ligero et al. (1987) reported a positive correlation between NO_3^- concentrations and the quantity of ethylene released from roots of alfalfa inoculated with *R. meliloti* in a mineral solution. The ethylene release was increased in alfalfa after application of nitrogenous fertilizer (Ligero et al. 1991). In another study, Ligero et al. (1999) compared NO_3^- and inoculation-induced ethylene biosynthesis in soybean genotypes Bragg (wild type) and its supernodulating *(nts 382* and *nts* 1007) and nonnodulated *(nod* 49 and *nod* 139) mutants. They found that regardless of the NO_3^- treatment, inoculation with *B. japonicum* significantly increased the release of ethylene in roots. The highest production of ethylene was observed between 24 and 48 h after inoculation. They suggested that the response could be related to the infection process and nodule development as the treatment with Ag^+ at the time of inoculation substantially increased nodule numbers of Bragg under both low and high NO_3^- concentrations. The availability and development of legume mutants varying in sensitivity and responsiveness against ethylene or lacking autoregulation provide excellent tools to explore the role of ethylene in nodulation (Guinel and Sloetjes 2000).

5.2.3 Effect of Exogenously Applied Ethylene on Nodulation

The effects of exogenously applied ethylene gas or ethylene-releasing compounds such as ACC and 2-chloroethylphosphonic acid (CEPA) (ethephon/ethrel) on nodule formation and development have been investigated by many workers (Tamimi and Timko 2003; Goormachtig et al. 2004; Sun et al. 2006). These studies have documented strong inhibitory effects of exogenous ethylene on nodulation (Table 5.1). For example, low concentration (0.01 ppm) of ethylene applied exogenously inhibited nodulation of *Pisum sativum* (Lee and LaRue 1992b) while in

Table 5.1 Effect of ethylene gas or ethylene-releasing compounds on nodulation of legume crops

Plant	Treatment	Responses	References
Glycine max L.	Ethephon	No effect on nodule number	Hunter (1993)
	Ethylene gas	Decrease in nodule number	Xie et al. (1996)
	ACC	Stunted root growth but no effect on nodulation	Schmidt et al. (1999)
	ACC/ethephon	Decrease in nodule number	Caba et al. (1999)
	ACC	No effect on nodule number	Nukui et al. (2000)
Lotaus japonicum L.	ACC	Decrease in nodule number	Nukui et al. (2000)
Macroptillium atropurpureum L.	1 μM ACC	Suppress nodulation	Yuhashi et al. (2000)
	ACC	Decrease in nodule number	Nukui et al. (2000)
Medicago sativum L.	ACC	Decrease in nodule number	Nukui et al. (2000)
	ACC	Decrease nodulation	Charon et al. (1999)
	ACC	Negatively effect on the nodulation	Penmetsa and Cook (1997)
	ACC	Control of the position of nodule primordium formation and hyperinfection	Penmetsa et al. (2003)
	ACC	Blockage of Ca spiking in root cells, fewer infection threads and decrease in nodule number	Oldroyd et al. (2001)
Melilotus alba L.	Ethylene gas	Decrease in nodule number	Lee and LaRue (1992c)
Phaseolus vulgaris L.	Ethephon	Decrease in nodule number	Tamimi and Timko (2003)
	2-chloroethyl phosphonic acid	Decrease in nodule number	Drennan and Norton (1972)
	10 ppm ethylene	Decrease in nodule number	Goodlass and Smith (1979)
	0.01 ppm ethylene	Decrease in nodule number	Lee and LaRue (1992a)
	0.07 μL ethylene	Decrease in nodule number	Lee and LaRue (1992c)
	Ethrel	Decrease in nodule number	Drennan and Norton (1972)
	Ethylene gas	Blockage of infection thread elongation in inner cortex and decrease nodule number	Heidstra et al. (1997)
Sesbania rostrata L.	Ethephon/ACC	Decrease in nodule number and induction of indeterminate nodule	Fernandez-Lopez et al. (1998)
Trifolium repens L.	Ethylene gas	Decrease in nodule number and decrease in nitrogen fixation	Goodlass and Smith (1979)
Vicia sativa L.	Ethephon	Tsr (thick, root and shoot) phenotype	van Spronsen et al. (1995)
Vigna radiate	Ethephon	Decrease in nodule number	Duodu et al. (1999)

Modified from Okazaki et al. (2004)

other investigation, they found that nodule numbers were reduced to half when pea was grown in the presence of 0.07 ppm ethylene applied continuously to the roots for 3 weeks (Lee and LaRue, 1992c). Exogenous ethylene also inhibited nodulation of sweet clover and pea mutants that were hyper-nodulating or had ineffective nodules. The exogenous ethylene though did not decrease the number of infections per cm of lateral pea roots, but nearly all of the infections were blocked when the infection thread was in the basal epidermal cell or in the outer cortical cells (Lee and LaRue 1992c). Similarly, Duodu et al. (1999) reported a significant reduction in the number of mature nodules on roots of mung bean (*Vigna radiata*) upon treatment of 100 μM ethephon. Inhibition in nodule formation has also been observed in response to exogenous application of ethylene precursor, ACC. Penmetsa and Cook (1997) found that nodulation in *M. truncatula* was suppressed when ACC was applied during the primary infection phase (24–48 h) but not when ACC was applied after the emergence of nodule primordia (72 h). The study demonstrated that time of application of ACC was also critical in inhibiting nodulation. Similarly, Schmidt et al. (1999) reported that ACC decreased the number of nodules formed on soybean wild-type Hobbit 87 roots, but had nonsignificant effect on the ethylene insensitive mutant *etrl-l* line. However, from this study it was suggested that control of nodule numbers is independent of ethylene signaling and the effect of ACC on nodule numbers may be attributed to the stunted growth of Hobbit 87 roots. In a similar experiment, Yuhashi et al. (2000) assessed the effect of 1.0 μM ACC on nodulation of *Macroptillium atropurpureum* inoculated with *Bradyrhizobium elkanii*. The results revealed that the number of nodules formed in the presence of ACC, 8 days after inoculation and thereafter were significantly fewer than the number of nodules formed in the absence of ACC. The addition of ACC also suppressed nodulation in the control and transgenic plants of *Medicago truncatula*, implying that ethylene negatively regulates nodulation (Charon et al. 1999). Caba et al. (1999) conducted a comprehensive study on differential sensitivity of nodulation to ethylene in soybean cv. Bragg and its super-nodulating mutants. Both ACC and ethephon reduced nodule numbers per plant, nearly two fold more in wild-type than in the supernodulating mutants, strongly suggesting the involvement of ethylene in such inhibition. Valverde and Wall (2005) investigated regulatory function of ethylene in nodulation in the actinorhizal symbiosis between *Discaria trinervis* and Frankia BCU110501. Roots of axenic *D. trinervis* seedlings showed abnormal growth and reduced elongation rate in the presence of ethylene-releasing compounds ACC and CEPA in growth pouches studies. In contrast, AVG or Ag^{+} did not modify root growth, indicating that the development of *D. trinervis* roots is sensitive to elevated ethylene levels. Although drastic response to higher ethylene levels did not result in a systemic impairment of root nodule development, however, changes in the nodulation pattern of the taproots were detected. As a result of exposing the roots to CEPA, less nodules developed in older portions of the taproot while a slight increase in nodulation of the mature regions of the taproot was observed in response to chemical inhibitors of ethylene. These results suggest that ethylene is involved in modulating the susceptibility for nodulation of the basal portion of *D. trinervis* seedling roots. In another study, to determine whether

ethylene has a regulatory effect on spontaneous nodulation of *snf* mutants of *M. loti*, different concentrations of ACC were applied in nodulation plate tests (Tirichine et al. 2006). Five weeks after germination, nodule numbers in *snf* mutants and *M. loti* inoculated wild type declined with increasing concentrations of the ethylene precursor ACC. Spontaneous nodulation was totally inhibited at 10 μM ACC, while nodulation of wild-type plants was reduced to 50% (Tirichine et al. 2006). Goormachtig et al. (2004) also found that ethylene affects root-hair invasion process in *S. rostrata* and thus nodulation. The addition of ACC reduced root-hair invasion. They observed 100% inhibition of root-hair infections before inoculation with *A. caulinodans*. Thus, root-hair curl invasion in *S. rostrata* is sensitive to ethylene, similar to the situation described for *M. truncatula* and for several other legumes (Oldroyd et al. 2001).

5.2.4 *Effect of Ethylene on Nod* Factor(s)

The rhizobia signals that initiate development of the nodule organ are specific lipochitin oligosaccharides called *Nod* factors (Arshad and Frakenberger, 2002). *Nod* factors induce root-hair deformation by inducing tip growth in existing root hairs and also activate cortical cells to resume mitosis resulting in nodule primordia (Truchet et al. 1991). Perception of *Nod* factor in the plant leads to the activation of a number of rhizobial-induced genes (Middleton et al. 2007; Oldroyd and Downie 2008). Ethylene is known to have negative effect on *Nod* factors as well as tip growth and cell division in roots of dicotyledonous plants (Sun et al. 2006; Ding and Oldroyd 2009). Nonetheless, mechanisms involved in the regulation of nodule development are poorly understood, and to-date, very few regulatory genes have been cloned and characterized (Vernie et al., 2008).

Penmetsa and Cook (1997) reported that nodule number is regulated by ethylene locally in the root, as the *M. truncatula* sickle (skl) mutant is insensitive to ethylene and shows supernodulation, while a similar phenotype was also observed in a *L. japonicus* line expressing a mutated ethylene receptor gene (Nukui et al. 2004). Charon et al. (1999) examined the effect of alteration of *enod 40*, a nodulation gene associated with the earliest phases of nodule organogenesis, on nodule development in transgenic *M. truncatula*. They observed that *enod 40* actions could be partially imitated by treatment of the infected root with the ethylene inhibitor, AVG. Similarly, Vernie et al. (2008) investigated the role of EFD, a gene that is upregulated during nodulation in *M. truncatula*. EFD is an ethylene response factor required for nodule differentiation and also involved in *Nod* factor signaling. The studies indicated that EFD is a negative regulator of root nodulation and infection by Rhizobium. Goormachtig et al. (2004) reported plenty of root hairs in *S. rostrata* roots under nonaquatic conditions in contrast to hydroponic roots. Root-hair infection was inhibited by ethylene and required more stringent Nod factor features than intercellular invasion. The addition of AVG enhanced the number of nodules. Similar results were obtained with Ag_2SO_4. On the other hand, ethylene has been

shown to have no or a negative effect on the root-hair invasion process (Guinel and Geil 2002). D'Haeze et al. (2003) reported that ethylene mediates *Nod* factor responses and is required for nodule initiation. It was found that application of purified Nod factors triggered cell division, both *Nod* factors and ethylene-induced cavities, and cell death features in the root cortex. Thus, in *S. rostrata*, ethylene acts downstream from the *Nod* factors in pathways that lead to formation of infection pockets and initiation of nodule primordia (D'Haeze et al. 2003).

It has been observed that some strains of *Rhizobium* induce the formation of thick, short roots (Tsr) in common vetch (*Vicia sativa)* just like the exogenous ethylene and this response is eliminated by AVG, an inhibitor of ethylene synthesis (Zaat et al. 1989). Such type of root phenotype as well as root-hair induction and root-hair formation are induced by a factor(s) produced by the bacterium in response to plant flavonoids. Root growth inhibition and root-hair induction but not root-hair formation could be mimicked by an ethephon treatment (Zaat et al. 1989). The addition of AVG to bacterized vetch plants suppressed the development of Tsr and restored nodulation. Similarly, van Spronsen et al. (1995) reported the development of Tsr phenotype in *Vicia sativa* sp. *nigra* plants upon inoculation with *R. leguminosarun* br *viciae*. The Tsr phenotype can be mimicked by addition of ethephon and inhibited by AVG, suggesting that the Tsr phenotype is caused by excessive ethylene production. The ethylene related localized changes were also observed during infection thread formation. These phenomena inhibit nodulation of the main root by preventing formation of preinfected threads and by reducing formation of root nodule primordia. By using AVG and Ag^{+}, Heidstra et al. (1997) reported that ethylene has no required positive role in the reinitiation of root-hair tip growth induced by *Nod* factors. They also reported that ethylene is a negatively active factor controlling the position where nodule primordia are formed.

5.3 Nodulation Improvement Through Chemical/Biological Inhibitors of Ethylene

Any factor or substance that causes a reduction in the concentration of ethylene in plant tissues may have a positive effect on nodule formation and development. Both chemical and biological means have been investigated by the researchers to suppress ethylene levels in plants and their subsequent effect on nodulation, which are discussed in the following section.

5.3.1 Effect of Chemical Inhibitors on Nodulation

The chemical inhibitors of ethylene biosynthesis have been used by many scientists to elucidate the regulatory role of ethylene on nodulation of legumes (Table 5.2).

Table 5.2 Effect of chemical inhibitors of ethylene synthesis or action on nodulation of legume crops

Plant	Treatment	Responses	References
Glycine max L.	STS	Restoration of nitrate inhibition of nodulation	Ligero et al. (1999)
	STS	Stunted root growth but no effect on nodule number	Schmidt et al. (1999)
	AVG/STS	No effect on nodule number	Nukui et al. (2000)
Lotaus japonicum L.	AVG/STS	Increase in nodule number	Nukui et al. (2000)
	AVG/STS	Enhancement of *NIN* gene expression	Nukui et al. (2004)
Macroptillium atropurpureum L.	AVG/STS	Increase in nodule number	Nukui et al. (2000)
Medicago sativum L.	AVG	Increase in nodule number	Sato-Nara et al. (1999)
	AVG	Restoration of nitrate inhibition of nodulation	Ligero et al. (1991)
	STS	Restoration of nitrate inhibition of nodulation	Caba et al. (1998)
	AVG/STS	Increase in nodule number	Nukui et al. (2000)
Medicago truncaltula L.	AVG	More infection threads and nodule number	Oldroyd et al. (2001)
Phaseolus vulgaris L.	AVG/AOA/ Cobalt	Increase in nodule number	Tamimi and Timko (2003)
Pisum sativum L.	AVG/Ag_2SO_4	Control of the position of nodule primordium formation	Guinel and Sloetjes (2000)
	AVG/Ag_2SO_4	Increase in nodule number and changed nodule distribution on root	Lorteau et al. (2001)
	AVG/Ag_2SO_4/ $Co(NO_3)_2$	Restoration of nodule in *sym*5 mutant	Fearn and LaRue (1991)
	AVG/Ag_2SO_4	Restoration of nodule in *Brz* mutant	Guinel and Geil (2002)
	AVG	Acceleration of nodule development	Guinel and Sloetjes (2000)
Sesbania rostrata L.	Ag_2SO_4	Induction of determinate nodule	Fernandez-Lopez et al. (1998)
Vicia sativa L.	AVG	Restoration of normal nodulation in *tsr* mutant	Zaat et al. (1989)
	AVG	Restoration of normal root phenotype	van Spronsen et al. (1995)
Vigna radiate	STS/Cobalt	Increase in nodule number	Duodu et al. (1999)

AOA Aminooxyacetic acid, *AVG* Aminoethoxyvinyl glycine, *STS* Silver thiosulfate

Silver [Ag(I)] is a well-established chemical inhibitor of ethylene action, while AVG and AOA (aminooxyacetic acid) are well-known inhibitors of ethylene biosynthesis in higher plants (Guinel and Sloetjes 2000; Tirichine et al. 2006; Ding and Oldroyd 2009). Ag^+ blocks binding of ethylene to ethylene receptors

(Abeles et al. 1992), thus inhibiting ethylene action. Application of Ag^+ restores partially or completely the NO_3^- or ethylene suppressed nodulation. For example, Caba et al. (1998) in a study found that Ag^+ treatment increased nodulation in alfalfa at all NO_3^- concentrations. Later on, Ligero et al. (1999) confirmed the above findings and observed maximum stimulation of nodulation in soybean plants when Ag^+ was applied in combination with *Bradyrhizobium* culture. Similarly, Ag^+ at rate of 10 μM significantly increased nodulation in common bean cv. OAC Rico, but had no effect on its mutants R69 and R99 (Shirtliffe et al. 1996), while Markwei and LaRue (1997) reported that Ag^+ promoted nodule numbers in a pleiotropic mutant (El32) of pea cv. Sparkle.

Similarly, inhibitor of ethylene biosynthesis, AVG stimulated nodule formation by *R. meliloti* on *Medicago sativa* when it was added along with the inoculum (Peters and Crist-Esters, 1989). Stimulation of nodule formation by AVG showed a similar concentration-dependent inhibiting effect on endogenous ethylene biosynthesis, suggesting that the primary action of AVG is in the inhibition of the endogenous ethylene biosynthesis. Tirichine et al. (2006) reported a nonsignificant increase in nodule numbers in *Lotus japonicus* when seedlings were grown in the presence of 1.27 nM AVG. Maekawa-Yoshikawa et al. (2009) observed that the different doses of AVG as well as Ag+ treatment enhanced nodulation in *L. japonicus*. Likewise, Fearn and LaRue (1991) reported an increase in nodulation when roots of poorly nodulating *symS* mutants of pea cv. Sparkle were treated with AVG, Ag^+, and Co^{2+}.

Rhizobitoxine (Rtx), an enol-ether amino acid [2-amino-4-(2-amino-3-hydroxypropoxy)-trans-3-butenoic acid] is a structural analog of AVG and inhibits ACC synthase (Tittabutr et al., 2008). It is synthesized by the legume microsymbiont like *Bradyrhizobium elkanii* and because of its inhibitory effects on ethylene (Owens et al. 1972), Rtx has been shown to enhance nodulation on legumes (Sugawara et al. 2006). However, the effect of Rtx on nodulation has been contradictory and has been found both legumes- and rhizobia dependent. For example, nodulation of *G. max* is generally not sensitive to ethylene (Xie et al. 1996; Schmidt et al. 1999), while nodulation of *V. radiata* is sensitive (Duodu et al. 1999). Similarly, some reports have shown that there is not a significant difference in nodule number between plants inoculated with *B. elkanii* USDA61 and plants inoculated with rhizobitoxine-deficient mutants during nodulation of *G. max, G. soja, Vigna unguiculata,* and *Macroptilium atropurpureum* (Xiong and Fuhrmann, 1996). Among bradyrhizobia able to produce Rtx, *B. elkanii* accumulates Rtx in cultures and in nodules, while *B. japonicum* does not (Kuykendall et al. 1992). In this regard, nodulation experiments using *B. elkanii* USDA61 and its Rtx -minus mutants revealed that the efficient nodulation occurring in *A. edgeworthii* but not in *A. bracteata* is highly dependent on Rtx production (Parker and Peters 2001). Therefore, such variation in the performance of Rtx seems probably be due to the differences in the abilities of the legume genotypes and rhizobial strains forming symbiosis with their host plant.

5.3.2 *Plant Growth-Promoting Bacteria Containing ACC Deaminase as a Biological Inhibitor of Ethylene Biosynthesis*

Bacteria can influence plant growth through multifarious mechanism (s) of actions (Bajgiran et al. 2008; Khalid et al. 2009; Lugtenberg and Kamilova 2009; Khan et al. 2009). Recently, plant growth-promoting rhizosphere bacteria possessing ACC deaminase activity are receiving worldwide attention. These bacteria influence plant growth by altering the synthesis of endogenous levels of ethylene in plant tissues by producing an enzyme ACC deaminase (Glick et al. 2007; Farajzadeh et al. 2010). Production of ethylene in plant tissues has, however, been found directly related to the amount of ACC synthesized (Penrose and Glick 2001). Being precursor of ethylene, ACC is immediately cleaved to ethylene by the enzyme ACC-oxidase. However, the uptake and cleavage of ACC by the bacterium containing ACC deaminase outside the germinating seeds or growing roots reduce the amount of ACC as well as ethylene, by acting as a sink for ACC. Thus, bacteria exhibiting ACC deaminase activity may serve as a biological inhibitor of ethylene biosynthesis (Shaharoona et al. 2007). It is well established that higher concentration of ethylene suppresses plant growth and hence, reduction in ethylene levels in plant tissues as a result of bacterial ACC deaminase activity could promote plant growth (Andrea et al. 2007). Furthermore, plants inoculated with bacteria containing ACC deaminase have been found resistant to the harmful effects of stress ethylene, generated under undesirable environments (Mayak et al. 2004; Bonfante and Anca 2009). Several bacteria including both rhizobia and free-living rhizobacteria have been found to facilitate plant growth and nodulation through ACC-deaminase activity (Saleem et al. 2007; Yang et al. 2009; Musarrat et al. 2009).

5.3.2.1 Effect of Co-inoculation with Free-Living Rhizobacteria Containing ACC Deaminase on Nodulation

Inoculation with PGPR other than rhizobia has been shown to increase nodulation in legumes either by changing root architecture to facilitate root infection with rhizobia or by suppressing ethylene biosynthesis in legume roots. Several authors have reported that coinoculation with rhizobacteria containing ACC deaminase promote nodulation of legumes by lowering ethylene concentrations. As an example, Shaharoona et al. (2006), while evaluating the effectiveness of PGPR possessing ACC deaminase activity on nodulation of mungbean, demonstrated that the coinoculation of PGPR with *Bradyrhizobium* enhanced the nodulation to an extent of 48% compared with only *Bradyrhizobium* inoculated legume. It was, therefore, concluded from this study that improvement in nodulation was most likely due to lowering of ethylene as a result of ACC deaminase activity of the PGPR. Similarly, of the total nine bacterial strains containing ACC deaminase, three *Pseudomonas* strains (PGPR1, PGPR2, and PGPR4) resulted in a significantly higher pod yield,

N and P contents, of peanut in a pot trial experiment (Dey et al. 2004). Under field conditions, these PGPR significantly enhanced nodule dry weight (up to 24%) over the control in 3 years trials. Other biological traits like root length, pod numbers, and nodule numbers were also enhanced. Three rhizobacterial strains with ACC deaminase were evaluated for improving nodulation in chickpea (*Cicer arietinum* L.), both under pot and field conditions (Shahzad et al. 2008). Inoculation with ACC deaminase producing bacteria resulted in a highly significant increase (87%) in number of nodules per plant compared to control. Similarly, Belimov et al. (2009) reported the effect of root-associated bacterium *Variovorax paradoxus* 5C-2 carrying ACC deaminase on pea plants grown in dry soil. Inoculation with *V. paradoxus* 5C-2 improved nodulation in peas under water stress conditions. Significant increase in nodulation of common bean upon inoculation with bacteria containing ACC deaminase has also been reported by Remans et al. (2007).

5.3.2.2 Relative Effectiveness of Rhizobial Strains Containing ACC Deaminase for Nodulation

Rhizobial strains with ACC deaminase have been found more effective in nodulating the host, most likely, by lowering ethylene concentration in legumes (Okazaki et al. 2004; Musarrat et al. 2009). Ma et al. (2003) found that ACC deaminase producing *R. leguminosarum* bv. *viciae* 128C53K enhanced the nodulation in *Pisum sativum* L. cv. Sparkle by modulating ethylene levels in the plant roots during the early stages of nodule development. It has been reported that ACC deaminase producing rhizobial cells reduce ethylene concentrations in the infection threads and increase the persistence of infection threads by suppressing the defense signals in the plant cells (Ma et al. 2004). Consequently, greater numbers of nodules are formed in the inoculated plants. However, relatively low ACC deaminase activities have been observed in rhizobia compared to free-living rhizobacteria (Glick et al. 2007). Further, the extent of ACC deaminase activity in different strains of rhizobia varies greatly (Duan et al. 2009).

5.3.2.3 ACC-Deaminase Encoding Gene (*acd*S) and Nodulation

Genes encoding ACC deaminase have been reported in many bacterial species (Contesto et al. 2008; Duan et al. 2009; Farajzadeh et al. 2010). The studies on *acd*S gene encoding ACC deaminase activity show that presence of such gene improves symbiotic efficiency and increases nodulation in legumes. For example, *Sinorhizobium meliloti* containing ACC deaminase gene (*acd*S) derived from *R. leguminosarum* showed increased ability to nodulate alfalfa (Ma et al. 2004). Tittabutr et al. (2008) studied the effect of ACC deaminase on nodulation and growth of *Leucaena leucocephala*. The *acd*S genes encoding ACC deaminase were cloned from *Rhizobium* sp. strain TAL1145 and *Sinorhizobium* sp. BL3 BL3 in multicopy plasmids, and transferred to TAL1145. The BL3-*acd*S gene greatly enhanced

ACC deaminase activity in TAL1145 compared to the native *acd*S gene. The resulting transconjugants of TAL1145 containing the native and BL3-acdS genes formed greater (in number) and bigger nodules on *L. leucocephala* than by TAL1145 besides yielding higher root mass. Similarly, Ma et al. (2003) isolated ACC deaminase gene to examine its regulatory role in nodulation. Mutants with bacterial ACC deaminase gene (*acd*S) and a leucine-responsive regulatory protein-like gene (*lrp*L), were constructed to assess their abilities to nodulate *Pisum sativum* L. cv. Sparkle (pea). Both mutants were then unable to synthesize ACC deaminase. A decrease in nodulation efficiency was observed in response to inoculation with both mutants compared with parental strain. The study demonstrated that the presence of ACC deaminase activity in bacteria enhanced the nodulation of *P. sativum* L. cv. Sparkle, by modulating ethylene levels in the plant roots during the early stages of nodule development. Nukui et al. (2006) in yet another experiment examined the regulation of the *acd*S gene encoding ACC deaminase in bacteria during symbiosis in *Lotus japonicus*. A glucuronidase (GUS) gene was introduced into *acd*S to show GUS under control of the *acd*S promoter. Another mutant was generated with mutation in a *nif*A gene (a nitrogen-fixing regulatory gene). Two homologous *nif*A genes, mll5857 and mll5837, (designated as *nif*A1 and *nif*A2 respectively) were observed in the symbiosis island of *M. loti*. The *nif*A2 disruption resulted in considerably reduced expression of *acd*S, *nifH*, and *nif*A1 in bacteroid cells while *nif*A1 disruption slightly promoted expression of the *acd*S transcripts and suppressed *nif*H. The study illustrated that the *acdS* gene and other symbiotic genes were positively regulated by the NifA2 protein, but not by the NifA1 protein, in *M. loti*. Furthermore, it was suggested that *M. loti acd*S participates in the establishment and/or maintenance of mature nodules by interfering with the production of ethylene. Uchiumi et al. (2004) found that inactivation of the *acd*S gene in *M. loti* reduced the number of nodules on *Lotus japonicus* compared with the number of nodules formed by the wild-type strain.

5.4 Transgenic Legumes with ACC deaminase

Biotic and abiotic stresses result in accelerated production of ethylene in plant issues. Moreover, the use of genomic technologies leads to products with more predictable and consistent effects. Following this concept, the genetically engineered plant species with the expression of bacterial ACC deaminase activity have been reported to exhibit greater tolerance to various stresses (Nie et al. 2002; Stearns and Glick, 2003) and, thus, attenuate the impact of "stress ethylene". As ethylene inhibits nodulation in legumes, constructing transgenic plants with low ethylene sensitivities or with the gene that lowers ethylene biosynthesis will be a promising approach to enhance nodulation process. On this aspect, very little work has been done so far. As an example, Nukui et al. (2004) prepared a transgenic plant of *L. japonicus* with low ethylene sensitivity. They transformed *L. japonicus* B-129 with a mutated ethylene receptor gene Cm-ERS1/H70A, which abolished its

ethylene-binding ability, resulting into reduced ethylene sensitivity. The transgenic *L. japonicus* produced markedly higher numbers of infection threads and nodule primordia on their roots when inoculated with *M. loti*, than did either wild-type or azygous plants without the transgene. Thus, it is very likely that nodulation could be substantially increased by transforming ACC deaminase gene in legumes.

5.5 Conclusion

Ethylene plays significant regulatory roles in nodule organogenesis including its effects on root growth and *Nod* factors. Endogenous ethylene synthesis is increased upon infection by the microsymbiont, which affects nodulation negatively. Reduction in nodulation in response to exogenous application of either ethylene gas or ethylene-releasing compounds confirms that ethylene negatively regulates the formation of nodules. Similarly, it has been found that the chemical inhibitors of ethylene action or synthesis restore nodulation. Moreover, these inhibitors have been found to eliminate the effects of NO_3^- and light on nodulation, as these factors are known to stimulate ethylene production. Since all the phytohormones interact with each other and a physiological response is usually a result of a hormonal balance, it is very likely that the ethylene effects on nodulation may be created due to changes in the balance of other hormones. Very recently, several authors reported that hormone signaling is integrated at several levels during growth and development (Santnerl and Estelle, 2009; Ding and Oldroyd 2009; Stepanova and Alonso 2009; Yoo et al., 2009).

Plant growth-promoting bacteria containing ACC deaminase can help plants to tolerate a range of biotic and abiotic stresses and thereby enhance nodulation by decreasing ethylene levels in roots. Finally, if some rhizobial strains lack the ability to decrease ethylene levels in host legumes, the introduction of genes for ACC deaminase into such rhizobia could enhance their symbiotic interactions with host legumes. The ethylene-decreasing strategies of rhizobia could be useful for positive plant–microbe interactions and a promising tool for enhancing nodulation, leading thereby to improve the fertility, especially the N pool of soil.

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Chapter 6
Microbial Biofilms: How Effective in *Rhizobium*–Legume Symbiosis?

G. Seneviratne, M.L.M.A.W. Weerasekara, and J.S. Zavahir

Abstract Diverse genera of bacteria live as microbial communities called biofilms on biotic or abiotic surfaces, or interfaces. They exhibit elevated microbial action, as a result of symbiosis in biofilm structure and physiological adaptation. The formation of fungal–bacterial biofilms by bacterial colonization on biotic fungal surfaces gives the biofilms enhanced microbial effectiveness compared to monocultures. When the bacteria include rhizobia, they are called fungal–rhizobial biofilms. The role of biofilm formation in *Rhizobium*–legume N_2-fixing symbiosis contributes to effective root colonization by rhizobia and provides an effective mode for defense and helping rhizobia to survive under harsh and nutrient-limiting environments. Biofilms also indirectly promote the symbiosis by assuring a healthy root system, preserving rhizospheric moisture, and modifying soil pH, leading to enhanced nutrient cycling, biocontrol etc. Poor survival of rhizobial monocultures in inoculant technology can be overcome by using biofilmed inocula called biofilmed biofertilizers (BBs), which could improve legume production. The use of BBs is also likely to develop cooperative symbioses between *Rhizobium*–legume interaction and microbe–microbe interactions in the biofilm, which will make an improved effect on the former, leading to increased legume production.

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6.1 Introduction

Microbial communities are an intrinsic component in nature whose functions and activities have a direct impact on ecosystem functioning. Microbes are able to flourish under varying environmental settings, including habitats with extreme conditions through a variety of life strategies. It is increasingly apparent that in nature, bacteria function less as individuals and predominantly as coherent groups or communities that are able to create multiple ecological niches to permit their survival (Lazdunski et al. 2004). Microbial biofilms are such communities of microorganisms (algal, fungal, bacterial, and/or other microbial) adherent to biotic or abiotic surfaces or associated with interfaces. Microbes in biofilms are in intimate contact with each other, and encased in a self-produced extracellular polymeric substances (EPS). Newly developed microbiological and molecular methods have clearly shown that most bacteria live as biofilms, formed on a range of surfaces (Costerton et al. 1995; Harrison et al. 2005; Romanova et al. 2006). The adherent nature of microbial cells in the biofilm exhibits an elevated antimicrobial tolerance as a consequence of symbiosis in biofilm structure and physiological adaptation (Gilbert et al. 1997; Stoodley et al. 2002). The surrounding EPS matrix of a biofilm is the key component in increased resistance to various environmental stress factors such as UV radiation, extreme pH, osmotic shock, desiccation, predators, etc. (Costerton et al. 1987; Stewart and Costerton 2001; Romanova et al. 2006). Thus, microorganisms prefer to exist in the biofilm mode rather than freely swimming planktonic stage in nature.

The microbes, however, undergo profound changes during their transition from planktonic organisms to cells that are part of a complex, surface-attached biofilm. Genetic and molecular approaches have identified that the biofilms are considerably different in gene expression (Davies et al. 1993; Vilain and Brözel 2006), physiology and functions (Dow et al. 2007), compared to that of their individual microbes in planktonic life style. As a consequence, biofilms encompass an enormous and significant impact in industrial, medical, and agricultural settings, exhibiting both harmful and beneficial activities. Beneficial biofilms can further be engineered in vitro for various biotechnological applications (Seneviratne 2003). The distinctiveness of action of the beneficial biofilms developed in vitro has already shown numerous favorable effects on agricultural and biotechnological applications (Seneviratne et al. 2008a). For example, formation of fungal–bacterial biofilms/fungal–rhizobial biofilms (FBBs/FRBs) by colonizing bacteria on biotic fungal surfaces (Fig. 6.1) gives the biofilms enhanced metabolic activities compared to monocultures, through a range of mechanisms. As such, diverse forms of the FBBs/FRBs inocula have been shown to improve nodulation and N_2 fixation in *Rhizobium*–legume symbiosis (Jayasinghearachchi and Seneviratne 2004a), colonize nonlegume plant roots, improve growth (Seneviratne et al. 2009), increase soil nitrogen (N) and phosphorus (P) availabilities (Jayasinghearachchi and Seneviratne 2004b), biosolubilize rock phosphate (Jayasinghearachchi and Seneviratne 2006; Seneviratne and Indrasena 2006), produce higher acidity and plant

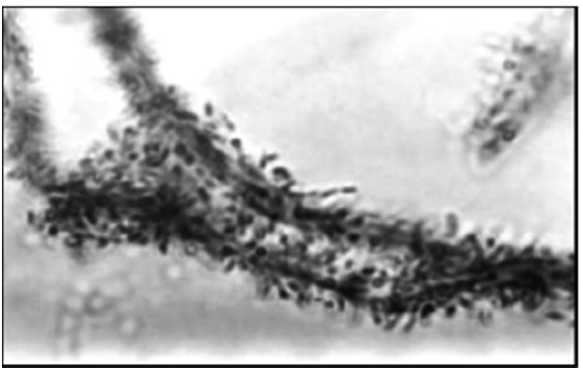

Fig. 6.1 *Azorhizobium caulinodans* ORS 571 colonized on a *Mucor* spp., forming fungal–rhizobial biofilms (FRBs)

growth-promoting hormones (Bandara et al. 2006), generate bioactive compounds (Zavahir and Seneviratne 2007), and increase the biodegradability of synthetic polymers (Seneviratne et al. 2006). Even though numerous recent studies have shown both the interactions of biofilms (commensal, mutualistic, antagonistic, or parasitic/pathogenic) with a variety of counterparts (living and nonliving) and their applications in diverse fields, more interactions are yet to be discovered. Of these, *Rhizobium*–legume symbiosis is an important interaction, which has shown to be mediated directly or indirectly by biofilm formation. This chapter focuses on new insights into the role of microbial biofilms in *Rhizobium*–legume symbiosis. Prospects and potentials of biofilms in yield improvement of legumes are also discussed.

6.2 Biofilms in the Rhizosphere of Leguminous Plants

The rhizosphere, the few millimeters of soil surrounding a plant root, is characterized as a complex and highly dynamic front for plant–root interactions with soil microbes and invertebrates (Hirsch et al. 2003). It is a preferential site for soil microbial colonization, since it contains great amount of C and nutrients, released as root exudates and/or by the death and lysis of cortex cells during root growth (Chin-A-Woeng et al. 2000). A number of multifaceted processes consisting of physical, chemical, and biological modifications occurring at the root–soil interface result in the formation and development of the rhizosphere. Microbe–microbe interactions are crucial to understand the dynamic processes characteristic of rhizosphere establishment and maintenance affecting plant growth and health (Barea et al. 2005). In order to address the role of microbial biofilms in enhanced *Rhizobium*–legume symbiosis, it is important to discuss the various survival modes of biofilms that persist in the rhizosphere. There are three major types of biofilms

that may occur in the rhizosphere, namely, bacterial biofilms (including Actinomycetes), fungal biofilms, and FBBs/FRBs. The bacterial and fungal biofilms, which consists of either a single species or multiple species, are formed on biotic or abiotic surfaces in the soil. The FBBs/FRBs are somewhat different from bacterial or fungal biofilms in the sense that during this biofilm formation fungi act as the biotic surface to which the bacteria adhere (Seneviratne et al. 2008a). In the case of nonfilamentous fungi, both bacteria and fungi can act as the biotic surfaces. These interactions benefit both partners during metabolic cooperation, etc. In addition to the surface-attached mass colonies of bacterial biofilms, less complex biofilms with lower numbers of cells, variably described as microcolonies, aggregates, or cell clusters (Bloemberg and Lugtenberg 2001; Morris and Monier 2003; Ramey et al. 2004) persist in the rhizosphere (Whipps 2001).

Numerous studies have indicated that a number of different bacteria exist in the rhizosphere as bacterial biofilms (Ramey et al. 2004). For examples, the plant pathogen *Agrobacterium tumefaciens* persists as a surface-associated population of cells or biofilms on soil particles and living plant tissues (Ramey et al. 2004), *Rhizobium leguminosarum* bv. *viciae* and *Sinorhizobium meliloti* establish biofilms on both roots and abiotic surfaces in the soil (Fujishige et al. 2006a,b) and species of *Pseudomonas*, a primary model in biofilm research, form dense biofilms on both biotic and abiotic surfaces in the rhizosphere (Parsek and Fuqua 2004). Further, *Pseudomonas putida* can respond rapidly to the presence of root exudates in soils by establishing stable biofilms at root colonization sites (Espinosa-Urgel et al. 2002), *Azospirillum brasilense*, a free-living N_2 fixer, colonizes root elongation zones and root hairs, forming dense biofilms (Assmus et al. 1995) and Gram-positive, biocontrol agents such as *Bacillus cereus* have the ability to form dense surface-associated biofilms (Bais et al. 2004). Furthermore, a range of species from diverse bacterial genera, including members of *Pseudomonas*, *Bacillus*, *Paenibacillus,* and *Burkholderia*, have often been found to occur attached to the surfaces provided by soil fungi, such as hyphae, mycorrhizal roots, spores, and interior of fruiting bodies (Elsas et al. 2006), forming FBBs. As revealed by microscopic techniques (Nurmiaholassila et al. 1997), it has been reported that the biofilms of bacteria are associated with hyphal surfaces of ectomycorrhizal fungi. Furthermore, such microbial associations between bacteria and mycorrhizal fungi have also been observed to occur naturally in the soil (Artursson and Jansson 2003), promoting plant–mycorrhizal symbiosis (Frey-Klett et al. 2007). Pearce et al. (1995) discussed the role of rhizosphere biofilms in the complex web of interactions in the rhizosphere, which govern plant growth and microbial dynamics by means of chemical, physical, biotic, and nutritional interactions. The evolutions of such mutualistic interactions have successfully benefited both the biofilm and the plant. For example, Foster and Wenseleers (2006) suggested that the phenotypic feedbacks were more important explanation for success between-species cooperation than the mere development of genetic correlations among species. Therefore, it is clear that such simple or complex consortia of biofilms exist in the rhizosphere and play an important role in directly and/or indirectly supporting plant–microbes interactions, including *Rhizobium*–legume symbiosis.

6.3 How are Biofilms Important in *Rhizobium*–Legume Symbiosis?

Rhizobium–legume interaction, a symbiotic relationship, occurs between leguminous plant roots and the bacterial genera, rhizobia. Rhizobia fix atmospheric N_2 for the host plant and in return receive carbohydrates from the host plant. Though, rhizobia are well known for their ability to form nodules on the roots of legume plants, only a few reports thus far have indicated the biofilm formation by rhizobial species (Matthysse and Kijne 1998; Matthysse and McMahan 2001; Gage 2002, 2004; Ramey et al. 2004; Fujishige et al. 2006a,b; Rodríguez-Navarro et al. 2007; Williams et al. 2008). Also, most of the studies have not described that this relationship is influenced by biofilm formation. However, there is growing interest to know how *Rhizobium*–legume symbiosis is influenced by biofilm formation and how both symbionts are benefited from such symbiotic relationships (Rinaudi and Giordano 2009). In this regard, it has been reported that living within a biofilm could offer many advantages to rhizobia in comparison with the planktonic state. For instance, the biofilm may act as an effective mode for bacterial attachment to the roots of legumes, provide defense or help rhizobia to survive in nutrient-poor soil environments, in cell-to-cell communication and growth (Morris and Monier 2003; Fujishige et al. 2006a, b; Russo et al. 2006). Though the role of biofilms in *Rhizobium*–legume symbiosis has not been adequately explained, the discussion below indicates some possible direct and indirect benefits.

6.3.1 Rhizobial Biofilms as an Effective Mode of Bacterial Attachment

Rhizobial attachment to root in nodulation process during *Rhizobium*–legume symbiosis has been extensively studied and reviewed (Hirsch 1999; Schauser et al. 1999; Long 2001; Hirsch et al. 2001) and is not discussed here. Therefore, this section is confined to a discussion on studies describing the rhizobial attachment and biofilm formation. Attachment of rhizobia to legume roots (Fig. 6.2) is the earliest step essential for *Rhizobium*–legume interaction (Fujishige et al. 2006b; Williams et al. 2008). It is also a prerequisite for the formation of microbial biofilms. In a study, Rodríguez-Navarro et al. (2007) stated that it is reasonable to suppose that the molecular mechanisms operating in bacterial attachment to roots might also be relevant for biofilm development. The formation of microcolony mode of biofilms in the rhizosphere is common and the attachment and aggregation in the microcolonies is the basis of plant root colonization by rhizobacteria (Lugtenberg et al. 2001; Ongena and Jacques 2008). Moreover, in view of the overlap in genes important for adherence to abiotic and biotic surfaces, Fujishige et al. (2006b) suggested that bacterial colonization on roots is equivalent to biofilm formation. They also have investigated the biofilm formation in the early stages

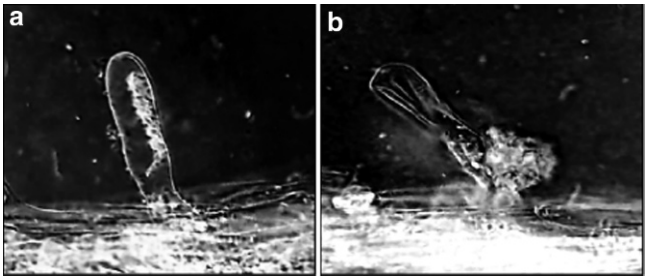

Fig. 6.2 (**a**) *Rhizobium leguminosarum* bv. phaseoli cells attached initially on to a root hair of bean (*Phaseolus vulgaris* L.) and (**b**) a developed rhizobial biofilm on the base of a root hair

of the *Rhizobium*–legume symbiosis and have proven that *S. meliloti* establishes biofilms on the roots of its legume hosts, *Medicago sativa* L. and *Melilotus alba* Desr. In addition, bacterial surface polysaccharides (e.g., exopolysaccharides (EPS)) are involved in the attachment process (Kijne et al. 1988; Pueppke et al. 1980; Williams et al. 2008) and production of EPS is characteristic to biofilmed form of bacteria including rhizobia. Several other studies have also confirmed that EPS during biofilm formation enhance the attachment of bacteria onto roots and root appendages (Hirsch 1999; Bianciotto et al. 2001a,b; Rudrappa et al. 2008; Williams et al. 2008). In other study, biofilm formation at root sites has been reported to be triggered by a plant-derived component similar to that involved in *Rhizobium*–legume and other bacterial interactions (de Ruijter et al. 1999). It was revealed that legume lectins, for example, located at the root-hair tip recognize and bind to specific carbohydrate structures that are present on the bacterial surface (Matthysse and Kijne 1998), thereby promoting attachment and biofilm formation (Pérez-Giménez et al. 2009). Microscopic observations of rhizobial cells within curled root hairs disclosed small biofilm-type aggregates that help the inocula for root invasion (Gage 2002). In general, rhizobial cells migrate down the infection threads as biofilm-like filaments toward the root interior. For example, *R. leguminosarum* bv. *viciae* and *S. meliloti* form biofilms on inert surfaces and legume roots (Fujishige et al. 2006b). Clusters of *S. meliloti* are concentrated in the root-hair zone and are often trapped within curling root hairs in the initial nodulation step (Fujishige et al. 2006b). Bacterial growth patterns inside infection threads can influence whether nodules contain single or mixed strains of bacteria (Gage 2002), possibly forming bacterial biofilms.

Microbial attachment and colonization is generally regulated in a population density dependent manner by a process called quorum sensing (Whitehead et al. 2001), in plant-associated microbial communities (Elasri et al. 2001). Quorum sensing is the regulation of bacterial gene expression via the production and sensing of specific signaling molecules in response to fluctuation in cell densities within the population and involves cell-to-cell signaling in a microbial assemblage (Elsas et al. 2006). Cell-to-cell communication, thus, may play a significant role in the attachment of rhizobia to leguminous roots, forming biofilms. This has important

implications in the bacterial competitiveness to colonize the root. Thus, it seems that formation of rhizobial biofilms is important for effective root colonization and possibly nodulation in the *Rhizobium*–legume symbiosis.

6.3.2 Rhizobial Biofilms: Helping Survival in Harsh and Nutrient-Limiting Environments and Defense

Since rhizobia are nonspore-forming bacteria, how they survive in soil in the absence of the host plant is very important for the establishment of the *Rhizobium*–legume symbiosis (Pueppke et al. 1980). In the absence of a host plant, rhizobia are directly exposed to diverse environmental conditions. And hence, formation of biofilms has been suggested as the most likely protective mechanism against adverse environmental conditions (Parsek and Tolker-Nielsen 2008). It was demonstrated that biofilm formation of rhizobia with common soil fungi forming FRBs could be a plausible strategy for rhizobial survival (Seneviratne and Jayasinghearachchi 2003). In that, the biofilm formation provides a number of survival benefits to resident microbes under stressful conditions (Seneviratne et al. 2008a). For example, inocula of FRBs were observed to help their rhizobia survive at high salinity (400 mM NaCl) and tannin concentrations (0.4 mM tannic acid) by 10^5-fold and 12-fold, respectively, compared to rhizobial monocultures (Seneviratne et al. 2008a). Their higher tolerance than the monocultures for low pH, chromium, and predation by earthworms was also noted in the studies. Biofilm formation has further been reported as an effective mode of survival in nutrient-limiting conditions. For example, *S. meliloti* formed larger biofilms when the bacterium was grown in *Rhizobium* defined medium, compared to nutrient rich media such as Luria Bertani or Tryptone Yeast Extract (Raman and Sambandan 1998), which indicated that bacteria tend to exist as biofilms rather than planktonic state under nutritionally limited environments. In addition, it has been reported that the formation of microcolonies and the production of toxins are effective mechanisms, which may allow bacterial biofilms (e.g., *Pseudomonas aeruginosa*) to resist protozoan grazing and to persist in the environment (Matz et al. 2004). In a similar study, Burmolle et al. (2006) revealed that in multispecies biofilms, the synergistic interactions cause an enhancement of biofilm formation and increased resistance to antimicrobial agents. Bacterial cells are protected from antimicrobial agents in biofilms through the formation of persisted cells, a highly protected state adopted by a small fraction of the outermost cells of a biofilm (Roberts and Stewart 2005). Enhanced bioremediation of antimicrobial agents by biofilms also assists the better survival of biofilm microbes under stressful environmental conditions (Singh et al. 2006). Research on biofilm structures suggests that biofilms exist as a mass of microcolonies in a single layer or as three-dimensional structures with vertical and horizontal channels permitting liquid flow and dispersion of nutrients and waste components (Ramey et al. 2004), which allow enhanced bioremediation. Presence of biofilms in the

rhizosphere demand a higher amount of C sources than their absence. Being a C sink, they maintain a diffusion gradient of C from the root, thus stimulating rhizodeposition (Pearce et al. 1995). This helps increase soil C, which in turn is beneficial to survival of the microbes. It appears from these studies that rhizobial biofilm formation on legume and nonlegume roots, fungi, and soil particles ensures the effective *Rhizobium*–legume symbiosis while protecting the rhizobia from adverse environmental conditions in the absence of the host plant. The question arises here is whether or not the rhizobial biofilm formation occurs naturally at an adequate rate to make a significant effect on the symbiosis. If not, inoculation of the developed BBs could be important at field level (Seneviratne et al. 2008b, 2009). However, this needs further investigations which are explained later in the chapter.

6.3.3 *Indirect Effects of Biofilms on Rhizobium–Legume Symbiosis*

In spite of the direct effects of biofilm lifestyle of rhizobia in *Rhizobium*–legume symbiosis, some indirect effects could also affect the symbiosis. For example, increased production of indoleacetic acid (IAA) by an inoculated biofilm of *Penicillium* spp.–*Bradyrhizobium* spp. increased root growth of soybean (*Glycine max*) (Jayasinghearachchi and Seneviratne 2004a). When root growth abrasion occurs against the soil aggregates, it results in leakage of cell plasma content and finally retards the growth of root cells (Pearce et al. 1995). The lubrication effect on the rhizosphere by the biofilms through the release of lubricants such as EPS, however, facilitates the smooth growth of the plant-root system. Therefore, presence of biofilms in rough soils could assure a healthy root system, which in turn favorably influences the *Rhizobium*–legume symbiosis. Most leguminous crops thrive in neutral or slightly low soil pH (i.e., slightly acidic soil conditions). Under moderate and high acidic conditions, rhizobial survival and nodulation are limited (Ibekwe et al. 1997). Soil pH also affects indirectly the rhizobial attachment to roots by altering Ca^{2+} availability (Rinaudi et al. 2006), because Ca^{2+} acts as a bridge between negatively charged active groups of organic molecules on the root and bacterial surfaces. However, higher levels of Ca^{2+} negatively affect rhizobial growth. The rhizospheric pH is modified by existing biofilms in response to proton fluxes toward the root tip during growth (Pearce et al. 1995). Therefore, biofilmed communities in the rhizosphere may indirectly affect *Rhizobium*–legume interaction. The other important aspect of biofilm suggests that it can fill soil porous spaces and also can form a thin water layer in between the rhizoplane and biofilm (Pearce et al. 1995). Clogging the porous spaces leaving smaller pores in the channels of the biofilm structure reduces permeability (Cunningham et al. 1991) and water movement in the rhizosphere, thus preserving water from evaporation, which is important under dry land conditions. Further, very high water retention ability in EPS of the biofilms (Sutherland 2001) makes biofilm microbes more moisture stress

tolerant than planktonic cells. Both of these phenomena help survive rhizobia under soil moisture stress, thus promoting *Rhizobium*–legume symbiosis.

6.4 Potential of Biofilmed Biofertilizers in Improving Legume Performance

Biofertilization with improved rhizobial inocula is particularly necessary as most of the agriculturally important leguminous species sometime fail to perform well in regions beyond their origin, probably due to the absence of appropriate rhizobia (Bottomley and Myrold 2007). Effective microorganisms like inoculation of N_2-fixing bacteria, thus, have long been used into field practices worldwide (Bashan 1998) as inocula for biofertilization (Bloemberg and Lugtenberg 2001) as reviewed by Triplett and Sadowsky (1992). It was revealed that most field experiments failed to improve N_2 fixation even with the use of superior rhizobial strains, probably due to poor/lack of survival and inability to compete with well-adapted indigenous microbial strains. Therefore, in conventional inoculant technology of microbial monocultures, a major problem yet to be addressed is the poor survival of introduced microorganisms in the soil due to various environmental stress factors. Thus, it is vital to find competitive inocula, which can overcome less-effective native populations. In an attempt to solve this problem, in an experiment, it was reported that plant-associated biofilms have a high ability to protect themselves from external stress and microbial competition that are characteristic of the rhizosphere, and also to produce beneficial effects in plant growth promotion (Ramey et al. 2004). Moreover, it was clearly shown that the use of FBBs/FRBs as biofilmed inocula is more effective in terms of their biological performance than mono- or mixed microbial cultures (Seneviratne et al. 2008a). For example, BBs applied to soybean significantly increased seed yield by ca. 35%, compared to conventional rhizobial inoculant under field conditions (G. Seneviratne, unpublished). Furthermore, the biofilms can also be used to successfully introduce rhizobial strains into the soil (Seneviratne et al. 2008b, 2009).

Apart from environmental stresses, P deficiency in soil has been identified as one of the important limiting factors in the N_2-fixation process, which eventually affects the legume crop production (Zahran 1999; Vance 2001). Arbuscular mycorrhizal (AM) fungi produce extensive network of external hyphae, which help obtain mineral nutrients, primarily P (Lum and Hirsch 2003). The *Rhizobium*–AM (RAM) symbiosis, possibly forming FRB (Seneviratne et al. 2008a) plays an important role in *Rhizobium*–legume symbiosis (Lum and Hirsch 2003; Chalk et al. 2006; Kaschuk et al. 2009). This intergeneric interaction improves the nutrient availability where AM fungi supplies P while rhizobia provides N which together leads to increase in photosynthetic rates and concurrently the plant growth. The RAM associations have been found to improve the performance and yields of legumes compared to nonsymbiotic plants (Kaschuk et al. 2009). Similar effects can also be

achieved by inoculating FBBs/FRBs, as observed under soil environments (Seneviratne and Jayasinghearachchi 2005). In addition, introduced biofilms from the BBs and *Rhizobium*–legume symbiotic system interact with each other, rendering more beneficial effects to both symbionts and the environment. Therefore, in vitro production and application of efficient BBs could serve as an alternative to chemical fertilizers and help to augment legume production in eco-friendly and sustainable manner.

6.5 Conclusion

Understanding the biofilm interactions with legume roots is challenging due to the difficulty in studying underground processes under controlled yet realistic conditions. Thus, developing novel methodologies to study the effect of biofilm interactions on *Rhizobium*–legume symbiosis under natural conditions is desirable and collaboration among plant biologists, ecologists, and soil microbiologist would be a decisive factor. It is clear that our general understanding of the symbiosis as an individually performing system needs rethinking as cooperative symbiosis actions with plant–microbe as well as microbe–microbe symbioses in an interactive environment. It also seems that attachments to plant roots and mycelia by rhizobia are comparable events, which may be governed by common factors. Further, it can now be speculated that leguminous roots play a vital role, facilitating communication between the plant and, not only planktonic microbes, but also biofilms in the soil. Current knowledge on biofilms indicates that the interactions could potentially be translated to mediate the activities of the whole system in the rhizospheric environment. What does this mean at the rhizosphere? Why are these important in *Rhizobium*–legume symbiosis? Evidently, the possible answer is that cooperation between symbioses makes an immense effect on the symbionts as well as the environment. Individualism has become outdated in more efficient systems across both biological and physical worlds. Finally, it is clear that well-managed biofilm interactions are likely to lead to the development of better legume plants capable of not only efficient N_2 fixation, but also absorbing more nutrients from the surroundings, detoxifying soils more efficiently, tolerating pathogens and better-coping adverse environmental conditions.

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Chapter 7
Potential of Rhizobia as Plant Growth-Promoting Rhizobacteria

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Abstract Bacteria collectively known as rhizobia are widely studied due to their ability to associate with legumes, establishing a symbiotic relationship in which the fixation of atmospheric nitrogen is the main benefit to the plant. These bacteria are capable of colonizing the rhizosphere of nonhost plants, as well as living inside the plant tissues as endophytes. These traits, along with their ability to produce phytohormones, solubilize, and bind nutrients, besides eliciting plant defense reactions against pathogens, turn rhizobia into organisms with high potential to act as plant growth-promoting rhizobacteria (PGPR). This review intends to present research results obtained so far concerning to the application of rhizobia as PGPR, highlighting the benefits of this practice adopted to increase the plant growth and yields and nutrient uptake, besides focusing on other mechanisms used by rhizobia to promote plant health. These microorganisms, due to the diverse range of activities as well as the number of rhizobia stored in different culture collections around the world, may provide an important resource to rationalize the use of fertilizers and chemicals in agriculture.

7.1 Introduction

Bacteria that form root nodules and fix atmospheric nitrogen (N) in association with legumes are collectively known as rhizobia, irrespective of genus (Lindstrom and Martinez-Romero 2005). Many different bacteria share this feature. There are

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reports accounting that the designation rhizobia could currently consist of more than 70 species distributed over 13 genera (Weiss and Ori 2007), including some betaproteobacteria such as *Burkholderia* and *Cupriavidus* (Chen et al. 2007; Barrett and Parker 2006). However, most authors have considered rhizobia as alphaproteobacteria, which includes the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium,* and *Azorhizobium* (Sahgal and Johri 2003). These five genera are considered the traditional rhizobial phylogenetic lineage (Willems 2006) and the present text will focus on them.

Rhizobia are largely studied due to their highly efficient symbiotic association with legumes and hence, the use of rhizobial inoculants is considered an important application of microbial technology in sustainable agriculture. Only in Brazil for example, inoculation of soybean (*Glycine max*) fields has been found to supply up to 300 kg N ha^{-1}, resulting in savings of N fertilizers estimated at US$ 3 billion (Santos et al. 2006). Nevertheless, besides its undisputed role in agriculture as N provider to legumes and companion crops, rhizobia may also benefit plants by other means, acting as PGPR, a concept introduced in the late 1970s (Kloepper et al. 1980; Suslow et al. 1979; Kloepper and Schroth 1978). Since then it has grown and well established and gained worldwide attention. Plant growth-promoting rhizobacteria can simply be defined as root colonizing bacteria that exert beneficial effects on plant development by direct mechanisms, indirect mechanisms, or a combination of both. There are many different genera of bacteria that have proved to be efficient PGPR. Perhaps the most widely studied PGPR genus are *Azospirillum* (Bashan et al. 2004), *Pseudomonas* (Adesemoye and Ugoji 2009; Cattelan et al. 1999; Kloepper et al. 1980), *Bacillus* (Recep et al. 2009; Probanza et al. 2001), and *Burkholderia* (Joo et al. 2009). More recently, some rhizobial strains have also called the attention due to their positive effects both on host legume plants and on nonlegumes. Rhizobia strains are reported to posses many desirable plant growth-promoting traits and exert diverse positive effects on many important crops. Based on the data generated in recent times for rhizobia, the large numbers of strains stocked in culture collections around the world, the great genetic variability among strains and the fact that they are harmless microorganisms, rhizobia is considered as one of the most promising groups of PGPR for its ultimate application in legume improvement.

7.2 Root Colonization

Considering how microbial communities affect plants, soil microorganisms can be deleterious (including pathogens), neutral or beneficial. In the last group are included the PGPR which includes largely Gram-negative bacteria (Arora et al. 2001) including rhizobia. In order to effectively promote plant growth, rhizobacteria must be able to occupy niches in rhizosphere and roots in competitive conditions (Kloepper 2003). Thus, to facilitate plant growth, by direct or indirect mechanisms, a rhizobacterium has to be in intimate contact with the plant, which

occurs by rhizosphere colonization or by penetrating and establishing itself inside the plant as an endophyte (Vessey 2003). Consequently, the interaction between plant and microorganism is of major practical importance, because rhizobacteria on one hand play a role as plant growth promoter while plants on other hand may exert a selective control over bacterial diversity and abundance in rhizosphere. By releasing organic compounds as exudates, which may either stimulate or inhibit the rhizospheric biota species, plants create a very selective pressure in a low diversity environment (Barriuso et al 2008). So, root colonization is one of the first steps during which PGPR can express their characteristics (Kloepper and Beauchamp 1992) and rhizobia are known to be competent rhizospheric bacteria able to survive in soil for too long, regardless of the presence of host legume. For example, Giongo (2007) observed that a population of bradyrhizobia, with high genetic diversity, nodulating soybean, was able to survive in a field kept in fallow for more than 30 years without reinoculation and in absence of the host plant. In a shorter period experiment Batista et al. (2007) also noticed the competitive ability of a *Bradyrhizobium japonicum* strain CPAC 15 (same serogroup as USDA 123), characterized as having high saprophytic capacity and competitiveness. The ability of rhizobia to survive and multiply in nonlegume rhizosphere was explored even as an alternative way to increase the desirable population of efficient bradyrhizobia in soil, by means of inoculating winter cereals seeds, prior to soybean sowing (Domit et al. 1990). But, instead of just increasing its population in soil, the inoculation of nonlegumes may result in intense colonization of roots by rhizobia. As an example, Chabot et al. (1996) inoculated maize (*Zea mays*) and lettuce (*Lactuca sativa*) with two strains of *Rhizobium leguminosarum* bv. *phaseoli*. They observed that rhizobial populations on root surfaces averaged log 4.1 CFU g^{-1} (fresh weight) on maize roots 4 weeks after seeding and log 3.7 CFU g^{-1} (fresh weight) on lettuce roots 5 weeks after seeding. A similar result was obtained by Schloter et al. (1997), who studied the colonization of different roots of nonlegumes by a *R. leguminosarum* bv. *trifolii* strain. The authors observed that, in maize roots, an overexpression of bacterial lipopolysaccharides was suggested as an indicative of plant–microbe interaction. Unlike Chabot et al. (1996), Schloter et al. (1997) observed that rhizobia not only colonized root surfaces, but also lyzed cells of the root cortex and intracellular space of central root cylinder cells. The endophytic colonization of nonlegumes by rhizobia was also reported by Sabry et al. (1997), who during an interaction study between *Azorhizobium caulinodans* and wheat (*Triticum aestivum*) observed the invasion of azorhizobia between cells of the cortex. Yanni et al. (1997) also noticed a natural endophytic establishment of *R. leguminosarum* bv. *trifolii* in rice (*Oryza sativa*) roots in a rotation with berseem clover (*Trifolium alexandrinum*). They isolated two endophytic rhizobial strains that in subsequent experiments increased rice production under gnotobiotic as well as field conditions. Similarly, Zamora and Romero (2001) identified the natural endophytic *Rhizobium etli* in maize plants grown in association with common bean.

It is important to emphasize here that the endophytic association of rhizobia and nonlegumes occurs without the involvement of genetic signals related to nodulation process (Reddy et al. 1997). Rhizobial penetration inside nonlegume plant tissues

occurs mainly through cracks in epidermal cells of the roots and in fissure sites where lateral roots have emerged (Dazzo and Yanni 2006; Prayitno et al. 1999). Nonetheless, the rhizobial endophytic establishment is a dynamic process, beginning with colonization at lateral root emergence, crack entry into the root interior through separated epidermal cells, followed by endophytic ascending migration up to the stem base, leaf sheath, and leaves where they grow transiently to high local population densities (Chi et al. 2005). Once inside the plant and established in high numbers, rhizobia may influence plant growth by different PGPR mechanisms.

7.3 Mechanisms of Plant Growth Promotion by Rhizobia

PGPR can affect plant growth via direct or indirect mechanisms. The direct promotion of plant growth by PGPR entails either providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of certain nutrients from the environment (Glick 1995). The most usual direct mechanisms of plant growth promotion include atmospheric nitrogen fixation, the production of plant growth regulators, and the solubilization of phosphates (P). Nonsymbiotic nitrogen fixation is also one of the most desirable PGPR traits and some workers suggested that rhizobia may also fix N in association with nonlegumes. For example, Sabry et al. (1997) described high level of nitrogenase activity in wheat inoculated with an *A. caulinodans* strain while Chaintreuil et al. (2000) in other study detected acetylene reduction activity (ARA) in rice plants inoculated with a photosynthetic bradyrhizobia. However, in most of the cases of plant growth promotion of nonlegumes by rhizobia, nitrogen fixation is rare and negligible, if not inexistent. And hence, plant growth promotion in such cases is the result of an improved uptake of the soil mineral N, rather than from BNF (Dazzo and Yanni 2006). In contrast, the indirect mechanisms of plant growth promotion occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms (Gandhi Pragash et al. 2009). It is important to mention here that the separation in direct or indirect mechanisms of plant growth promotion is artificial and sometimes difficult to delimit. Productions of siderophores, for instance, may be considered a direct factor, since siderophores solubilize and sequester iron from soil and provide it to plant cells. But it can also be considered an indirect factor, since it is linked to suppression of plant pathogens by iron deprivation. Anyway, PGPR may affect plant growth and development by using any one or combination of these mechanisms.

7.3.1 Production of Plant Growth Regulators

Plant growth regulators are organic molecules analogous to plant hormones, which, at low concentrations, cause a physiological response and influence plant development. They are divided into five general groups of compounds based on

their chemical structures and effects on plants: auxins, gibberellins, cytokinins, ethylene, and a group called inhibitors, which includes abscisic acid (ABA), phenolics, and alkaloids (Ferguson and Lessenger 2006). All these compounds are produced by soil bacteria, but vary in concentration. The production of auxins and ethylene by bacteria is considered a common trait, while the synthesis of cytokinins is less common, and the gibberellin secretion at high concentrations is very rare (Solano et al. 2008). It been estimated that more than 80% of the soil bacteria are able to produce auxins, especially indoleacetic acid (IAA), indolebutyric acid, or similar compounds derived from tryptophan metabolism (Solano et al. 2008; Loper and Schroth 1986). Auxins are plant growth regulators that stimulate cell division and elongation and its production by PGPR is one of the most studied and, perhaps, the most effective mechanism of plant growth promotion by rhizobia (Schlindwein et al. 2008; Hafeez et al. 2004; Biswas et al. 2000).

Many rhizobial strains are reported to produce auxins in variable amounts. For example, Antoun et al. (1998) working with 266 rhizobial strains, from different species and genera, found that 58% of the strains produced IAA, while Vargas et al. (2009) in a similar study found a considerably lower frequency of auxin producers (23%) among populations of clover nodulating *R. leguminosarum* bv. *trifolii*. However, they noticed a very distinct behavior between strains isolated from arrow leaf clover and those isolated from white clover nodules. In the first group, IAA production was much more frequent accounting for more than 90% of the isolates. On the contrary, IAA production was considerably less frequent (only 15%) in rhizobia isolated from white clover nodules. Auxins produced by rhizobia may be related to nodulation, and hence, IAA synthesizing rhizobia have been found to nodulate more intensely than IAA negative mutants (Boiero et al. 2007). In nonlegumes, IAA produced by rhizobia may stimulate plant root system, increasing its size and weight, branching number and the surface area in contact with soil, resulting in the development of more expansive root architecture (Dazzo and Yanni 2006). Inoculation with auxin-producing bacteria may also result in the formation of adventitious roots that derive from the stem of the inoculated plant (Solano et al. 2008). All these changes in root system increase its ability to prospect the soil for nutrient exchange, therefore improving plant nutrition and growth capacity (Gutiérrez Mañero et al. 1996). Noel et al. (1996) verified that the inoculation with IAA-producing strains of *R. leguminosarum* accelerated the germination of canola and lettuce. Similarly, Biswas et al. (2000) observed that the inoculation of rice with *R. leguminosarum* bv. *trifolii* increased dry matter and grain production, besides an increment in N, P, K, and Fe content in plant tissue. All these effects were credited to IAA accumulation in rhizosphere by rhizobial inoculation, resulting in physiological changes in root system with consequent improvement in nutrient uptake.

However, this positive effect depends on the amount of IAA produced by the bacterium, since an IAA overproduction is considered deleterious to plants (Schlindwein et al. 2008; Ahmad et al. 2005). Similar to other phytohormones, IAA exerts a stimulatory effect on plant growth within a narrow concentration range only, outside of which the plant is either unresponsive or its growth is inhibited. In this context, Barazani and Friedman (1999) found that deleterious

rhizobacteria (DRB) produced high IAA, about 77 μmol L^{-1} after 84 h of incubation, while a consortia of beneficial rhizobacteria produced much less IAA (16 μmol L^{-1}) during the same period. In a similar way, while analyzing the production of IAA by rhizobial strains, Schlindwein et al. (2008), found that the *R. leguminosarum* bv. *trifolii* strain TV-13 produced 171.1 μg mL^{-1} IAA in media enriched with tryptophan. Lettuce seeds treated with this strain did not germinate normally and those that germinated were without radical protrusion and with precocious opening of the cotyledons (Fig. 7.1). These toxicity symptoms were similar to those observed for herbicide suggesting that auxins may act as herbicides for dicotyledons. On the other hand, strains of *Bradyrhizobium* sp. isolated from black wattle roots (T6–4, T6–12, V-10, and C3), which produced between 1.2 and 3.3 μg mL^{-1} IAA, increased seedlings vigor in relation to uninoculated control. The amount of IAA produced by a given bacterium strain, however, varies with the composition of the growth medium and is tryptophan-dependent. The deleterious effect of inoculation with TV-13 was ceased when the isolate was grown in yeast mannitol (YM) medium without tryptophan supplementation (Schlindwein et al. 2008). In such condition, IAA production by TV-13 was not detected and germination rates equaled to the uninoculated control. Sridevi et al (2008) also observed that IAA production by rhizobia occurred only when tryptophan was added to YM and observed that the isolates produced maximum amount of IAA in medium supplemented with 2.5 mg mL^{-1} tryptophan concentration.

Like auxins, cytokinins not only affect cell division and cell enlargement, but also affect seed dormancy, flowering, fruiting, and plant senescence (Ferguson and Lessenger 2006). Production of cytokinins by PGPR is considered to be less usual than the production of auxins, perhaps because there is no foolproof method available to evaluate cytokinins as is reported for the production of auxins. So, the report on the production of cytokinins is scarce, and hence, it is inadequately studied. For rhizobia, the production of cytokinin, however, must be a frequent trait, since this is instrumental in nodule developmental process, as it is required to initiate the cortical cell divisions necessary to form a root nodule, and may also

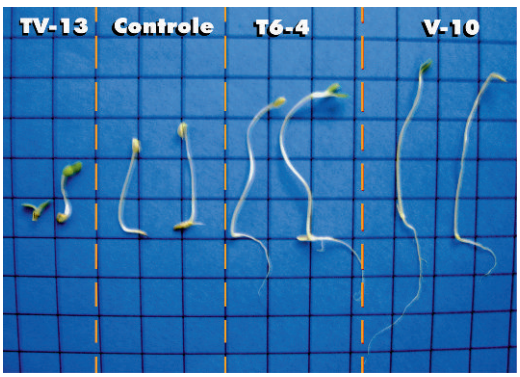

Fig. 7.1 Seeds of lettuce inoculated with *R. leguminosarum* bv. *Trifolii,* IAA overproducing strain TV-13 and *Bradyrhizobium* sp. T6-4 and V-10, producers of small concentrations of IAA (adapted from Schlindwein et al. 2008)

mediate rhizobial infection in legumes (Frugier et al. 2008). Gibberellins are other plant growth regulators that enhance seed germination (Miransari and Smith 2009), stimulate extensive growth of plants, and delay aging (Ferguson and Lessenger 2006). The production of gibberellins at high concentrations is considered very rare and has only been reported for two strains of *Bacillus* isolated from the rhizosphere of *Alnus glutinosa*, the amounts being 1,000 times higher than those produced by *Rhizobium* when forming the nodule (Solano et al. 2008; Gutiérrez Mañero et al., 2001). The concentration of gibberellins in nodules is, however, generally higher than in nearby root tissue as supported by the fact that free-living rhizobial bacteria have the capacity to produce some amount of gibberellin-like substances. However, it is not known whether bacteria contribute significantly to the amount of gibberellins within the nodule or it is just imported from some remote host plant tissue (Dobert et al. 1992). In spite of this, role of gibberellin in *Rhizobium*–legume symbiosis that may have important implications in endophytic colonization of nonlegumes by rhizobia is described. For example, *A. caulinodans* infects the semiaquatic legume *Sesbania rostrata* via the intercellular crack entry, by a process mediated by gibberellins. Considering that crack entry is the main process of endophytic colonization of nonlegumes by rhizobia, the production of gibberellins by the bacterium is reported to facilitate this process (Lievens et al. 2005). In contrast, ABA inhibits growth and germination and promotes seed dormancy (Ferguson and Lessenger 2006), acting adversely to gibberellins (Miransari and Smith 2009; Yang et al. 2009). Besides, ABA plays an important function in mediating plants tolerance to abiotic stresses. When plants are exposed to drought stress, they change their plant hormone balance, increasing ABA content in the leaves, accompanied by a reduction in endogenous cytokinin levels, which in turn elicits stomata closure (Yang et al. 2009). Cohen et al. (2009) in a similar study suggested that ABA produced by an *Azospirillum* strain, along with bacterial gibberellins, significantly contributed to water-stress alleviation of maize plants. Some rhizobial strains, such as *B. japonicum* USDA110, are also reported to produce ABA (Boiero et al. 2007) and contribute in the same way as do other PGPR.

Similar to ABA, ethylene acts as a messenger of biotic and abiotic stresses, acting as a negative regulator of vegetal growth. Ethylene also affects ripening and senescence in plants (Ferguson and Lessenger 2006). Its biosynthesis starts from methionine with 1-aminocyclopropane-1-carboxylic acid (ACC) as the key intermediate, which is converted to ethylene through the action of enzyme ACC oxidase. Some bacteria are able to produce ethylene from methionine. Boiero et al. (2007) found that the strains of *B. japonicum* E109, USDA110, and SEMIA5080, the most commonly used for inoculation of soybean and nonlegumes in USA, were able to produce ethylene, in yeast extract mannitol medium amended with methionine. On the other hand, some bacteria are able to decrease the levels of ethylene in plant root tissue mediated by the bacterial enzyme ACC deaminase, which competes with plant ACC oxidase. According to Glick et al. (1998), the bacterial enzyme acts in rhizosphere and degrades ACC exuded by plant roots to ammonia and α-ketobutyrate, resulting in lowering the level of ACC outside of the plant, forming a gradient

from the interior of the plant to its exterior. In order to maintain the equilibrium between internal and external ACC levels, the plant must exude increasing amounts of ACC. As a consequence, the level of ACC within the plant is reduced and hence, inhibitory action of ethylene is decreased. Thus, plants influenced by ACC deaminase positive PGPR are supposed to have longer roots and possibly shoots as well (Glick et al 1997).

The reduction of ethylene levels in plant tissues derived from the ACC deaminase activity can cause significant morphological changes in root tissue, such as changes in root-hair length and increases in root mass, accompanied by the consequent improvement in nutrient uptake. The morphological changes are greater when ACC deaminase action is combined with production of auxins by PGPR. It has been observed that some rhizobia may reduce plant ethylene levels by means of ACC deaminase activity and results in enhanced nodulation in host legumes (Ma et al. 2003) or modifications in root system of nonlegumes. For instance, strains of *R. leguminosarum* bv. *viciae* and *Mesorhizobium loti* increased the number of lateral roots in *Arabidopsis thaliana* because of this plant growth-promoting mechanism (Contesto et al. 2008). Rhizobitoxine (Rtx) is another metabolite produced by bacteria including *B. elkanni* and acts in a way similar to ACC deaminase: it strongly inhibits ACC and decrease ethylene levels. Since ethylene is known to inhibit or down-regulate nodule development, Rtx plays a positive role in nodule development by inhibiting ethylene biosynthesis (Duodu et al. 1999). However, unlike ACC deaminase, Rtx is not expected to promote plant growth. Its most usual effect is deleterious, since it causes chlorosis in plant leaves (Okazaki et al. 2007; Duodu et al. 1999). The only presumed role of Rtx in growth promotion has been the protection of soybean roots from *Macrophomina phaseolina* infection, suggesting that Rtx may act as antifungal substance (Chakraborty and Purkayastha 1984).

7.3.2 Solubilization of Phosphates

Phosphorus (P) is one of the major mineral nutrients required by plants whose deficiency is extremely limiting for crop production. In nature, P is found in a variety of organic and inorganic forms that are very poorly soluble. It is considered as one of the less soluble elements in the natural environment, with less than 5% of the total soil P content being available to the plants (Dobbelaere et al. 2003). So phosphatic fertilization is needed to obtain optimum crop production. However, a large portion of the soluble inorganic P applied to soil as fertilizer is rapidly immobilized by the iron and aluminum oxides in acid soils and by calcium in calcareous soils soon after application, thus becoming unavailable to plants (Khan et al 2007; Chacon et al. 2006). Besides mineralizing organic P through the action of phosphatase enzymes (Garcia et al. 1992), many soil microorganisms can solubilize mineral P generally via the production of organic acids (Zaidi et al. 2009a), which acidify the surrounding soil. Due to this reason, solubilization of P is thought to be more efficient in basic soils than in naturally acid soils (Solano et al.

2008; Khan et al. 2010). A large number of P-solubilizing bacteria have been isolated from the rhizosphere of several crops (Zaidi et al. 2009b). It was estimated that these microorganisms constitute about 20–40% of the cultivable population of soil microorganisms and that a significant proportion of them can be isolated from rhizosphere soil (Chabot et al 1993). There have been a number of reports on plant growth promotion by bacteria that have the ability to solubilize P. However, the production of other metabolites beneficial to the plant by these microorganisms, such as phytohormones, antibiotics, or siderophores, among others, has created confusion about the specific role of P solubilization in plant growth and yield.

Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic P compounds, such as tricalcium phosphate (TCP), dicalcium phosphate (DCP), hydroxyapatite, and rock phosphate (RP). There are considerable populations of P-solubilizing bacteria in soil and in plant rhizospheres, including many genera of both aerobic and anaerobic strains. Commonly, the density of these bacteria is considerably higher in the rhizosphere than in adjacent nonrhizosphere soil (Zaidi et al. 2009b). According to Rodriguez and Fraga (1999), the genus *Rhizobium* is one of the major P solubilizers, along with bacteria belonging to the genera *Pseudomonas* and *Bacillus*, as also reported by Vargas et al. (2009). Among the PGPR traits evaluated for 252 isolates of *R. leguminosarum* bv. *trifolii*, solubilization of P was the most usual characteristics. This trait was identified in 42% of all the isolates and in 100% of the isolates from one of the sampling sites (Porto Alegre, Brazil). Like *Rhizobium* species, other rhizobia also posses this PGPR trait. For example, Alikhani et al. (2006) while working with 446 bacteria belonging to the genera *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium,* and *Rhizobium* evaluated the solubilization of inorganic and organic P under in vitro conditions. They observed that 44% of the isolates solubilized TCP while 76% solubilized inositol hexaphosphate. However, the rhizobial isolates differed in their P-solubilizing ability. Of these, *R. leguminosarum* bv. *viciae* was most prominent P solubilizer, which was followed by *M. ciceri*, *M. mediterraneum*, *S. meliloti,* and *R. leguminosarum* bv. *phaseoli*. However, none of the 70 strains of *Bradyrhizobium* tested were able to solubilize inorganic P, confirming the observation of Antoun et al. (1998) who also found only one P-solubilizer strain out of the 18 tested *B. japonicum* strains. The genus *Bradyrhizobium* is characterized by the production of alkali in growth media, a possible reason to explain poor P solubilization by this organism. On the other hand, when the authors analyzed the mineralization of organic P, a process mediated by phosphatases, *Bradyrhizobium* sp. strains were the most effective ones. But, even though, *B. japonicum* were the less efficient strains. It was concluded from this study that many rhizobia isolated from Iranian soils are able to mobilize P from both inorganic and organic sources and hence, the probable beneficial effects of such bacteria needs to be tested with crops before they are recommended for use in field environments.

The results of in vitro tests for P solubilization are, however, not always related to effects in vivo, which make difficult the screening for PGP activity. But many times in vitro results are corroborated by results of inoculation in plants. For example, Peix et al. (2001) selected P solubilizers among several species of

rhizobia, belonging to different genera, specific to chickpea (*Cicer arietinum*). They observed that strains nodulating only chickpea (including the type strains) were able to solubilize P and that the strain PECA21 was the most efficient one. This isolate later on when used as inoculant enhanced the growth and P content in chickpea and barley (*Hordeum Vulgare*) plants grown in soil with and without P in a growth chamber experiment. In soil treated with insoluble P, the strain PECA21 improved the P content of both plants, as well as their dry matter, N, K, Ca, and Mg content.

7.3.3 Biological Control of Plant Pathogens

Plant growth-promoting bacteria can also stimulate plant growth by producing and/or inducing the plant to release secondary metabolites, facilitating the uptake of nutrients, and/or inhibiting plant pathogenic organisms in the rhizosphere. The control of plant pathogens by PGPR can be achieved by involving different mechanisms, acting either alone or in combination with other compatible microbes. The most usual mechanisms of biological control by PGPR include the production of siderophores and antibiotic substances and the induction of systemic resistance. Rhizobia are among the most effective and promising biological control agent. There are many reports of plant disease control by different rhizobial genera. For example, Ozkoc and Deleveli (2001) in an in vitro experiment tested the effects of 23 *R. leguminosarum* bv. *phaseoli* isolates on the mycelium development of three phytopathogenic fungi (*Fusarium oxysporum*, *Pythium ultimum*, and *Rhizoctonia solani*) and observed that most of the isolates inhibited the pathogens. In a similar study, Chao (1990) tested six rhizobia, belonging to genera *Rhizobium*, *Bradyrhizobium*, and *Sinorhizobium* against ten fungi. Their abilities to inhibit the growth of fungal isolates varied tremendously, but *R. leguminosarum* bv. *phaseoli* 6–3 significantly reduced the mycelium dried weights of all the fungal isolates tested. In a recent investigation, Hossain and Mårtensson (2008) also found that some rhizobial strains are able to dissolve the fungal mycelium at the initial stage. However, one of the widely accepted mechanisms of pathogen suppression by rhizobia and PGPR is the production of siderophores. Siderophores are organic molecules with low molecular weight (400–1,000 Da) containing functional groups with high affinity for Fe_3^+, capable of binding iron in a reversible way (Solano et al. 2008; Gray and Smith 2005). Although iron is an abundant mineral in the soil, most of it is unavailable for direct assimilation by plants. In aerobic soils, it is found predominantly in the oxidized ferric form, Fe_3^+, constituting oxyhydroxide polymers with extremely low solubility. This precipitated form is also unavailable to microorganisms which, in order to obtain iron for their growth and development, releases iron-binding molecules, the siderophores. Once iron is chelated by siderophores, the stable complex is picked up by specific receptors located in the outer membrane of the bacterium and, once inside the microbial cell, iron is ready to be metabolized by the microorganism and by the plant. The production of siderophores by PGPR

can thus promote plant growth either by directly improving plant iron nutrition or by inhibiting growth of pathogens in rhizosphere by limiting the iron availability (Solano et al 2008). Another possible positive effect of siderophore production is the complexation of toxic aluminum. In a study, Roy and Chakrabartty (2000) evaluated the production of sidrophores by a *Rhizobium* sp. influenced by the concentration of Al_3^+. Besides increasing iron availability, rhizobial siderophores also reduced Al_3^+ toxicity to the bacterium through complex formation mechanism. Similarly, Rogers et al. (2001) proved the effectiveness of the hydroxamate siderophore vicibactin produced by *R. leguminosarum* bv. *viciae* in alleviating aluminum toxicity. The complex siderophore-aluminum may eventually be taken up into the bacterial cytoplasm. However, it is unlikely to become toxic intracellularly, because aluminum cannot be released from the complex by reduction and the complex, therefore, simply accumulates as a nontoxic species or even, if it is released, Al_3^+ will precipitate as $Al(OH)_3$ at the slightly alkaline cytoplasmic pH (Rogers et al. 2001; O'Hara et al. 1989).

There are many reports of synthesis of siderophores and its consequent effect in the suppression of plant pathogens by siderophore-producing rhizobia (Ahemad and Khan, 2010; Chandra et al. 2007; Sessitsch et al. 2002). Later on, Deshwal et al. (2003) evaluated ten strains of peanut nodulating *Bradyrhizobium* and found three strains (AHR-2, AHR-5, and AHR-6) able to produce siderophores, besides synthesizing IAA and P solubilization in vitro. These strains also efficiently fixed N and showed antagonistic action against *M. phaseolina*, the causal agent of charcoal rot of peanut. So, the inoculation with selected rhizobia may not only provide N to the host legume plants, but also promote plant sanity by controlling pathogens. All the three bradyrhizobial strains inhibited radial growth of the fungus in vitro and declined its population in rhizosphere soil of bradyrhizobia inoculated peanut. Since all strains were siderophore producers, the inhibition of *M. phaseolina* may be imputed to this PGPR mechanism. However, a clear relation between the production of siderophores and the suppression of plant pathogens is not clear. For example, Vargas et al. (2009) tested ten isolates of *R. leguminosarum* bv. *trifolii* for antagonism against a phytopathogenic fungus *Verticillium* sp. isolate. All rhizobial isolates showed some degree of antagonism against the test fungus (Fig. 7.2). The greatest level of inhibition was achieved by the isolates CXS-12, AGR-3, ELD-15, VAC-12, and DPE-12. Two isolates (CXS-12 and AGR-3) with greatest antagonistic activity that decreased mycelial growth (by about 65%) were also siderophore producers. However, two other siderophore-producing isolates (IRG-17 and SBO-3) displayed less pronounced antagonistic effect compared to other nonsiderophore producers. The variation in antagonisms could probably be due to the differences in the type of siderophores produced by each isolate. Accordingly, Matthijs et al. (2007) observed that *Pseudomonas fluorescens* ATCC 17400 produced two siderophores, pyoverdine and thioquinolobactin, with thioquinolobactin showing a much more intense antifungal activity than pyoverdine.

A very similar result was obtained by Omar and Abd-Alla (1998) while evaluating the antagonism of 20 isolates of *Bradyrhizobium* and *Rhizobium* against the phytopathogenic fungi *Fusarium solani*, *M. phasolina*, and *R. solani*. All the

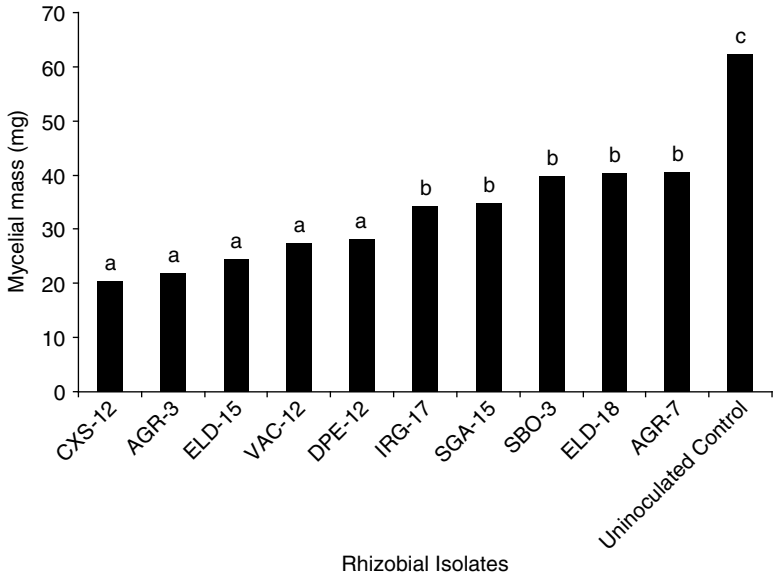

Fig. 7.2 Inhibition of *Verticillium* sp. mycelial mass production by rhizobial isolates. Means followed by the same letter did not differ significantly at $P \leq 0.05$ (Scott-Knot test) (adapted from Vargas et al. 2009)

isolates possessed antagonistic activity against fungi in both iron-deficient and iron-rich media. In such cases, ability to produce siderophores seems to act more as a competitive advantage, which allows the PGPR to colonize the rhizosphere more efficiently, than as a direct suppressive mechanism against pathogen by iron deprivation. Thus, when iron is not limiting, other mechanisms are more important in plant pathogen suppression than siderophore production. The secretion of metabolites such as antibiotics (Robleto et al. 1998) and hydrocyanic acid (HCN) (Ahemad and Khan 2009; Chandra et al. 2007) or the production of β-1,3-glucanase, proteases (Compant et al. 2005), and chitinases (Kacem et al. 2009) are important mechanisms of biological control of plant pathogenic microorganisms by PGPR.

Many rhizobia can produce antibiotics, especially bacteriocins, proteinaceous toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strains, conferring competitive advantage to bacteriocin-producer strains (Hafeez et al. 2005; Yanni et al. 2001). Robleto et al. (1998) described the effects of the antibiotics trifolitoxin, a bacteriocin produced by a *R. etli*, on the microbial composition in the rhizosphere of common bean. They observed a significant reduction in the genetic diversity of alphaproteobacteria, with little apparent effect on most microbes. Though bacteriocins are a narrow-spectrum antimicrobial compound, yet it is an effective metabolite that inhibits bacterial plant pathogens (Cladera-Olivera et al. 2006). *Rhizobium* sp. strains ORN 24 and ORN 83, isolated from Algerian soil, were found to produce bacteriocins with antimicrobial activities

against *Pseudomonas savastanoi*, the agent responsible for olive knot disease (Mourad et al. 2009).

Additionally, it has been shown that rhizobia are able to elicit reactions of plant defense against pathogens, as demonstrated by Elbadry et al. (2006). The authors verified the occurrence of induced systemic resistance (ISR) against bean yellow mosaic potyvirus (BYMV) in faba bean (*Vicia faba*) inoculated with *P. fluorescens* FB11 and *R. leguminosarum* bv. *viceae* FBG05. Plants inoculated showed a pronounced and significant reduction in percent disease incidence and a significant reduction in virus concentration. Since the PGPR inoculants and the pathogen remained spatially separated, it could be concluded that the tested *Pseudomonas* or *Rhizobium* strains induced systemic resistance in faba bean against BYMV. The activation of ISR by PGPR can be optimized when more than one microorganism are used as elicitors as reported by Dutta et al. (2008), who evaluated the occurrence of the process in pigeonpea (*Cajanus cajan*). They exposed, separately, part of plant root systems to the pathogenic fungus *Fusarium udum* and part to PGPR *B. cereus* or *P. aeruginosa*, and also evaluated the interaction of these PGPR with *Rhizobium* sp. It was evidenced by an enhancement of resistance in treated plants, mainly when PGPR strains were used with *Rhizobium*. Plants with mixture of PGPR and *Rhizobium* survived longer and showed higher level of defense-related enzymes than individual organism and nonbacterized control. Plant resistance to pathogens is, however, based on the deployment of a multicomponent defense response, which includes the hypersensitive response, chemical weapons, and structural defensive barriers. Signals for activation of these various defenses are initiated in response to recognition, by plant receptors of pathogen avirulence determinants, the elicitors, including PGPR (Dixon et al. 1994). Arfaoui et al. (2007) in an experiment analyzed the effect of rhizobial inoculation on chickpea, and noticed the activation of plant defense response against wilt caused by *F. oxysporum*. As a result, there was an accumulation of phytoalexins and a consequent activation of the defense enzymes such as ammonia-lyase, chalcone synthase, and isoflavone reductase. These findings complemented previous work of the same research group, who reported that the inoculation with rhizobial strains induced defense responses, reduced disease severity in chickpea plants infected with *F. oxysporum* (Arfaoui et al. 2006), and increased the activity of other defense-related enzymes, such as peroxidases and polyphenoloxidases, as well as led to the accumulation of phenolic compounds (Arfaoui et al. 2005). Similarly, Mishra et al. (2006) observed that the inoculation of rice plants with *R. leguminosarum* bv. *phaseoli* caused an increase in the production of phenolic compounds in relation to untreated plants. The increase in the production of phenolic compounds, which are indicative of plant defense response, was more remarkable in the presence of *R. solani*. Moreover, rhizobia are reported to reduce infections by the parasitic weed *Orobanche crenata* (Mabrouk et al. 2007). As an example, *R. leguminosarum* strains were able to promote pea development and simultaneously controlled *O. crenata*, notably by inducing necrosis in the parasite. Besides, *R. leguminosarum* can also elicit IRS of pea plants, as indicated by the accumulation of toxins and phenolic compounds in plant tissues.

7.4 Conclusion

Rhizobia are widely studied due to their undisputable role as legume symbionts. More recently, research works are suggesting that rhizobia can be beneficial to crops through their action as PGPR for both nonlegumes and host legume plants. It is possible to find rhizobia possessing virtually all desirable PGPR trait, without any harm to human and plant health. Though there has been a tremendous focus on rhizobia as PGPR, but further more research is required to achieve maximum benefits of such a naturally gifted organism. Rhizobia, thus, offers great opportunity to researchers/scientists to reveal its unexplored plant growth-promoting potentials as PGPR.

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Chapter 8
Engineering Nodulation Competitiveness of Rhizobial Bioinoculants in Soils

Gattupalli Archana

Abstract In field conditions, inoculated strains of rhizobia are at a survival disadvantage as compared to indigenous strains that are well adapted to local environment. Consequently, nodulation by unwanted strains is a major problem in enhancement of legume growth by rhizobial bio-inoculants. Competitiveness determinants include motility, chemotaxis, cell surface components, ability to use certain substrates, storage polymers, and production of antimicrobial compounds, higher growth rates, and ability to bring about faster infection. More recently, the involvement of other factors such as quorum sensing, the ability to form biofilms, and presence of protein secretion machinery has been shown to be important. Using genomics-based approach, numerous competitiveness genes have been identified. Variation in competitiveness traits among different legume-microsymbionts is becoming apparent. Approaches for the development of competitive bioinoculants by genetic engineering employ the following strategies (a) production of antimetabolites to inhibit nodule occupancy of native rhizobia, (b) interference with the regulation of plant–microbe signaling molecules to ensure efficient nodulation, (c) specific adaptation of the inoculated strain to environmental stresses, and (d) improved nutrition of the inoculant strain for competitive sustenance in soil or rhizosphere including root-derived compounds as well as other soil metabolites such as siderophore iron complexes. Engineering rhizobia for enhanced competitiveness is a challenging aspect of developing effective bioinoculants and ability to utilize heterologous siderophores could provide them with better iron acquisition ability and consequently, rhizosphere stability.

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8.1 Introduction

Competitiveness for nodulation is a trait that confers upon the rhizobial strains the ability to dominate in the nodulation of a particular legume host when other nodulation proficient strains also inhabit root rhizosphere (Dowling and Broughton 1986). Practically, when two or more distinct rhizobial strains, capable of nodulating the same legume species, are applied to the seed, competitiveness is measured by relative nodule occupancy by the individual strains (Beattie et al. 1989). Competition is also reported between nodulating and nonnodulating rhizobia, where nonnodulating strain prevents establishment of infective strain (Dowling et al. 1987). This is usually tested using axenic systems with known mixture of pure cultures of specific strains. Alternatively for testing in soils, the prospective inoculant strain is allowed to compete with native soil bacterial population to occupy the nodules. Although a plant may get nodulated by several strains simultaneously, each nodule usually harbors clonal populations of bacteria derived from a single strain, which initiates the infection (Simms et al. 2006; Pobigaylo et al. 2008; Shimoda et al. 2008). Thus, strain identification from independent nodules helps determine the successful events for different strains. The bacteria recovered from individual nodules are identified on the basis of their natural phenotypic characteristics, engineered marker genes (Sessitsch et al. 1998), or on the basis of DNA fingerprinting using ERIC, REP, or SP-PCR (Law et al. 2007; Ampomah et al. 2008a; Bogino et al. 2008; Duodu et al. 2009), ITS-RFLP (Simms et al. 2006), or ELISA (Spriggs and Dakora 2009). It should be noted that competitive ability is typically considered only between nodule bacteria. However, in fields, interactions are also possible between rhizobia and other soil bacteria, which could either be positive (Dashti et al. 1998) or negative (Mrabet et al. 2006). If interaction is negative, it may affect the saprophytic survival of the inoculant strain and/or lower the nodule occupancy of the inoculant strain. Hence, such interactions may complicate the interpretation of field results of rhizobial inoculations.

Slattery et al. (2004), while screening large number of Australian soils, reported that nearly 30–60% of the soils had sufficient naturalized rhizobia, which could nodulate faba beans, lentils, field pea, and vetch. Lowest populations of resident rhizobia were, however, found in acid soils, whereas in alkaline soils, the population size was often large. Generally, inoculated strains show large variation in nitrogen fixation ranging between 20 and 105% compared to an appropriate N-supplied control plant (Howieson et al. 2000). In spite of providing the commercial inoculant strain a numerical as well as positional advantage, applied rhizobial inoculant fails to outcompete naturalized rhizobia for nodule occupancy (Denton et al. 2003) as also reported for recombinant strains with improved nitrogen-fixing ability (Bosworth et al. 1994). It has been suggested that 90% of all commercially developed rhizobial inoculants applied are of no practical benefit to the productivity of legumes (Brockwell and Bottomley 1995).

Although in some cases, numerical superiority does not correlate with dominance in nodule occupancy, this has been considered one of the important factors

in nodulation competitiveness. Poorly competitive strains may nodulate even if another highly competitive strain is present but in lesser numbers, as was shown for *Sinorhizobium meliloti* indigenous populations (Bromfield et al. 1995; Hartmann et al. 1998; Velasquez et al. 1999). It is estimated that the field response to rhizobial bioinoculants diminishes when the number of competing rhizobia in the soil exceed above 10 cells/g. Careful consideration is given to indigenous rhizobial population in predictive models, which can be used for estimation of expected inoculation response (Thies et al. 1991).

8.1.1 Scope of the Chapter

Rhizobia are a phylogenetically diverse group of Gram-negative bacteria, belonging to α- and β-proteobacteria, representing currently 12 genera and over 70 species, able to establish symbiosis with leguminous plants (Franche et al. 2009; Masson-Boivin et al. 2009). The legumes (*Fabaceae*) are a diverse family and display high to moderate specificity toward microsymbionts (Sprent 2007). With the appropriate host, rhizobial strains form specialized organs, called nodules, wherein they reduce atmospheric nitrogen and make it available to plants. Rhizobial–legume symbiosis research dates back to over a century, from the time the role of bacteria in nodule formation was unambiguously demonstrated. Extensive data regarding the molecular basis of nodulation shows that it is an elaborate process involving clustered *nod* (nodulation) and *nif* (nitrogen fixation) genes, active molecular communication between the two partners and is controlled by bacterial as well as plant genes determining secretion of small signaling molecules, cell surface determinants, catabolism of legume, or bacterial metabolites (Cooper 2007; Garg and Geetanjali 2007; Gibson et al. 2008; Oldroyd and Downie 2008; Sprent 2008; Masson-Boivin et al. 2009).

Most soils have indigenous populations of rhizobia, and the resident population may be sufficient to effectively nodulate introduced legumes. However, the paucity of suitable rhizobial strains in certain soils or the poor symbiotic efficiency of native strains has led to the practice of application of selected well-characterized effective rhizobial strains as inoculants (Catroux et al. 2001; Deaker et al. 2004). The aim of inoculation is to provide sufficient numbers of symbiotically effective rhizobia to induce a rapid colonization of the rhizosphere and develop nodules on the plant. This is easily achieved by treating the seed with a powder or liquid formulation of the inoculants strain before planting, a technology globally followed for the last century (Bashan 1998). However, several strains that effectively increase symbiotic nitrogen fixation under controlled experimental conditions fail to do so in fields. Inconsistency in yield improvements plagues the rhizobial bioinoculant technology (Brockwell et al. 1995). Lack of thorough understanding of the abiotic as well as biotic factors that affect field performance limits the development of effective inoculant strains and, thus, results in poor crop response to laboratory-tested, highly efficient nitrogen-fixing rhizobial strains (Cummings et al. 2006).

An important factor that operates under field conditions is the competition barrier posed by native rhizobial strains, which outcompete introduced rhizobia for nodule formation, since the latter are far better adapted to the local edaphic conditions. Since nodulation efficiency does not necessarily entail symbiotic efficacy, strains vary in their symbiotic potential ranging from highly beneficial to completely ineffective, and nodulation by indigenous strains reduces the benefit offered by the inoculant. This has led to development of the concept of nodulation "competitiveness," a property that is believed to be independent of symbiotic efficiency. Knowledge regarding the determinants of competitiveness is vital for development of effective bioinoculants either by strain selection or by genetic engineering. Although an extensively researched area, yet very little is known about the molecular mechanisms that determine the relative competitiveness between the nodulating strains. The nodulation competiveness phenotype is complicated to understand because it involves root colonization ability, antagonistic properties, efficient nodulation ability as well as saprophytic survival abilities of the bacteria in the soils, and the final outcome of competition is determined by the strain genotype in combination with the host genotype as well environmental conditions (Streeter 1994; Toro 1996; Vlassak and Vanderleyden 1997; Sessitsch et al. 2002).

The availability of complete genome sequences of several rhizobial strains has drastically changed the approach to study rhizobial genetics (MacLean et al. 2007). Postgenomic researches including transcriptome, proteome, and interactome analyses on several rhizobia have helped understand the gene expression patterns under variety of conditions (Djordjevic et al. 2003 Yahyaoui et al. 2004; Sarma and Emerich 2005; Jones et al. 2008). Comprehensive mutagenesis studies have been conducted to examine physiological phenotypes of rhizobia under diverse conditions, including symbiosis with the host legume or nutrient-depleted conditions (Pobigaylo et al. 2008). The present chapter highlights the recent developments regarding nodulation competitiveness in rhizobia and approaches adopted to increase competitiveness of inoculant strains.

8.2 Traits for Competitiveness

Nodulation competiveness has been largely understood with the help of mutants that are compromised in their competitive ability as compared to their wild counterparts (Triplett and Sadowsky 1992). These include motility and chemotaxis, cell surface components, ability to use certain substrates, production of antimicrobial compounds, higher growth rates, and faster infection ability (Sessitsch et al. 2002). Some bacterial traits are constitutive and required for general saprophytic survival, whereas others are expressed only during interaction with the legume host while some are dependent on functional *nif* genes (Triplett and Sadowsky 1992) and nod factors (Lamrabet et al. 1999) indicating competitiveness and nitrogen fixation to be linked. Onishchuk et al. (2001) suggested three types of competitiveness:

saprophytic, rhizospheral, and nodulation, supporting the idea that rhizobia have different gene sets determining fitness in planta and ex planta.

Recently, a large library of signature tagged mutants (STM) was constructed in *S. meliloti* strain 1021, a symbiont of *Medicago truncatula* (Pobigaylo et al. 2006). Using STM technique, pools of mutants are used to determine nodule occupancy and the mutant strains recovered were identified later on with the help of unique tags. Pobigaylo et al. (2008) compared the composition of tags recovered from nodules with the composition of tags in the culture used to inoculate the plants in order to identify mutants, which were attenuated or overrepresented in symbiosis. Over a 100 mutants were attenuated (i.e., not recovered efficiently from nodules) while 29 mutants were the dominant genotypes recovered. The attenuated mutants were further grouped as fix^-, nod^-, or inf^-, those which did not fall in any of these categories, but gave normal nodules were considered competitiveness deficient. The mutants identified as competitiveness mutants, but no other obvious nodulation or nitrogen-fixation defect, carried disruptions in 23 novel genes whose involvement was not known previously. The STM technique thus, allowed identification of large number of genes which were hitherto not known to affect symbiotic competitiveness and was reasonably accurate, since out of 38 mutants showing reduced competitiveness, all but three were validated as being truly positive when tested one to one with the wild type. The competitiveness genes detected through this approach are involved in diverse functions such as uptake of iron, P and amino acids, biosynthesis of tryptophan, *myo*-inositol metabolism, others involved in DNA repair such as *lex*A, Clp protease, among others. It should be noted, however, that the representation of a particular mutant in the output pool depends not only on the number of nodules occupied by this mutant but also on the size of the nodules and the density of the bacterial population in each nodule. Thus, bacteria that initiate fewer nodules might not be effectively distinguished from those that are unable to remain viable in large numbers within infected nodules. Whether attenuated phenotypes of these mutants originate from disruptions in specific plant–microbe interaction pathways or simply reflect general reductions in growth rate and ability to adapt to stress conditions is not known. It will be interesting to compare the gene pool involved in competitiveness across different rhizobia–legume partnerships. Indeed, Shimoda et al. (2008) have recently reported the construction of an STM library for the lotus symbiont, *Mesorhizobium loti*, screening of which is likely to uncover additional genes important for competitiveness. The following account provides current understanding of some of the earlier discovered determinants of nodulation competitiveness as well as new ones whose involvement has been understood more recently. For many of them a detailed picture of their role in bacterial symbiotic fitness has emerged. It is becoming increasingly clear that competitiveness traits ensure either increased population in root zone or increased chances of establishment in the nodules. The first could be achieved by efficient chemotaxis and movement toward root exuded substances; the efficiency to attach to roots and show movement along the growing root tip, high rates of multiplication by the efficient utilization of root-derived substances as growth substrates, increased ability to survive in the bulk soil, tolerance to environmental stresses such as

fluctuations in temperature, dessication, tolerance to antimicrobial substances, and reduction of density of competing rhizobia by antibiosis. The increased chances of establishment in nodules could be achieved by a quicker ability of rhizobia to sense and respond to signals such as flavonoids, greater invasion ability into the plant tissue, superior survival in infection threads, reduced elicitation of plant defense response, or enhanced protection from plant defense response. Many of these traits are intimately associated with the nodulation process and cannot be considered clearly as affecting competitiveness exclusively.

8.2.1 Chemotaxis and Motility

Chemotaxis and motility are key features that help plant associated bacteria to recognize and respond to chemical signals emanating from their host plants (Brencic and Winans 2005). Several workers have reported chemotaxis and motility in different rhizobial strains including *S. meliloti* (Ames and Bergman 1981), *Bradyrhizobium japonicum* (Chuiko et al. 2002), *Rhizobium leguminosarum* (Miller et al. 2007), and cowpea rhizobia (Pandya et al. 1999). For example, *S. meliloti* shows positive chemotactic movement toward a variety of amino acids, dicarboxylic acids, and sugars. These molecules are generally good substrates for growth of the bacteria and hence attraction toward them could be considered important for saprophytic fitness. Since most of the attractants are also expected to be components of plant root exudates, and that root exudates can directly stimulate attractive chemotaxis (Barbour et al. 1991; Pandya et al. 1999), this property could also be important for rhizospere colonization. However, rhizobia are also attracted toward flavonoids released by their host, and require functional *nod* genes to be responsive to the same (Caetano-Anolles et al. 1988). Thus, aspects of host recognition and specificity are established before the bacterium and its host physically interacts. Motility and chemotaxis are, therefore, important for saprophytic, rhizospheric, as well as nodulation competitivity. Wei and Bauer (1998) observed that under C, N, or P starved situation, there was a rapid drop in motility of *S. meliloti* strains, even though the cells remained viable for weeks. Although there was an observable loss of flagellar integrity, the reduced motility was attributable to the inactivation of flagellar motors, since motility seemed to be lost earlier than the flagellar loss. Addition of glucose as well as a nonmetabolizable chemoattractant, however, restored motility of the starved cells partially. Interestingly, the rates of motility loss differed by several folds among different strains indicating that starvation-induced regulation of motility may proceed differently among rhizobial strains. As motility is considered an important determinant of competitivity, the subtle differences in the strains could account for variation in competitiveness between them. Behavioral regulation of motility of flagellated bacteria may vary between different species and also within strains. For instance, in certain strains of *Pseudomonas* (Wrangstadh et al. 1990), starvation resulted in increased chemoattraction and motility, while in *Vibrio* strains motility was lost upon nutrient deprivation

(Malmcrona-Friberg et al. 1990). Thus, it is important to study starvation effects on motitlity of rhizobial inoculants as well.

In order to obtain a supernodulating rhizobial strain, Althabegoiti et al. (2008), by using an iterative selection of rhizobial colony spreading on soft agar plates, obtained a highly motile derivative of *B. japonicum* USDA 110. The mutant strain displayed multiple thin peritrichous flagella (thin flagella may be used for bacterial adhesion and displacement upon the root surface) in addition to the thick subpolar one found (thick flagella is used for swimming) in the wild type. The presence of additional flagella was correlated with the derepressed expression of an additional flagellar protein not found to be expressed by the wild-type strain. This strain was found to show greater chemotaxis toward various amino acids and sugars. The derived strain also showed superior behavior as compared to the parental strain in terms of greater adsorption to the roots and nodulated earlier when tested in liquid growth media.

The improved nodulation characteristics of the highly motile mutant of Althabegoiti et al. (2008) were not observed when inoculated on plants grown on vermiculite with field capacity or in soils as seed-coated inoculants. When the highly motile mutant strain was inoculated in sowing furrows, the superiority of the mutant over the wild type was more obvious indicating that the introduced rhizobial strains can compete best with indigenous strains when they are inoculated in-furrow, an observation also made by others (Lopez-Garcia et al. 2002; Bogino et al. 2008). The effect of bacterial positioning is also reported in nodulation of soybean, a legume infected by rhizobia through an infection thread (Althabegoiti et al. 2008) as well as for peanut, a legume infected by rhizobia by crack entry (Bogino et al. 2008). Recently López-García et al. (2009) in field studies conducted in Argentinian soils found that the mutant strains of *B. japonicum* with increased motility increased nodule occupancy by twofolds compared to the wild type. However, this modest increase did not lead to significant increase in grain yields and N content.

Limitation of movement of bacteria in unsaturated porous media might put rhizobia inoculated on the seeds at a disadvantage compared with the soil population that is already evenly distributed in the soil. This distribution profile might constitute a bottleneck for the competitiveness of inoculants (Wadisirisuk et al. 1989; Lopez-Garcia et al. 2002). Therefore, the improvement of rhizobial attachment to and movement along growing roots is an important target for selection of strains with enhanced competitiveness for nodule occupancy in soils. In this regard, Mongiardini et al. (2009) have overexpressed the adhesion protein Rap1 in *R. leguminosarum* bv *trifolii* and found that it increases nodulation competitivity.

Swarming is another means of movement that helps rhizobia to migrate toward the growing root tip. The development of rhizobial biofilms on plant roots is an aspect being recently explored (Danhorn and Fuqua 2007). Biofilm formation by rhizobia was first reported by Seneviratne and Jayasinghearachchi (2003) and this ability has been found to be influenced by nutritional status, environmental conditions as well as plant-derived compounds such as lectins (Rinaudi et al. 2006; Perez-Giménez et al. 2009) and flavanoids, since functional *nod* genes are also required for biofilm formation (Fujishige et al. 2008). Biofilm formation has been

implicated in competitive nodule infection (Williams et al. 2008). Flagella-less mutants of *S. meliloti* fail to show biofilm formation and delayed nodulation phenotype (Fujishige et al. 2006). Swarming motility is important for establishment of symbiosis (Braeken et al. 2007), which depends on presence of functional flagella and also needs intact quorum-sensing system and exocellular polysaccharides (EPS) as reported in *S. meliloti* (Bahlawane et al. 2008).

8.2.2 Cell Surface Components

Surface polysaccharides produced by rhizobia which include exocellular polysaccharides (EPS), capsular polysaccharides (KPS), lipopolysaccharides (LPS) and the cyclic β-glucans, are considered the second key molecules in legume infection, after nod factors (Fraysse et al. 2003). Rhizobia secrete large amounts of acidic EPSs that constitute species-specific heteropolymers consisting of common sugars substituted with noncarbohydrate residues (Skorupska et al. 2006). Bhagwat et al. (1991) reported a mutant of *B. japonicm*, deficient in acidic polysaccharide and lipopolysaccharide production. The mutant was motile, grew normally on minimal medium, and fixed almost as much N as did the wild type during symbiosis, yet it was defective in competitive nodulation as compared to the wild type. Parniske et al. (1993) reported that *exoB* mutants of *B. japonicum* are compromised in their nodulation competitivity. Araujo et al. (1994) described a Tn5 mutant of *Rhizobium etli* that showed altered colony morphology and had a hydrophobic cell surface. This mutant showed decrease in nodulation competitiveness and was impaired in competitive growth in the rhizosphere. The wild-type gene responsible for this phenotype was cloned by Bittinger et al. (1997) and found to be *ros*R, a transcriptional regulator involved in EPS production, as shown for *R. leguminosarum* bv. *trifolii* homologue (Janczarek and Skorupska 2007). Interestingly the *ros*R mutant of *R. leguminosarum* bv. *trifolii* also formed dry, wrinkled colonies and induced fix-minus nodules on clover. Multiple copies of *ros*R increases EPS production. One of the genes controlled by *ros*R is *pss*A, encoding the glucose-1P-transferase, which initiates repeating unit synthesis, whose expression was downregulated in the *ros*R mutant. Mutation in *pss*A causes a complete loss of EPS and defects in LPS synthesis and these mutants induce empty nodules in some biovars. This information was effectively used by Janczarek et al. (2009) to develop a *R. leguminosarum* bv. *trifolii* strain that harbored multiple copies of *ros*R and *pss*R, which showed high levels of EPS and enhanced nodule competitiveness in nodulating clover. Since the *ros*R regulon comprises more than 50 genes of different functions, including those involved in polysaccharide production, carbohydrate metabolism, and plant infection (Bittinger and Handelsman 2000), the effect of multicopy *ros*R may affect the cellular physiology in many pathways, EPS overproduction being one of them.

EPS is an important factor required at multiple stages of functional symbiosis for (1) bacterial attachment (2) root hair curling (3) infection thread formation (4) for bacteroid development, and (5) protection of bacteria from host defenses (D'Haeze

and Holsters 2004; Jones et al. 2007). Using microarray experiments, it was shown that *M. truncatula* inoculated with succinoglycan-deficient *S. meliloti* more strongly express an unexpectedly large number of genes involved in plant defense responses (Jones et al. 2008).The EPS of *S. meliloti* is structurally different from that of *R. leguminosarum* EPS (Skorupska et al. 2006). Yet somewhat similar observations have been made for *S. meliloti* mutants deficient in succinoglycan (EPSI) synthesis, which form aberrantly small nodules devoid of bacteria and deficient in nitrogen fixation on *Medicago* hosts (Leigh et al. 1985). In these mutants, infection thread formation proceeds from only 10% of bacterially colonized root hairs and those that do form terminate prematurely before they reach nodule primordium (Cheng and Walker 1998). In *S. meliloti*, EPS production is under the control of nodulation regulator *nod*D (Machado and Krishnan 2003). Another control is exerted by a r*os*R equivalent gene of *S meliloti*, *muc*R, which controls EPS production (Keller et al. 1995). A screen for reactive oxygen species-sensitive *S. meliloti* mutants that simultaneously display aberrant symbiosis phenotypes revealed, among others, mutants defective in EPS production indicating both these properties to be controlled by EPS (Davies and Walker 2007). In *M. loti* cyclic β glycans are required to suppress high level production of antimicrobial phytoalexins during symbiotic development with *L. japonicus* (D'Antuono et al. 2008). *M. loti* β (1–2) cyclic glucan synthase (*cgs*) mutant induces white, empty, ineffective pseudonodules in *Lotus tenuis*, it induces normal root hair curling but is unable to form infection threads. *M. loti cgs* mutant also displayed motility impairment. Disruption of *cgs* equivalent gene *ndv*B in *S. meliloti* blocks symbiosis at the stage of bacterial attachment to root hairs and infection thread initiation, and also results in the formation of aberrantly small and empty nodules (D'Antuono et al. 2005). In *Sinorhizobium fredii* also, *cgs* mutation results in the abortion of nodulation at the very early stages of invasion, resulting in empty nodules (Crespo-Rivas et al. 2009). In contrast, a *B. japonicum ndv*B mutant is able to elicit normal nodule development and invade host tissues, although the resulting nodules do not fix nitrogen (Dunlap et al. 1996). These are candidate genes for increasing nodulation efficacy and competitiveness.

LPS is another surface polymer that is important in nodulation competitiveness in *S. meliloti–Medicago sativa* (indeterminate nodules) (Lagares et al. 1992) and in *M. loti–Lotus glaber* symbiosis (determinate nodules) (D'Antuono et al. 2005) and for progress of infection (Mathis et al. 2005). In both cases, the LPS-minus mutants showed reduced competitiveness compared with the wild type, but induced normal nodules. LPS-deficient mutants are more sensitive to cationic peptides and a correlation was observed between competitiveness and sensitivity to these agents (Lepek and D'Antuono 2005). Bacteroid LPS has increased hydrophobicity compared to that of free-living bacteria, suggesting that LPS differences in planta may contribute to symbiosis (Kannenberg and Carlson 2001; D'Haeze et al. 2007). LPS mutant displays normal host infection thread invasion and is taken up into the host cell cytoplasm, however, it undergoes rapid senescence and is degraded within the symbiosome compartment. The lipidA portion of the LPS is a strong suppressor of the host defense reaction (Scheidle et al. 2005), suggesting its role in rhizobial

adaptation and persistence within the host cell cytoplasm. The exact role of LPS in nodulation competitiveness is, however, poorly investigated.

8.2.3 Metabolic Fitness

The ability of microbes to efficiently utilize nutrients in soil and exclusively do so seems to be an essential requisite for competing in soil, where nutrient limitation is the rule rather than exception. By far the largest number of competitiveness traits identified fall in this category. They include determinants for catabolism of various sugars, sugar derivatives, amino acids; biosynthesis and catabolism of storage compounds or specific molecules that functionally resemble opines of *Agrobacterium* sp. The precise nature of C and energy sources that support proliferation of rhizobia during growth in the rhizosphere, infection and nodule formation in legumes is largely unknown. Amino acids and N-derivatives of amino acids (betaines and related compounds), rhamnose, *myo*-inositol, and rhizopines (modified derivatives of *myo*-inositol) have been identified as potential C sources for rhizobia in the rhizosphere and during plant infection (Prell and Poole 2006). Rhizobia can also grow on a variety of unusual compounds found in the rhizosphere such as stachydrine, homoserine, trigonelline, mimosine, calystegines, and rhizopines. In soil, individual C sources are not sufficiently present but a variety of them are available, that too at varying levels. Indeed, the nutritional status of soil and rhizosphere determines the size of rhizobial populations as well as the competition for nodulation. A small difference in the use of compounds might, therefore, determine the population that stays in the growth zone at the end of the infection thread and finally colonizes the nodule tissue. Accumulation of poly-β-hydroxybutyrate (PHB) and glycogen are important traits that provide rhizobia the fitness to survive under conditions of starvation. In order to understand the role of metabolic fitness in nodulation competitivity, Wielbo et al. (2007) screened a large number of strains and found only nine of them to be highly competitive for nodulation on the appropriate host. The competitive strains utilized more C and energy sources than uncompetitive ones as detected using Biolog microplate test. A major difference was in the utilization of amino and organic acids, which were metabolized by most of the competitive and only a few uncompetitive strains, whereas sugars and their derivatives were commonly utilized by both groups of strains, indicating that these metabolic properties may be essential traits determining the competitiveness of rhizobia. The current information on genetic and physiological role of each of factors related to metabolic fitness is highlighted in the following section.

8.2.3.1 Poly-β-hydroxybutyrate Metabolism

PHB is accumulated by a wide range of microorganisms as a C and reductive power storage polymer, particularly synthesized during conditions when there is excess

C relative to N (Kadouri et al. 2005). Rhizosphere soils, where the C:N ratio is estimated to be as high as 20, provide conditions conducive for PHB synthesis. Many rhizobia including *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, and *Azorhizobium* species produce PHB and most rhizobia accumulate the polymer during free-living reproductive growth but not all of them are able to store it during symbiotic life (Trainer and Charles 2006). In rhizobia, PHB reserves are particularly needed for survival under starvation (Ratcliff et al. 2008).

Aneja et al. (2005) studied the competitive abilities of *S. meliloti* mutant strains containing lesions in the PHB synthesis (*phb*C) and degradation (*bdh*A) genes. They found that the *bdh*A mutant showed no noticeable symbiotic defects on alfalfa host plants when inoculated alone but was compromised when coinoculated with the wild-type strain. Interestingly, the mutant had survival disadvantages as compared to the wild type when both were cultivated separately in alternating C-limiting and C-excess conditions suggesting that this ability may be important under fluctuating conditions of C. The mutant unable to synthesize PHB showed more severe defect in competition for growth and nodule occupancy. The authors concluded that the ability to build up PHB stores is a key factor influencing competitive survival, and the ability to use the accumulated PHB is important under conditions of fluctuating nutrient availability. In other study, Povolo and Casella (2004) constructed a disruption mutant of *pha*C gene of *Rhizobium tropici* involved in PHB synthesis and found this mutant was unable to produce PHB under free living as well as symbiotic conditions. This mutant was unaffected in its ability to elicit nodules on *Phaseolus vulgaris* and showed as much specific nitrogenase activity as the wild type. Since the wild-type *R. tropici* does not accumulate PHB in bacteroids, it is not surprising that the mutant was unaffected in symbiotic properties. Results from coinoculation experiments suggested that the wild-type bacteria outnumbered PHB mutant bacteria by more than 200 to 1 (Willis and Walker 1998). Since EPS and PHB production are linked and share common regulatory systems (Lakshman and Shamala 2003), the effects of PHB-minus strains may also be due to changes in EPS as also reported by Povolo and Casella (2009) for *S. meliloti phb*C-minus mutant. This is further complicated by strain-specific differences, for example *S. meliloti* MTCC 100 unable to produce EPS was shown to accumulate more PHB than the parental strain (Lakshman and Shamala 2003), an observation contrary to that of Povolo and Casella (2009) who found both the polymers to be down-regulated in PHB-minus mutant of another strain of *S. meliloti*. In addition, defects in the biosynthesis of glycogen, which is upregulated in PHB-minus mutants, showed enhanced symbiotic performance (Marroquì et al. 2001). On the contrary, glycogen synthase mutations (*glg*A) constructed by Wang et al. (2007), when combined with a *phb*C mutation in *S. meliloti* resulted in strains unable to synthesize PHB or glycogen. These were still able to form nodules and fix N; however, nodule formation on *M. truncatula* was delayed than on *M. sativa*. The competitiveness of these mutants is, however, not reported.

Unlike *S. meliloti* and *R. tropici*, *R. etli* accumulates PHB freely as well as during symbiosis (Cevallos et al. 1996). PHB is formed upto 70% of dry weight in bacteroids of nodules on bean and soya bean; however, this polymer does not

accumulate in nodules of alfalfa, pea, and chickpea. PHB also accumulates in rhizobial cells in infection threads (Lodwig et al. 2005) suggesting that a plentiful C supply is available for bacteria during growth while present within infection threads. Role of PHB may be different for same symbiont when associated with different plants. For example, bacteroids of determinate nodules (e.g., bean nodules) accumulate high levels of PHB, and the accumulation of PHB requires nitrogen fixation. However, bacteroids of indeterminate nodules (e.g., alfalfa nodules) do not accumulate PHB. During the formation of bacteroids in indeterminate nodules, the PHB granules are broken down. In indeterminate pea nodules inoculated with a *R. leguminosarum* mutant unable to make PHB, more plant starch was consumed during bacteroid development (Lodwig et al. 2005). The role of PHB in *R. leuminosarum* bv *viciae* as well as bv *phaseoli* seems to be different as growth of the pea plants nodulated with the *pha*C mutant was decreased while the bean plants were unaffected. The role of PHB, thus, cannot be generalized for all legume-rhizobial symbiosis. It has been shown that PHB accumulated in *B. japonicum* can be used to support nitrogen fixation. As against the supportive role played by PHB as a reductant for nitrogen fixation, PHB synthesis can also divert reductant from nitrogen fixation when synthesis and nitrogen fixation occur simultaneously. In accordance with this, *R. etli* mutant affected in *pha*C gene, the product of which catalyzes the PHB polymerization step, produced increased nitrogenase activity in symbiosis and a modest increase in seed yield in comparison with wild-type strain (Cevallos et al. 1996). Peralta et al. (2004) expressed a chimeric *Nif* HDK construct in *R. etli* in which the *Nif H*DK operon was engineered to be regulated under the strong *nif*Hc promoter, resulting in the overexpression of nitrogenase, in a background of PHB synthesis-defective mutant strain. The engineered strain showed significant increase in growth and yields of common bean (*P. vulgaris*) supporting the conclusion that excess reducing power available in the *pha*C mutation was used to energize nitrogenase catalysis (Cevallos et al. 1996).

8.2.3.2 Rhizopine and *myo*-Inositol Catabolism

Several *Rhizobium* strains have evolved the ability to synthesize simple C compounds, the function of which resembles that of opines associated to the *Agrobacterium*–plant interaction. For this reason, they have been termed rhizopines. These molecules are inositol derivatives such as L-3-O-methyl-scyllo-inosamine (3-OMSI) and scyllo-inosamine (Murphy et al. 1995). In alfalfa nodules, *S. meliloti* strains have been shown to produce 3-OMSI. The genes for synthesis and catabolism of 3-OMSI are located on the *nod–nif* containing Sym plasmid, and expression of genes for synthesis (but not catabolism) is regulated by the symbiotic regulator *nif*A and are, thus, expressed in the bacterial cell during endosymbiosis in the plant nodules. The rhizopine concept of Murphy et al. (1995) to enhance strain competitiveness suggests that presence of 3-O-MSI in the environment may constitute a selective pressure favoring the growth of the *Rhizobium* strains harboring the Sym-plasmid, hence genes essential for nitrogen fixation. Rhizopines confer a

competitive advantage at early stages of nodulation. To validate this hypothesis, Gordon et al. (1996) tested mutants of a *S. meliloti* strain defective in rhizopine synthesis or rhizopine catabolism in competition experiments with the wild-type strain using alfalfa as host plants. The ability to synthesize rhizopines was not apparently advantageous but the ability to catabolize these molecules provided a growth advantage to the rhizopine-degrading wild-type strain in comparison to the nonrhizopine-utilizing mutant strain. When inoculated alone, the mutants had a similar rate of growth and nodulation efficiency but occupied less than 30% of nodules when coinoculated with wild type. This competitive advantage of wild type persisted in soil 4 years after inoculation, even though there was turnover of nodules in that time (Heinrich et al. 1999).

Rhizopine synthesis and catabolism is also reported for *R. leguminosarum* bv *viciae* (Denton et al. 2002). Only approximately 10% of *S. meliloti* and *R. leguminosarum* strains, however, produce and catabolize 3-OMSI (Wexler et al. 1995). The synthesis takes place in bacteroids and they are utilized exclusively by the same strain when they reside in plants and are thus considered private nutritional source, which may broaden the conditions under which nitrogen fixation is advantageous to terminally differentiated bacteroids (Simms and Bever 1998). Low levels of rhizopines are also synthesized at the early stages of the symbiotic interaction, probably resulting from microaerobic induction in free-living bacteria. The genes involved in rhizopine synthesis and catabolism are termed *mos* and *moc* genes, respectively (Murphy et al. 1995). *moc* locus consists of four open reading frames (ORFs) involved in the catabolism, of which three *moc*A, *moc*B, and *moc*C are found to be sufficient to confer microbial rhizopine-catabolizing (Moc) activity onto otherwise Moc strains of *S. meliloti*. Rhizopine synthesis depends on three genes arranged in an operon: mosABC. Though gene sequences are known, the functions of the encoded proteins are not fully understood. The relatively low abundance of 3-OMSI-degrading microorganisms indicates that this is a novel resource for developing specific interactions.

Myo-inositol is an essential precursor of rhizopines and degradation of rhizopines requires a functional *myo*-inositol catabolism (Galbraith et al. 1998). Besides, *myo*-inositol is an abundant compound in nodules and bacteroids. *Myo*-inositol utilization has important influence on the efficiency of nitrogen fixation even in rhizobia that do not synthesize rhizopines. Jiang et al. (2001) constructed a mutant of *S. fredii* with defect in the *myo*-inositol dehydrogenase gene (*idh*A), the first enzyme responsible for inositol catabolism. The mutant was drastically affected in its ability to reduce nitrogen and revealed deteriorating bacteroids inside the nodules. In addition, the mutant was also severely affected in its ability to compete with the wild-type strain in nodulating soybean. Similar findings were also reported for a *myo*-inositol degrading but nonrhizopine synthesizing/degrading strain of *R. leguminosarum* bv. *viciae* (Fry et al. 2001). These researchers showed that mutants that were impaired in their ability to catabolize *myo*-inositol were able to nodulate pea and vetch at the same rate as wild type, differentiated into bacteroids and resulted in similar gains in plant growth. However, they suffered a severe disadvantage in competition for nodulation when coinoculated with the wild type.

By careful analysis of the rhizospheric growth rates and nodule initiation and formation kinetics, it was concluded that they may be affected in the early stages of nodulation and infection thread formation.

8.2.3.3 Utilization of Root-Derived Compounds

Proline utilization: Proline as well as compounds such as betaines and stachydrine that release proline upon degradation, are important components of the root exudates of the host plant alfalfa. The *put*A gene of *S. meliloti* encodes proline dehydrogenase, the enzyme catalyzing the oxidation of proline to glutamate. Mutants impaired in their ability to utilize proline (*put*A) or stachydrine (*stc*D) have weakened ability to colonize the root surface and/or in nodulation efficiency and competitiveness (Jiménez-Zurdo et al. 1997; Phillips et al. 1998) suggesting that proline acts as an important energy source for bacteria during early stages of the infection process. Complementation of the *put*A mutant with a genomic fragment containing the wild-type *put*A gene not only restored the competitiveness of the mutant, but improved the competitive ability to levels exceeding that of the wild type (Jiménez-Zurdo et al. 1995). Van Dillewijn et al. (2001) exploited this observation to overexpress *put*A by introducing extra copies of a modified *put*A gene on a multicopy plasmid and under the strong constitutive promoter of the kanamycin resistance gene (*npt*II) upstream of the proline-regulated native promoter. The resulting construct showed high constitutive as well as proline-induced expression. The increased nodule occupancy by the construct-bearing strain was observed in soils, particularly, in the first month after inoculation. However, after 3 months, number of nodules occupied by engineered strain and control reached equal proportions indicating that the benefit gained by *put*A overexpression is transitory and the modified *put*A gene tends to disappear with time. Subsequently, this group claimed that *put*A modified strain brought about moderate effect on native bacterial population in a field experiment conducted in Spain.

Erythritol and rhamnose catabolism: Mutation in erythritol catabolism impairs the nodulating competitiveness of a *R. leguminosarum* bv. *viciae* strain on pea plants (Yost et al. 2006). A Tn5-B22 mutant of *R. leguminosarum* unable to grow on erythritol was impaired in its ability to compete against wild-type nodulating pea plants but was still capable of forming nitrogen-fixing nodules. The mutation-affected erythritol uptake and, therefore, the reduction in competition ability is more likely due to inability to use this compound as C cource rather than due to a toxic effect of erythritol. The *ery*ABCD genes in *Rhizobium* strain NGR234 are found on a megaplasmid (Streit et al. 2004), whereas the homologous genes in *S. meliloti* 1021 and *M. loti* are chromosomally encoded.

Rhamnose is a methyl-pentose sugar found as a constituent of pectin in the form of rhamnogalacturonan within the cell walls of dicotyledonous plants and in mucilage of a number of legume plants (Knee et al. 2001). Mutants of *R. leguminosarum* bv. *trifolii*, unable to catabolize rhamnose, are at a severe disadvantage for clover nodulation, although this did not occur with sorbitol and adonitol utilizating

mutants (Oresnik et al. 1998). Rhamnose utilization genes are plasmid encoded and inducible with rhamnose. Data on population sizes of the individual strains in the rhizospheres suggests that the utilization of rhamnose does not bring about changes in the total population size. Thus, their uncompetitive nodulation phenotype is proposed to be due to the importance of rhamnose during infection thread initiation or development. This is supported by the fact that arabinose present in large proportions in pea plant exudates supports the growth of *R. leguminosarum* (Knee et al. 2001), but arabinose defective mutants are not at a competitive disadvantage compared to wild type (Poysti et al. 2007). However, the exact role of rhamnose metabolism in symbiosis remains unknown.

8.2.3.4 Iron Metabolism

Low-intracellular concentrations of iron in rhizobia result in lack of competitiveness for nodulation when coinoculated with inocula developed under high iron concentration (Battistoni et al. 2002). Mutants of *S. meliloti* affected in iron acquisition show deficiency in competitive nodule occupancy under iron-limited conditions. Siderophore-mediated high-affinity iron transport system comprises of an elaborate machinery involving genes for biosynthesis of siderophores, low-molecular weight iron chelating compounds, and specific receptors for the uptake of the siderophore–iron complex (Faraldo-Gomez and Sansom 2003). In soils, iron may be largely bound to siderophores secreted by various soil inhabitants. It has been shown that the siderophore ferrichrome, made by several fungi, is present in large amounts in soil solutions (Powell et al. 1980). Organisms that themselves do not synthesize ferrichrome may yet be able to utilize iron bound to this siderophore if they possess receptor for the same. Not only siderophores are important for iron scavenging, but they also mediate antagonistic effect on other strains by depriving them of iron (Joshi et al. 2006, 2008b). Majority of rhizobia fail to utilize ferri-ferrichrome and other related hydroxamate-type siderophores and are at competitive disadvantage in iron sufficiency (Khan et al. 2006; Carlton et al. 2007; Rajendran et al. 2007; Joshi et al. 2009).

Heterologous receptors for iron-ferrichrome uptake, (*fhu*A) from *Escherichia coli* or (*feg*A) from a *B. japonicum* strain proficient at utilizing ferrichrome, have been expressed in rhizobial strains nodulating pigeon pea (Rajendran et al. 2007; Geetha et al. 2009; Joshi et al. 2009) and ground nut (Joshi et al. 2008a) plants. Under laboratory conditions, the recombinant strains are at a growth advantage as compared to isogenic strains bearing the empty vector, when grown in a medium containing iron bound to ferrichrome as the only form of iron. The growth of the recombinants is also stimulated when they are grown as mixed cultures with *Ustilago maydis*, a fungus known to produce ferrichrome siderophore under iron-limited conditions. Plant inoculation studies with these strains done under gnotobiotic conditions using pure cultures, show greater nodule occupancy of the recombinant strains, bringing about net increase in plant growth when grown in autoclaved soils (Geetha et al. 2009; Joshi et al. 2008a). The competitive nodulation

ability was evident when the *feg*A-bearing strain was inoculated in unautoclaved soils with indigenous rhizobia that could initiate nodulation but were outcompeted by the recombinant strain (Joshi et al. 2009). Whether the competitive advantage of ferrichrome receptor expressing strains is restricted to the level of saprophytic survival alone or is also important during nodulation needs to be investigated, since Benson and associates (Benson et al. 2005) have observed a nodulation defect in mutants of *B. japonicum* 61A152 affected in *feg*A (ferrichrome receptor) and associated gene *feg*B.

8.2.3.5 Other Metabolic Traits

Glucose metabolism: S. meliloti is able to catabolize aldoses by an extracellular oxidative pathway, which results in the conversion of the sugars to the corresponding aldonic acids in the periplasm without the prior involvement of a phosphorylative step. This conversion is mediated by a periplasmic pyrroloquinoline quinone-linked glucose dehydrogenase (PQQ-GCD). Gluconate, formed from glucose by the action of this enzyme, can be further metabolized mainly via Entner-Duoderoff (ED) and pentose phosphate pathways. Bernardelli et al. (2009) have reported that a *gcd* mutant of *S. meliloti* showed a delay in nodule emergence and a reduced ability for nodulation at various inoculum dosages on alfalfa. The mutant was highly deficient in its competitive ability; 100 times higher inoculum of mutant was needed to get nodule occupancy equal to the wild type. In addition to the competiveness defect, the *gcd* mutant was also compromised in nodulation efficiency in pure culture inoculation experiments as evidenced by a decrease in nodule number as well as in delay in nodule formation. This indicates that probably the low competitivity maybe due to delay in nodule formation. The expression of *gcd* gene as studied using a *lac*Z fusion is required from initial colonization to late stages of symbiosis. The role of GCD is puzzling since *S. meliloti* is not normally able to use the product of GCD, that is, gluconate for further metabolism (Steele et al. 2009).

Biotin metabolism: Biotin is an essential growth requirement for many rhizobia (particularly *S. meliloti* strains) that are dependent on plant-derived biotin in the rhizosphere (Streit et al. 1996). Mutation in *bio*N eliminated growth of the mutant of on *M. sativa* roots (Entcheva et al. 2002) and this resulted in low competitiveness of such mutant strains (Guillen-Navarro et al. 2005).

Purine metabolism: Xie et al. (2009) performed the competitive nodulation tests with *S. fredii* containing different expression levels of *purL,* gene encoding a 5-phosphoribosylformyl-glycinamidine synthase, catalyzing the fourth step of the purine biosynthesis pathway. They monitored expression levels by means of the GUS activities from the purL-gusA transcriptional fusion and found nodule occupancy to be very well positively correlated with *pur*L expression. By adding the wild-type strain at different time intervals after inoculating plants with *pur*L-deficient strains, it was realized that the competition was at the very early stages of infection.

8.2.4 Stress-Related Genes

The saprophytic survival of rhizobia in soils is dependent on their ability to withstand a number of stresses other than starvation. These include dessication, salt stress and acid stress among others. Within plant tissues and nodules, bacteria also face osmotic stress, microaerobiosis, and oxidative stress caused by the induction of plant defense response (Nogales et al. 2002).

8.2.4.1 Trehalose Metabolism

The disaccharide trehalose is a well-known osmoprotectant, and trehalose accumulation through de novo biosynthesis is a common response of bacteria to abiotic stress. Trehalose is present at high concentrations in bacteroids at the onset of nitrogen fixation. Trehalose is available in the root environment and plays a role in stress adaptation in *S. meliloti* and in its interactions with alfalfa. Jensen et al. (2005) in a study showed that mutations in genes *thu*A and *thu*B of *S meliloti*, coding for a major pathway for trehalose catabolism, leads to impairment of competitive colonization of *M. sativa* roots. However, these strains were more competitive than the wild type in infecting alfalfa roots and forming nitrogen-fixing nodules. Subsequently, this group (Ampomah et al. 2008b) found that the *thu*B mutants formed more nodules than their parent strains on two of the three alfalfa lines tested and on one of the two *M. truncatula* lines tested and thus could increase competivity on some hosts. Their competitiveness for nodule occupancy did not correlate with their ability to colonize these roots but correlated well with the levels of *thu*B. McIntyre et al. (2007) studied the role of endogenous trehalose synthesis in desiccation tolerance in *R. leguminosarum* bv. *trifolii*. Double mutants affected in *tre*YZ and *ots*AB pathways failed to accumulate any trehalose. The double mutants were more sensitive to the effects of drying, and their survival was impaired compared to the wild type and showed lower competitiveness. These studies indicate that trehalose protects the bacterial cells against desiccation stress and against stress encountered during nodulation. Genes coding for three possible trehalose synthesis pathways are present in the genome of *S. meliloti* 1021 and at least one functional trehalose biosynthesis pathway is required for optimal competitiveness of *S. meliloti* to nodulate alfalfa roots (Domínguez-Ferreras et al. 2009). Overexpression of trehalose-6-phosphate synthase in *R. etli* showed more nodules on *P. vulgaris* with increased nitrogenase activity and higher biomass and enhanced drought tolerance compared with plants inoculated with wild-type *R. etli* (Suárez et al. 2008).

8.2.4.2 ACC Deaminase and Rhizobitoxine

1-Aminocyclopropane-1-carboxylate (ACC) deaminase has been found in various plant growth-promoting rhizobacteria, including rhizobia. This enzyme degrades

ACC, the immediate precursor of ethylene, and thus decreases the biosynthesis of ethylene, a plant stress hormone. In rhizobial symbiosis, ethylene is an inhibitor of nodulation (Nukui et al. 2000), nod factor signaling (Oldroyd et al. 2001), and infection thread formation (Penmetsa and Cook 1997). Ma et al. (2003a, b) reported the presence of ACC deaminase in *R. leguminosarum* bv. *viciae* and upon isolating the gene (*acd*S) involved, found a leucine-responsive regulatory protein (LRP)-like gene (*lrp*L), which is located immediately upstream of *acd*S, and is required for the expression of ACC deaminase. Both the *acd*S and *lrp*L gene knockout mutants showed approximately 30% lower nodulation ability than the wild type. The *R. leguminosarum* bv. *viciae acd*S-*lrp*L gene region was subsequently introduced into *S. meliloti* Rm1021, which does not have inherent ACC deaminase activity, and the influence of these genes on the ability of *S. meliloti* to nodulate alfalfa was reported (Ma et al. 2004). The resulting ACC deaminase-producing *S. meliloti* derivatives formed approximately 40% more nodules in symbiosis with *M. sativa*. This indicated that the reduced ethylene production may diminish the host defenses and thus allow the infection threads produced by the ACC deaminase-producing strains to be extended without abortive disruption before nodule formation (Vasse et al. 1993). Interestingly, the engineered strains were much more competitive in nodulating alfalfa than the wild-type strain. More than 80% of the nodules were occupied by Acd^+ strain even when the plants were inoculated with a mixed inoculum of wild type and Acd^+ at a ratio of 5:1 indicating it to be highly competitive. Since coinoculation with the Acd^+ strain provides no advantage to the wild-type strain, the authors suggest that reduction of ethylene by the engineered strain may be localized making it more likely for the acd+ strain to exclusively enter the root cortex.

Synthesis of an ethylene biosynthesis inhibitor, rhizobitoxine (2-amino-4-(2-amino-3-hydropropoxy)-trans-but-3-enoic acid), is yet another strategy utilized by *Rhizobium* spp. to reduce ethylene levels in legumes during nodulation (Okazaki et al. 2004; Sugawara et al. 2006). Nonrhizobitoxine-producing mutants of *Bradyrhizobium elkanii* formed fewer mature nodules on *Vigna radiata (*mung-bean) than did the wild-type strain (Duodu et al. 1999). Yuhashi et al. (2000) also reported that *B. elkanii* producing rhizobitoxine decreases the levels of ACC synthase in plant roots and enhances nodulation on *Macroptilium atropurpureum*. It is seen that in *V. radiata,* rhizobitoxine is required for normal nodulation also, whereas in *M. atropurpureum* it is required only for competitiveness. Recently, it has been shown that rhizobitoxine production by rhizobia decreased legume growth, but benefited relative to an isogenic, nonproducing strain on the same plant by accumulating more PHB (Ratcliff and Denison 2009)

8.2.4.3 Acid Tolerance

An acid tolerant strain of *R. tropici* is a good competitor for nodule occupancy of common bean plants in acid soils. The acid sensitive variants are usually severely affected in nodulation competitiveness (Riccillo et al. 2000; Vinuesa et al. 2003;

Rojas-Jiménez et al. 2005). For several of the mutants, the wild-type genes responsible for the acid-sensitive phenotype have been identified. One mutant was found to be affected in *gsh*B gene, encoding the enzyme glutathione synthetase (Riccillo et al. 2000). The *gsh*B mutant strain showed a delayed-nodulation phenotype coupled to a 75% reduction in the nitrogen-fixation capacity and abnormal nodule development. In one of the mutants studied by Vinuesa et al. (2003), the *atv*A gene (acid tolerance and virulence) was affected, which is likely to be involved in the formation of the membrane lipid lysyl-phosphatidylglycerol (LPG) (Sohlenkamp et al. 2007). Rojas-Jiménez et al. (2005) identified the gene *syc*A, a homolog chloride channel and Cl^-/H^+ exchange transporter and a second gene (*ols*C), found downstream of *syc*A, which is involved in modification of ornithine-containing lipids. Lipid changes in *S. meliloti* also have defects in symbiosis including formation of less number of nodules most of which were devoid of bacteria (Vences-Guzmán et al. 2008). Changing the properties of the bacterial membrane in response to stress is important for acid tolerance (Roy 2009) and this seems to be important for nodulation competitiveness.

8.2.5 Antagonism

8.2.5.1 Trifolitoxin and Bacteriocin Production

Trifolitoxin (TFX), a potent antirhizobial peptide that is produced by some strains of *R. leguminosarum* bv. *trifolii*, inhibits members of α-proteobacteria including *Rhizobium* and other plant and animal pathogens and its production is important for strain competitiveness (Triplett and Barta 1987). Genes involved in TFX synthesis when expressed in *R. etli* resulted in increased competitiveness in the heterologous system (Robleto et al. 1997). The recombinant, TFX-producing *R. etli* strain significantly increased nodule occupancy in nonsterile growth chamber experiments as well as under agricultural conditions. The recombinant strain exhibited at least 20% greater nodule occupancy than the wild-type strain. The TFX production was seen to confer ability to produce and resist trifolitoxin in other biovars of *R. leguminosarum* as also *S. meliloti*. However, trifolitoxin affects bacteria other than rhizobia and causes a reduction in the diversity of indigenous α-proteobacteria (Robleto et al. 1998).

Bacteriocins are peptide antibiotics, active against closely related strains or species. *Rhizobium* bacteriocins have been characterized as small, medium, and large, based on their size and diffusion characteristics. Bacteriocins are related to RTX proteins, which include hemolysin and leukotoxin. Only a few strains produce medium bacteriocins (Sridevi and Mallaiah 2008) and genes encoding them are plasmid associated. Oresnik et al. 1999 reported insertional mutants of the bacteriocin locus and found mutants to be non-competitive with wild type. Similar finding is reported for the bacteriocin of *Cicer–Rhizobium* (Nirmala et al. 2001). However, not all bacteriocins contribute to nodulation competitiveness (Venter et al. 2001).

8.2.6 Involvement of Other Loci in Competitiveness

Recently, Patankar and Gonzalez (2009) described a mutant of *S. meliloti* affected in *nes*R, which is compromised in competing with the wild-type strain for plant nodulation. When inoculated as a 50:50 mixture with the wild type, the mutant occupied only 35% of the nodules. *nes*R is a gene belonging to LuxR-type response regulator family, members of which are involved in quorum-sensing in gram-negative bacteria and hence this property seems to be important for nodulation competitiveness. The *nes*R is an orphan *lux*R since no *lux*I homologue is associated with it and it does not seem to be responsive to the quorum sensing molecules made by the *S. meliloti* strain (Patankar and Gonzalez 2009), therefore it is suggested that it may respond to external signal molecules produced by other species. Disruption of the *nes*R locus apart from affecting competitivity renders the bacteria unable to cope with specific nutritional, environmental, and other stresses indicating that its effect on competitivity is due to its disturbance of stress tolerance, as is seen in case of mutants directly affected in stress-related genes (Sect. 8.2.4)

Sanchez et al. (2009) showed that mutation of the *M. loti* homolog of *rhc*N, a gene encoding Type III secretion systems (T3SS) protein, affected its competitiveness on *L. glaber*, where nearly 70% of the nodules were occupied wild type and only approximately 30% were with the *rhc*N mutant. The *rhc*N locus is important in nodulation outer proteins (Nops) secretion and mutation in this gene decreases the symbiotic capacity of *S. fredii* with soybean (deLyra et al. 2006). They also identified Nops secreted by *rhc*N by comparing proteins secreted by wild-type and the *rhc*N mutant. Mutation in one of the effector encoding genes, *mlr6361*, also showed reduced competitiveness for nodulation. Mutation of the T4SS of *M. loti* R7A or its effectors reduced competitiveness on *Lotus corniculatus* (Hubber et al. 2004).

You et al. (1998) identified a gene (*slp*) of *R. etli* by complementation of a mutant defective in competitiveness. The *slp* gene encodes a stomatin-like protein and deficient mutant shows reduced growth in presence of high NaCl concentrations. The stomatin is a membrane-bound protein and is involved in ion channel regulation and signal transduction.

Among the STM mutants affected in competiveness reported by Pobigaylo et al. 2008, was a mutant-affected *feu*Q, which had severe defects in nodulation competitiveness. *Feu*Q is a positive regulator which acts in conjunction with its cognate response regulator *Feu*P. Although the *feu*Q/*feu*P system controls many genes, its effect on the *ndv*A locus is considered most important (Griffitts et al. 2008). The *ndv*A gene is involved in controlling the ATP-dependent extrusion of cyclic glucan from the cytoplasm. The symbiotic phenotype of a *feu*P mutant is suppressed by ectopic expression of *ndv*A. *feu*Q mutant is impaired in secretion of the cyclic glucan, which is just enough to support effective nodulation under ideal conditions but not enough to efficiently occupy nodules under competitive conditions.

The above examples show the importance of regulatory and secretory systems in correct manifestation of the competitiveness traits.

8.3 Competitiveness Variation in Legume–Rhizobial Partnerships

Factors affecting nodulation competitivity vary with different legume-rhizobial partnership. This variation may be due to differences in the (1) metabolic capacity of bacterium (Prell and Poole 2006) (2) infection process, or (3) life history of bacteria inside nodules. Recent evidence points that rhizobia have evolved different strategies to form an effective symbiosis and each partnership may have a distinct set of genes for competitiveness. Genome analysis has shown that different rhizobia share less than 10% genes (considering a genome equivalent to the mean of all sequenced rhizobial genomes) exhibiting variability in their metabolic capacity (Amadou et al. 2008) leading to grouping rhizobia into fast- and slow-growing rhizobia. Furthermore, a large variation in the *nif* gene homologs in different species is known. For instance, *Bradyrhizobium*, representative of slow-growing rhizobium, has 15 *nif* genes while the fast-growing *R. leguminosarum* and *S. meliloti* have 8–9 *nif* genes (Masson-Boivin et al. 2009) indicating that variation in *nif* gene number might correlate with the physiology of a bacterium.

Regulation of nitrogen fixation in different genera is also likely to differ since the way *nif*A transcription is regulated, varies markedly among rhizobia. Nod factors are essential for symbiosis for all rhizobia (except for the phostosynthetic bradyrhizobial symbionts of *Aeschynomene* species; Masson-Boivin et al. 2009), yet some bradyrhizobia synthesize additional nodule development factors such as cytokinins (Gonzalez-Rizzo et al. 2006) and IAA (Theunis et al. 2004). The process of infection also shows that all rhizobia do not infect in the same way (Gage 2004). Some penetrate the epidermal and cortical layers by root hair infection leading to the formation of infection thread that elongates along the root hair, ramifies and penetrates inside the emerging nodule. Other rhizobia enter by crack entry, a cruder process in which bacteria enter into plant parts through loose cellular junctions of emerging lateral roots. The life histories of rhizobia are also dependent on the legume host. It is becoming increasingly clear that in some legume hosts such as *Medicago*, rhizobia differentiate into bacteroids which swell and lose reproductive viability while in certain other plants they remain similar to free-living forms and continue to reproduce (Oono et al. 2009). Thus, it is not surprising that the factors affecting competitivity may not be equally relevant to all the symbiosis. It is possible that in fast-growing *R. leguminosarum* strains, antagonism may be important for competitiveness, while in slow-growing *Bradyrhizobium* species, competitiveness is correlated primarily with metabolic adaptation to local environments.

8.4 Competition Influences at Different Stages of Nodulation

A comprehensive understanding of which stage (s) in the nodulation process is/are most critical for the success or failure of a given strain to occupy the nodule is only recently being explored. Recently, Duodu et al. (2009) showed that greater nodule

occupancy by a native competitive strain of *R. leguminosarum* bv *trifolii* was due to higher number of infections induced by this strain than by better colonization of the root rhizosphere. Difference in the initial multiplication and colonization of the root surfaces is not the cause for differential nodulation competitiveness of the strains tested, as suggested by Leung et al. (1994). Since none of the strains tested by Duodu and co-workers produced antibiotics, the competitive ability of this strain is probably due to other factors. Moreover, the competitiveness of the efficient strain is possibly expressed either at infection thread initiation or during bacterial growth in the infection threads. Until additional information regarding the other properties of the competitive strain reported by Duodu et al. (2009) is available, it is not possible to extend these observations to other competitive strains. Jensen et al. (2005) studied time course of events during competition between wild-type and trehalose utilization mutant strains of *S. meliloti* on alfalfa roots. By using green fluorescence protein (gfp) under a promoter responsive to osmotic stress, they showed that osmotic stress was experienced by rhizobial cells while passing through the infection thread. Oxidative stress as monitored by specific staining was also noticed at this time. Thus, it is understandable that trehalose metabolism plays a role in early stages of infection when the bacteria have to survive the stress imposed by the active defenses of the plant cells lining the infection threads. Only a small percentage of newly formed infection threads actually penetrate the inner cortical cell layer, while majority of them get aborted which display characteristics of hypersensitive plant defense response (Vasse et al. 1993). Other factors that enable the bacteria to manage host-induced stresses during infection may also act during this stage. On the other hand, the factors that determine the ability to colonize the root such as motility, adhesion, etc. may be critical for competitive nodule occupancy before infection is established. Unlike such processes that occur at early stages of infection, nodulation competitiveness by rhizobitoxine occurs at a later stage as reported by Okazaki et al. (2003), who followed the time course of nodulation competitiveness between Rtx^+ and Rtx^- strains of *B. elkanii*. They observed that enhancement of competitiveness by Rtx accumulation occurred during late stages of nodule development and that it was not associated with an increase in the population size of the rhizobitoxine-producing strain. This correlated with the observation that increased amounts of ethylene were released from *S. meliloti* inoculated roots of *M. sativa* coincident with the time of nodule development and the beginning of nitrogen fixation (Ligero et al. 1986). However, it is interesting to note that strains that are poorly competitive often show delay in nodulation. Thus, subtle differences in the rates of the different events during infection may become exaggerated when competing strains are present. Using a simple approach of adding the wild-type strain at different time intervals after inoculation of the mutant onto the plants, Xie et al. (2009) showed that the *pur*L gene was involved in competition at early stages of nodulation. Developments in transcript quantitation can help determine the expression levels of genes at different stages of infection (Lohar et al. 2006). These studies can help understand the precise stage when the gene product is required. For example, using such an approach, genes involved in the catabolism of trigonelline and choline to glycine

betaine are found to be expressed at all stages of symbiosis, while stachydrine catabolism genes are required for nodulation.

8.5 Role of Plasmids

Rhizobial genomes are multipartite with several large plasmids along with the chromosome. The slow-growing bradyrhizobia generally lack plasmids, while fast-growing rhizobia have been found to harbor 2–6 plasmids (MacLean et al. 2007). In the fast-growing rhizobia, plasmids can make up as much as 40% of the total genome size. Symbiotically relevant genes in rhizobia are often clustered on large plasmids (pSym), or within genomic islands called symbiosis islands. For instance in *S. meliloti*, nod factor biosynthesis genes (*nod, nol, noe*) and nitrogen-fixation genes (*nif* and *fix)* are located on pSymA, whereas those for EPS biosynthesis (*exo*) and C4-dicarboxylic acid utilization (*dct*) are located on pSymB. The competitiveness traits are determined by the genomic as well as plasmid-borne determinants (Hynes and McGregor 1990). Some of the genetic determinants of nodulation competitiveness are localized on Sym plasmid, which also carries genes essential for nodulation and nitrogen fixation. For instance, the Sym plasmid of *R. leguminosarum* bv. *viciae* strains carries genetic determinants for the catabolism of organic compounds, which are exudated in the rhizosphere of peas (Murphy et al. 1995) besides genes for the synthesis and the catabolism of rhizopines. Using Sym plasmids in different genomic backgrounds, it has been concluded that both components are equally involved in competitiveness for nodule formation in *R. leguminosarum*, while the competitiveness for growth in the rhizosphere was mainly due to the genomic backgrounds.

In natural rhizobial populations, the sym plasmids of rhizobia are mobile and are not strictly associated with any particular chromosomal background (Nandasena et al. 2007). Barcellos et al. (2007) demonstrated the transfer of symbiotic genes from an inoculant strain into indigenous strains, even transfer between two different genera (*Bradyrhizobium* to *Sinorhizobium*). *Bradyrhizobium* is particularly prone to lateral gene transfer (Kunin et al. 2005). Yost et al. (2006) confirmed that the four largest of the six plasmids in *R. leguminosarum* bv *viciae* are necessary for competition for nodulation of peas and lentils. Since three of these plasmids, pRleVF39c, pRleVF39d, and pRleVF39e, are required for formation of nitrogen-fixing nodules (Hynes and McGregor 1990), their requirement for competition is not surprising. However, the largest plasmid, pRleVF39f, is not required for nodulation or nitrogen fixation. The plasmid-cured strains had reduced ability to catabolize a number of compounds in the seed exudates.

In several rhizobia, besides the sym plasmids, one or more accessory plasmids are present (Gonzalez et al. 2006). These may confer benefits with regard to overall fitness. Brom et al. (2000) constructed multiple plasmid-cured derivatives of *R. etli* in order to investigate their competitivity for nodulation. They found a drastic decrease in nodulation competitivity when cells were cured for multiple plasmids

which also had defect in cellular growth and viability, indicating that functional interactions among the pSym and accessory plasmids also exist. In other rhizobia ike *S. meliloti*, important traits for competition such as catabolism of proline (Jimenez-Zurdo et al. 1995), trigonelline (Goldmann et al. 1991), calystegines (Guntli et al. 1999), rhamnose (Oresnik et al. 1998), erythritol (Yost et al. 2006), and rhizopines (Heinrich et al. 1999) are plasmid borne. Stiens et al. (2006) sequenced the accessory plasmid of *S. meliloti* and found genes encoding various metabolic enzymes and transport systems. A gene encoding an ACC deaminase (*acd*S) known to be important for competitive nodulation was also identified.

8.6 Competition Between Species

Rhizobia able to nodulate soybean include six species belonging to three different genera, *Bradyrhizobium*, *Mesorhizobium*, and *Sinorhizobium* (Van Berkum and Eardly 1998). Bradyrhizobia of the "cowpea" miscellany that can nodulate both peanut and cowpea legumes (Strijdom 1998) but are not effective on both can outcompete peanut inoculant strain at least in some soils (Law et al. 2007). Bacteria isolated from *Mimosa* nodules may belong to α- or β-proteobacteria, the latter being *Burkholderia* and *Cupriavidus* strains and former being *R. etli* and *R. tropici* strains. While β-proteobacterial microsymbionts formed effective symbioses, the *Rhizobium* strains showed large variation in symbiotic phenotypes through ineffective to effective nodulation. These examples demonstrate the importance of interspecies competition, in addition to interstrain competition discussed above. Which species successfully nodulates may depend on other factors such as fixed N in the form of NH_3 or NO_3 and conditions (e.g., liquid or solid media) of experiments (Elliott et al. 2009). Although a single nodule is usually infected by a clonal population of a particular strain, plants are sometimes infected by more than one species of rhizobia resulting in different nodules on the same plant. This may happen regardless of whether they are proficient at nitrogen fixation or not. This means that rhizobia that supply their host with N may indirectly benefit competing ineffective strains of rhizobia infecting the same individual plant, creating a classic "tragedy of the commons" problem (Oono et al. 2009), and if the same legumes are inoculated with both fixing and nonfixing rhizobia, nonfixing rhizobia outcompete nitrogen fixers (Thrall et al. 2007).

8.7 Engineering Competitiveness

Several different strategies have been envisaged to improve the competitiveness of an introduced microorganism in the plant environment: promote its multiplication in the plant environment, impede growth of competing microorganisms, or interfere with some of the signals perceived by the microbes provided these signals control

(at least in part) the expression of functions central to microbial fitness (Savka et al. 2002). Because this is a triple interface (bacteria, plant, and soil) interaction, it is possible to modify one, two, or three of these factors to improve microbial colonization. An improvement of plant–microbe symbioses should involve the coordinated modifications in the partners' genotypes resulting in highly complementary combinations (Tikhonovich and Provorov 2007). Genetic manipulation of the bacteria should take into account genes which can be used to increase competitivity. While much of the effort has been directed to understand genes whose deficiency leads to loss of competitivity, only few studies deal with genes whose over-expression can improve competitivity. Successful strategies in this regard are (1) construction of a chimeric *Nif* HDK operon under the strong *Nif*Hc promoter and expression in PHB negative mutants of *R. etli* (Peralta et al. 2004) (2) construction of an acid-tolerant *R. leguminosarum* bv. *trifolii* strain (Chen et al. 1991) (3) expression of ACC deaminase gene in *S. meliloti* (Ma et al. 2004) (4) overexpression of putA (Van Dillewijn et al. 2001) (5) overexpression of trehalose 6-phosphate synthase (Suárez et al. 2008) (6) overexpression of rosR and pssR (Janczarek et al. 2009) (7) heterologous expression of ferrichrome siderophore receptor (Joshi et al. 2008a, 2009; Geetha et al. 2009), and (8) overproduction of the adhesin RapA1 (Mongiardini et al. 2009). Of these, the strategy of Peralta et al. (2004), explained that *R. etli* strains with the chimeric nitrogenase construct assayed in greenhouse experiments had increased nitrogenase activity (58% on average), plant weight (32% on average), N content in plants (15% at 32 days post inoculation), and most importantly, higher seed yield (36% on average), higher N content (25%), and higher N yield (72% on average) in seeds. Expression of the chimeric *nifHDK* operon in a PHB-negative *R. etli* strain produced an additive effect in enhancing symbiosis. Probably, this is the first report of increased seed yield and nutritional content in the common bean, obtained by using only the genetic material already present in *Rhizobium*. Mongiardini et al. (2009) on the otherhand, investigated the influence of adhesins on competitiveness of *R. leguminosarum bv trifolii* using clover as test plants. In this report, the *R. leguminosarum* bv *trifolii* adhesion protein RapA1 was overproduced from a pHC60-derived plasmid and expressed in R200 strain. When an overproducing strain and a control-carrying empty vector were coinoculated on clover plants, a positive effect of RapA1 on competition for nodule occupation was observed suggesting that optimization of RapA1 expression may be considered while improving the rhizobial competitiveness. Ability to cross-utilize heterologous siderophores is another trait that can be incorporated into bioinoculants to further improve their competitiveness.

The release of genetically improved strains is often restricted due to lack of regulation and proper guidelines of its release besides its potential ecological effects, as perceived by the public. However, despite these perceptions, some recombinant rhizobial strains have been commercialized, such as *S. meliloti* strain RMBPC-2, which was approved by the US Environmental Protection Agency in 1997. This genetically engineered bacterium contained additional copies of *nif*A and *dct*ABD to increase nitrogen fixation and when inoculated, enhanced the yield of alfalfa (Bosworth et al. 1994) planted at Arlington, Hancock, Lancaster, and

Marshfield, WI under N-limiting conditions. Later on, inoculation of alfalfa seeds with any of the three recombinant strains of *S. meliloti* significantly increased overall plant biomass compared with inoculation with the wild-type strains over a 3-year period during which high proportion of nodules were occupied by the inoculum strains (Scupham et al. 1996). However, the recombinant strains were ineffective in soils where the indigenous rhizobial population was more competitive, for instance, the yields at Marshfield site were much lower than at other locations because a low proportion of nodules were occupied by the inoculum strains at that site. Such strains can be further improved for nodulation competitiveness.

8.8 Conclusion

Bacteria may become competitively successful only if armed with the appropriate tools of efficient substrate acquisition, resistance mechanisms as well as competitive traits. Many important competitivity traits have been discovered in the recent past. Comparative genomics analyses have revealed the existence of competitivity genes that are conserved and many that are specific to each species. Signature-tagged mutant libraries constructed for *S. meliloti* and *M. loti* are an important and powerful resource for future functional genomics and such libraries of other strains may be useful in understanding and engineering rhizobia for better legume productivity in different agro-ecological regions of the world.

Molecular biology together with screening genotypes may help to identify and concurrently develop more effective inoculants strains. Even though the use of genetically modified microbial inoculants in agriculture is controversial (Amarger 2002), future demands on agricultural produce, ever-growing populations, and decreasing cultivable lands requires a regulated and lawful use of genetically engineered inoculants (Hirsch 2004). A very careful assessment of genetic modification of rhizobia have to be made so that after release into soil it does not have any deleterious impact on indigenous microbial communities of agronomic importance. The competitive advantage conferred by genes involved in metabolic fitness, nutrient acquisition, or motility are perhaps relatively harmless in nature, whereas overexpression of genes coding for the synthesis of antibiotic-like molecules may affect nontarget species and may alter bacterial diversity (Robleto et al. 1998). Recent interest in application of rhizobia to enhance growth of nonleguminous plants like rice, sugarcane, wheat, and maize either as associative symbionts or as endophytes extends the use of this group of microorganisms for plants other than legumes (Saikia and Jain 2007; Bhattacharjee et al. 2008). Applying rhizobial inoculation technology to the nonleguminous plants, however, may extend competition problem to such plants. Therefore, the factors affecting establishment of inoculated rhizobia as endophytes need to be considered and competitive effect of native population should be addressed. Whether increasing competitiveness for nodulation also enhances endophytic competiveness could be explored.

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Chapter 9
Growth Promotion of Legumes by Inoculation of Rhizosphere Bacteria

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Abstract Most plants grown in fields are colonized by diverse groups of rhizosphere bacteria that form beneficial or pathogenic relationships with their hosts. The root exudates encourage the development of beneficial bacterial communities in the root zone capable of producing secondary metabolites that improve plant growth and crop yield. These beneficial associations facilitate plant growth either by enhancing crop nutrition, releasing plant growth stimulating hormones, reducing damages caused by pathogens/pests by producing antibiotics, bacteriocins, siderophores, hydrolytic enzymes and other secondary metabolites or by improving resistance to environmental pollutants. Rhizosphere bacteria also supply biologically fixed nitrogen, solubilize bound phosphorus and may provide other nutrients, such as, potassium, iron and sulfur to plants. These beneficial associations hence, reduce the requirement of chemical fertilizers used for crop productivity. Moreover, some rhizobacteria are used to relieve the toxicity of metals and organic toxicants, either through stimulation of microbial degradation of pollutants in the rhizosphere, or by uptake of pollutants/toxicants by the plant. The inoculation of the legumes with such rhizosphere bacteria has often been found to increase symbiotic properties, plant biomass and yields under green house or field conditions. Tremendous progress has been made recently in characterizing the process of rhizosphere colonization, identification and cloning of bacterial genes involved in nitrogen fixation, phosphorus solubilization, production of plant growth regulators and in suppression of plant diseases. The interactions/relationships of rhizosphere bacteria with their hosts and performance of wild-type and genetically manipulated beneficial bacterial populations are discussed for their efficient utilization in legume production under sustainable agriculture systems.

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9.1 Introduction

The rhizosphere around the growing plant roots is a very dynamic environment and harbors a large number of total microorganisms, especially bacteria, greater than root-free soil. The heterogenous microbial populations interact with each other and with the plant through symbiotic, associative, neutralist or antagonistic effects. The outcome of colonization and penetration of the plant tissue with a microorganism varies from asymptomatic to disease and from associative to symbiosis, depending upon the mutual perception or recognition between the interacting cells. Such interactions are influenced greatly by the environment. The microbes that penetrate and colonize plants have evolved an elaborate system for subverting the plant defense system. The group of beneficial, root associative bacteria that stimulate the growth of a plant is termed as plant growth-promoting rhizobacteria (PGPR). Fluorescent pseudomonads and bacilli comprise the major group among PGPR along with other bacteria, like, *Acetobacter, Agrobacterium, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Cellulomonas, Clostridium, Enterobacter, Erwinia, Flavobacterium, Pasteuria, Serratia* and *Xanthomonas*. The beneficial rhizosphere microorganisms also include rhizobia and bradyrhizobia, which establish symbiotic relationship with leguminous plants. In the absence of appropriate microbial populations in the rhizosphere, plant growth may be impaired (Sturz et al. 2000).

Legumes are widely used for food, fodder, fuel, timber, green manure, and as cover crops in different agricultural systems. In developing countries, legumes are often an integral part of forest, pastures and agricultural ecosystems. On global scale, nitrogen-fixing legumes are the major source of soil N pool. Legume crops meet their N requirement through symbiotic N_2-fixation by forming nodules with rhizobia. Legumes and the rhizosphere provide most of the nutritional requirements of nodule bacteria and enhances the *Rhizobium* population several folds during plant growth. *Rhizobium*–legume associations are usually host specific, and a given rhizobial strain can infect only a limited number of hosts. Most of the characterized rhizobial strains have been isolated from the limited range of cultivated legume species.

The high-input agricultural practices of the more industrialized nations of temperate zones are rarely suitable for tropical conditions in most developing countries. Therefore, emphasis on biological processes which are able to improve agricultural productivity, while minimizing soil loss and ameliorating adverse edaphic conditions, are essential. A better understanding of rhizobial ecology, optimization of N_2-fixing conditions in legume-*Rhizobium* symbiosis and selection of rhizosphere bacteria having synergistic interactions with *Rhizobium* leading to growth-promoting effects on legumes are crucial for improving and sustaining agricultural ecosystems. The inoculation effects of diverse bacterial groups possessing plant growth-promoting traits on the performance of legumes are discussed. The fundamentals of the different processes involved in plant growth promotion are briefly introduced. The different strategies or biotechnological approaches adopted for enhancing biological N_2-fixation (BNF), P-solubilization, auxins production

and improving biocontrol activity are also described. Various constraints involved in crop improvement following inoculation with genetically engineered bacterial strains and the possibilities of deriving desired benefits by ensuring the establishment and survival of introduced microbial inoculants in soil are explored.

9.2 Mechanisms Involved in Plant Growth Promotion

Microbial ecology of the rhizosphere includes the study of the interactions of microorganisms with each other and the environment surrounding the plant root (Weyens et al. 2009). Rhizosphere microorganisms are of major interest due to their beneficial or detrimental effects on plant growth. It is therefore, important to understand the mechanism by which rhizosphere microorganisms impact plant growth in order to develop technologies that could enhance their activities. Microbial populations present in the rhizosphere of legumes have shown substantial effects on nodulation by *Rhizobium* spp. and on subsequent growth and yield of leguminous crops (Kloepper et al. 1989; Glick 1995). Microorganisms inhabiting rhizosphere of legumes may benefit plants in a variety of ways, like increased recycling, mineralization and uptake of nutrients; synthesizing vitamins, amino acids, auxins, gibberlins and plant growth regulating substances; reducing metal toxicity (bioremediation) in contaminated soils; antagonism with potential plant pathogens through competition and development of amensal relationships based on production of antibiotics, siderophores, and/or hydrolytic enzymes (Stockwell and Stack 2007; Sindhu et al. 2009c).

9.2.1 Increased Recycling, Mineralization and Uptake of Nutrients

Microorganisms in the rhizosphere influence the availability of mineral nutrients to the plants, sometimes by increasing the availability of inorganic nutrients to the plant, and in other cases, using limiting concentrations of inorganic nutrients before they could reach plant roots. Some rhizosphere bacteria, i.e., rhizobia, azotobacters, and azospirilla have the ability to fix atmospheric N into plant utilizable form, ammonia (Franche et al. 2009). Other microorganisms help plants by solubilizing bound P (Vessey 2003) and potassium or by providing iron and sulfur (Crowley et al. 1991; Scherer and Lange 1996; Crowley and Kraemer 2007).

9.2.1.1 Biological Nitrogen-Fixing Bacteria and Inoculation Responses

Sustainable agriculture involves the successful management of agricultural resources to satisfy the changing human needs, while maintaining or enhancing

the environmental quality and conserving natural resources. Consequently, sustainability considerations demand that alternatives to nitrogen fertilizers are sought. In this context, BNF offers an alternative in farming practices as it exploits the capacity of certain N_2-fixing bacteria to reduce atmospheric nitrogen into a compound (ammonia) mediated by enzyme nitrogenase (Bohlool et al. 1992; Burris and Roberts 1993). Legume crops meet their N requirement through symbiotic nitrogen fixation by forming root nodules with rhizobia (Brewin 2002; Gage 2004) which in turn reduce the dependency of agricultural crops on fossil fuel-derived nitrogenous fertilizers. Additionally, biologically fixed N is bound in soil organic matter and thus is much less susceptible to soil chemical transformations and physical factors that lead to volatilization and leaching. Therefore, BNF has an important role in sustaining productivity of soils.

Only some prokaryotes, a few bacteria and cyanobacteria, have acquired the ability to reduce atmospheric dinitrogen and add this essential nutrient to agricultural soils. Biological N_2 fixation occurs in a free-living state, in association with or in symbiosis with plants. Different N_2-fixing bacteria have been used to improve the supply of fixed N as nutrient to crop plants. Among the nitrogen-fixing systems, the legume-*Rhizobium* symbiosis alone accounts for 70–80% of the total N fixed biologically on global basis per annum and one-third of the total N input needed for world agriculture. The symbiotic rhizobia have been found to fix N ranging from 57 to 600 kg ha^{-1} annually (Elkan 1992). Annual inputs of fixed nitrogen are calculated to be 2.95 million tonnes (Tg) for the pulses and 18.5 Tg for the oilseed legumes (Herridge et al. 2008).

Rhizobium includes the fast-growing species, *Bradyrhizobium* includes slow-growing species and *Azorhizobium* includes those fast-growing species capable of forming both stem and root nodules on tropical water-logged legume, *Sesbania*. Chen et al. (1995) proposed a separate genus, *Mesorhizobium*, to indicate a growth rate intermediate between that of *Bradyrhizobium* strains and typical fast-growing *Rhizobium* strains. Subsequently, it was used to denote a phylogenetic position for rhizobia intermediate between these two genera. According to current taxonomic classification of root-nodule bacteria, 11 genera and 45 species have been defined (Sahgal and Johri 2006; Wiliems 2006).

In the rhizosphere of legumes and cereals, other diazotrophic bacteria could also contribute N to plants. Free-living diazotrophic bacteria contribute upto 15 kg ha^{-1} $year^{-1}$ fixed N and the root-associative bacteria fix N to a level of 15–36 kg ha^{-1} $year^{-1}$. Similarly, cyanobacteria, the free-living nitrogen fixers contribute about one-third of the N requirement of the crop and add about 15–80 kg ha^{-1} $year^{-1}$ to the rice cropping system (Elkan 1992) (Table 9.1). The free-living/associative diazotrophs, although have limited potential in terms of average N input on acreage basis but inhabit almost all ecological environments and contribute more in nutrient use efficiency and improvement in crop physiology (Pandey and Kumar 1989; Wani 1990; Fujiata et al. 1992).

The symbiotic effectiveness of different legume species and their microsymbionts has been found to be variable. In general, faba bean (*Vicia faba*), and

Table 9.1 Estimated average rates of biological nitrogen fixation by diazotrophs and associations

Organism/system	N_2 fixed (kg ha^{-1} $year^{-1}$)
Free living microorganisms – *Azotobacter, Clostridium* and *Derxia*	0.1–15
Associative symbioses – *Azoarcus* and *Azospirillum*	5–25
Cyanobacteria – *Nostoc*, *Anabaena,* and *Oscillatoria*	15–80
Azolla–Anabaena symbiosis	313
Rhizobium–legume symbiosis	57–600
Nodulated non-legumes – *Casuarina–Frankia, Parasponia–Rhizobium*	2–300

pigeonpea (*Cajanus cajan*) have been found to be very efficient; soybean (*Glycine max*), groundnut (*Arachis hypogaea*) and cowpea (*Vigna sinensis*) to be average; common bean and pea poor in fixing atmospheric N (Hardarson 1993). Among the legumes, soybean is the dominant crop legume, representing 50% of the global crop legume area and able to fix 16.4 million tones N annually, representing 77% of the N fixed by the legumes (Herridge et al. 2008). Inoculation of legumes with efficient strains of rhizobia has often resulted in significant increases in yields of various legume crops (Thies et al. 1991; Wani et al. 2007; Franche et al. 2009). Elsiddig et al. (1999) studied the inoculation effect of *Bradyrhizobium* strains TAL 169 and TAL 1371 (introduced) and strains ENRRI 16A and ENRRI 16C (local) on five guar (*Cyamopsis tetragonoloba*) cultivars in a field experiment. Most of the *Bradyrhizobium* strains significantly increased yield, protein, crude fiber and mineral content. The locally-isolated strains affected these parameters more than the introduced ones. Karasu et al. (2009) observed that inoculation of chickpea (*Cicer arietinum*) seeds with *R. ciceri* isolate had a significant effect on seed yield, plant height, first pod height, number of pods per plant, number of seeds per plant, harvest index and 1,000 seed weight. But, nitrogen doses (applied at 0, 30, 60, 90, and 120 kg ha^{-1} level as ammonium nitrate) had no significant effect on yield and yield components. Local population genotype as crop material gave the highest yield (2,149.1 kg ha^{-1}) among three chickpea genotypes used.

Sindhu et al. (1992) compared the potential of N fixed by *Rhizobium* strains in chickpea using non-nodulating genotype PM233 derived from normal nodulating genotype ICC640. The N fixed by the *Rhizobium* strains Ca534 and Ca219 in parent cultivar gave the plant dry weights more than those obtained by applying urea (80 kg N ha^{-1}) in the non-nodulating mutant PM233, suggesting that in chickpea effective symbiosis with rhizobia provides more than 80 kg N ha^{-1}. The benefits of N fixed in legumes to subsequent cereal crops are substantial and persist for several years due to progressively slow mineralization. The benefits obtained were of higher magnitude with green manuring crops and upto 532 kg N could be incorporated by 60 days green manuring crops where the rate of N accumulation were rapid upto 10.8 kg N ha^{-1} day^{-1} (Peoples and Herridge 1990).

In coinoculation experiments of N_2-fixing *Azotobacter vinelandii* with *Rhizobium* spp., it was found that coinoculation increased the number of nodules on the

roots of soybean, pea (*Pisum sativum*) and clover (*Trifolium pratense*) (Burns et al. 1981). Increased nodulation of soybean also occurred in field trial. Similarly, coinoculation of *Azospirillum brasilense* with *Rhizobium* strains showed synergistic effect on soybean and groundnut (Iruthayathas et al. 1983; Raverkar and Konde 1988). Compared to single *Rhizobium* inoculation, coinoculation of *Rhizobium* spp. and *Azospirillum* spp. was found more effective in enhancing the number of root hairs, the amount of flavonoids exuded by the roots and the number of nodules (Itzigsohn et al. 1993; Burdman et al. 1997; Remans et al. 2007, 2008b). The effect of *Azospirillum* on the legume-*Rhizobium* symbiosis was found to depend on the host genotype used. It was observed that *Azospirillum–Rhizobium* coinoculation increased the amount of fixed N and the yield of DOR364 genotype of common bean (*Phaseolus vulgaris* L.) across all sites on-farm field experiments, whereas a negative effect of *Azospirillum–Rhizobium* coinoculation on yield and N_2-fixation was observed in BAT 477 genotype on most of the sites as compared to sole application of *Rhizobium* (Remans et al. 2008b).

Field and greenhouse data indicated that increased nodulation of beans (*Phaseolus vulgaris*) by *R. phaseoli* occurred with coinoculation of *Pseudomonas putida* (Grimes and Mount 1984). However, bean yield and shoot weight were not significantly affected by coinoculation, demonstrating that increasing nodule number or infection by *Rhizobium* spp. may not affect plant productivity. Bolton et al. (1990) also demonstrated that nodulation of pea increased following the inoculation of mixtures of *R. leguminosarum* and a deleterious toxin-releasing *Pseudomonas* sp. However, nodules and dry matter accumulation in shoots were the same whether or not the *Pseudomonas* sp. was coinoculated. On the other hand, enhancement in nodulation, root length, plant biomass and yield by mixed inoculation of rhizobia with other rhizobacteria in different legumes have been reported. For example, Chanway et al. (1989) tested nine PGPR strains against single cultivar of lentil and pea in the field. None of the strains stimulated the growth of pea, but in lentil plots inoculated with one or more rhizobacterial strains, there were significant increase in emergence, vigor, nodulation, acetylene reduction activity and root weight. The enhanced nodulation and growth of chickpea along with reduction in wilt incidence was observed on coinoculation of rhizobacteria obtained from chickpea rhizosphere when these strains were coinoculated with an effective *R. ciceri* strain Ca181 (Khot et al. 1996).

Coinoculation of the five plant growth-promoting fluorescent pseudomonad strains, isolated from Indian and Swedish soils, and *R. leguminosarum* bv. *viceae* strains, recovered from Swedish soils, improved growth of pea cv. Capella (Dileep Kumar et al. 2001). In a similar study, Goel et al. (2002) observed that coinoculation of chickpea with *Pseudomonas* strains MRS23 and CRP55b, and *Mesorhizobium* sp. *cicer* strain Ca181 increased the formation of nodules by 68.2–115.4%, at 80 and 100 days after planting as compared to single inoculation of *Mesorhizobium* strain under sterile conditions. The shoot dry weight ratios of coinoculated treatments at different stages of plant growth varied from 1.18 to 1.35 times that of *Mesorhizobium*-inoculated and 3.25–4.06 times those of uninoculated plants. Similar synergistic effects on nodulation and plant growth have also been observed

for other legumes by dual inoculation of *B. japonicum* and *P. fluorescens* in soybean (Li and Alexander 1988; Nishijima et al. 1988; Dashti et al. 1998), *R. meliloti* with *Pseudomonas* in alfalfa (Li and Alexander 1988; Knight and Langston-Unkeffer 1988), *R. leguminosarum* with an antibiotic-producing *P. fluorescens* strain F113 in pea (Andrade et al. 1998) and *Mesorhizobium*/*Bradyrhizobium* strains with *Pseudomonas* sp. in greengram [*Vigna radiata* (L.) wilczek] and chickpea (Sindhu et al. 1999; Goel et al. 2000, 2002).

Similarly, bacterization of *Bacillus* species to seeds or roots altered the composition of rhizosphere, leading to increase in growth and yield of different legume crops (Holl et al. 1988). For instance, Halverson and Handelsman (1991) observed that seed treatment with *B. cereus* UW85 had 31–133% more nodules than untreated soybean plants after 28 and 35 days of planting in the field. In the growth chamber, in sterilized soil-vermiculite mixtures, UW85 seed treatments enhanced nodulation by 34–61% at 28 days after planting. It was suggested that UW85 affected the nodulation process soon after planting by stimulating bradyrhizobial infections or by suppressing the abortion of infections. In a follow up study, Turner and Backman (1991) reported that coating of peanut seeds with *B. subtilis* improved germination and emergence, enhanced nodulation by *Rhizobium* spp., enhanced plant nutrition, reduced levels of root cankers caused by *Rhizoctonia solani* AG-4 and increased root growth. In a similar study, Srinivasan et al. (1997) reported enhanced nodulation in *Phaseolus vulgaris* when coinoculated with *R. etli* strain TAL182 and *B. megaterium* S49. The mixed inoculation increased root hair proliferation and lateral root formation. The potential of *Bacillus* sp. to enhance nodulation, plant dry matter and grain yield on coinoculation with rhizobia has also been reported for pigeonpea (Podile 1995) and white clover (Holl et al. 1988). Sindhu et al. (2002a) found that coinoculation of *Bacillus* strains with effective *Bradyrhizobium* strain S24 caused enhancement in shoot dry mass of green gram ranging from 1.28 to 3.55 at 40 days of plant growth. Nodule promoting effect and increase in nitrogenase activity was also observed with majority of *Bacillus* strains at 40 days of plant growth.

Mishra et al. (2009a) showed that plant growth-promoting bacteria (PGPB) strain *B. thuringiensis*-KR1, originally isolated from the nodules of *Kudzu* vine (*Pueraria thunbergiana*), promoted plant growth of field pea and lentil (*Lens culinaris* L.) when coinoculated with *R. leguminosarum*-PR1 under Jensen's tube, growth pouch and non-sterile soil, respectively. Coinoculation with *B. thuringiensis*-KR1 (at a cell density of 10^6 c.f.u. ml^{-1}) had the highest and most consistent increase in nodule numbers, shoot weight, root weight, and total biomass, over rhizobial inoculation alone. The enhancement in nodulation due to coinoculation was 85 and 73% in pea and lentil, respectively, compared to *R. leguminosarum*-PR1 treatment alone. The shoot dry-weight gains on coinoculation with variable cell populations of *B. thuringiensis*-KR1 varied from 1.04 to 1.15 times and 1.03–1.06 times in pea and lentil, respectively to those of *R. leguminosarum*-PR1 inoculated treatment at 42 days of plant growth. The cell densities higher than 10^6 c.f.u. ml^{-1} had an inhibitory effect on nodulation and plant growth whereas lower inoculum levels resulted in decreased cell recovery and plant growth performance. Similarly,

enhanced nodule number and biomass yield were achieved after coinoculation of soybean with the *B. japonicum* SB1 and the plant growth-promoting *B. thuringiensis*-KR1 (Mishra et al. 2009b).

Inoculation of legumes with different rhizobial strains in general results in a 10–15% increase in yield of legumes. However, the desired impact of biofertilizer on legumes is usually not achieved under certain field conditions. The inoculation with commercial inoculants often fails to improve crop productivity (van Elsas and Heijnen 1990) probably due to the inability of rhizobial species to compete with the indigenous, ineffective and built in populations, which presents a competitive barrier to the introduced strains (Sindhu and Dadarwal 2000). In contrast, production of bacteriocins by rhizobia have been shown to suppress growth as well as nodulation by the indigenous non-producer strains, thus improving nodulation competitiveness of bacteriocin-producing inoculant strains (Goel et al. 1999; Sindhu and Dadarwal 2000). Transfer and expression of *tfx* genes (involved in trifolitoxin production) in various rhizobia showed stable trifolitoxin production and restricted nodulation by indigenous trifolitoxin-sensitive strains on many leguminous species (Triplett 1988, 1990). However, attempts to manipulate certain rhizobial genes in specific legume rhizosphere niches for improving competition have not been impressive (Nambiar et al. 1990; Sitrit et al. 1993; Krishnan et al. 1999).

Biotechnological approaches used to enhance N_2 fixation and crop productivity (Pau 1991; Hardarson 1993; Sindhu et al. 2009a) under field conditions have been of limited use. For example, recombinant constructs of *R. meliloti* and *B. japonicum* having increased expression of *nif*A and *dct*A genes although showed increase in the rate of N fixed but under field conditions, the same constructs did not show any significant increase in N_2-fixation or yields (Ronson et al. 1990). Manipulations of common nodulation genes to improve the bacterial competition have usually resulted in either no nodulation, delayed nodulation or inefficient nodulation (Devine and Kuykendall 1996). Mendoza et al. (1995) enhanced NH_4^+ assimilating enzymes in *R. etli* through genetic engineering, by inserting an additional copy of glutamate dehydrogenase (GDH), which resulted in total inhibition of nodulation on bean plants. However, nodule inhibition effect was overcome when *gdh*A expression was controlled by NifA and thereby, delaying the onset of GDH activity after nodule establishment (Mendoza et al. 1998). Similarly, attempts to engineer hydrogen uptake (Hup^+) ability by cloning hydrogenase genes into Hup^- strains of *Rhizobium* resulted in experimental successes only in areas where soybeans are cultivated and where the photosynthetic energy is limited (Evans et al. 1987). Attempts to develop self-fertilizing crops for N have also been a failure, mainly because of the complexity of the nitrogenase enzyme complex to be expressed in the absence of an oxygen protection system in eukaryotes (Dixon et al. 1997). Moreover, induction of nodule-like structures or pseudonodules using lytic enzymes or hormones treatment in wheat (*Triticum aestivum*) and rice (*Oryza sativa*) though showed nitrogenase activity and $^{15}N_2$ incorporation, but the activity expressed was $>1\%$ of the value observed for legumes (Cocking et al. 1994).

9.2.1.2 Phosphate Solubilization and Mobilization and its Agronomic Significance

In agriculture, phosphorus (P) is second only to N in terms of quantitative requirement for crop plants (Goldstein 1986; Fernandez et al. 2007). It is found in soil, plants and microorganisms in both organic and inorganic forms. However, the total P content in an average soil is 0.05% and only a very small fraction (~0.1%) of the total P present in the soil is available to the plants because of its chemical fixation and low solubility (Stevenson and Cole 1999). The pool of immediately available P is thus, extremely small and must be supplied regularly to offset plant demands (Bieleski 1973). Phosphorus may be added to soil either as chemical fertilizers or as leaf litter, plant residues or animal remains. The P fertilizers are the world's second largest bulk chemicals used in agriculture and therefore, the second most widely applied fertilizer (Goldstein et al. 1993; Goldstein 2007). However, 75% of phosphate fertilizers applied to soil are rapidly immobilized and thus become unavailable to plants (Rodriguez and Fraga 1999). Therefore, P deficiency is a major constraint to crop production and under such conditions, the microorganisms offer a biological rescue system capable of solubilizing the insoluble inorganic P of soil to make it available to plants and thus maintain the soil health and quality (Rodriguez and Fraga 1999; Richardson 2001; Deubel and Merbach 2005; Chen et al. 2006; Khan et al. 2007).

Phosphate solubilizing (PS) and mobilizing microorganisms include bacteria, actinomycetes as well as the fungi. The most important PS bacteria (PSB) belong to genera *Bacillus* and *Pseudomonas*, though species of *Achromobacter, Alcaligenes, Brevibacterium, Corynebacterium, Serratia* and *Xanthomonas* have also been reported as active P solubilizer (Venkateswarlu et al. 1984; Cattelan et al. 1999; Khan et al. 2007). In a study, Naik et al. (2008) screened 443 fluorescent pseudomonad strains for the solubilization of tricalcium phosphate(TCP) and reported that 18% formed visible dissolution halos on Pikovskaya agar medium plates. Based on phenotypic characterization and 16S rRNA gene phylogenetic analyses, these strains were identified as *P. aeruginosa, P. mosselii, P. monteilii, P. plecoglosscida, P. putida, P. fulva* and *P. fluorescens.* The P-solubilizing *Bacillus* species isolated from the rhizosphere of legumes and cereals included *B. subtilis, B. circulans, B. coagulans, B. firmus, B. licheniformis, B. megaterium* and *B. polymyxa* (Gaind and Gaur 1991; Rajarathinam et al. 1995). Other PSB include species of bacteria like, *A. chroococcum, Burkholderia cepacia, Erwinia herbicola, Enterobacter agglomerans, E. aerogenes, Nitrosomonas, Nitrobacter, Serratia marcescens, Synechococcus* sp., *Rahnella aquatilis*, *Micrococcus, Thiobacillus ferroxidans* and *T. thiooxidans* (Banik and Dey 1983; Kim et al. 1998; Bagyaraj et al. 2000). *Rhizobium* and *Bradyrhizobium* strains have also been found to solubilize rock phosphate (RP) or organic P compounds effectively through the production of organic acids and/or phosphatases (Halder et al. 1991; Abd-Alla 1994). The various fungi having efficient PS ability belong to genera *Aspergillus, Fusarium, Penicillium* and *Trichoderma* (Rashid et al. 2004; Khan et al. 2010). Ahmad et al. (2008) reported that out of 72 isolates obtained from rhizosphere soil and root

nodules, solubilization of P was commonly detected in *Bacillus* (80%) followed by *Azotobacter* (74%), *Pseudomonas* (56%) and *Mesorhizobium* (17%). The principal mechanism of increasing P availability is the microbial production of organic acids that may dissolve appetite, releasing soluble forms of P through acidification of rhizosphere soil. Additionally, the acidification of the rhizosphere environments through metabolic production of hydrogen ions alters the pH sufficiently to mobilize soil minerals (Rodriguez et al. 2006).

Phosphatic biofertilizers were first prepared in USSR, using *B. megaterium* var. *phosphaticum* as PSB and the product was named as "phosphobacterin." It was extensively used in collective farming for seed and soil inoculation, and reported to give 5–10% increase in crop yields. Subsequently, inoculation experiments using phosphobacterin and other PSM for legumes like, groundnut, peas, and soybean showed an average 10–15% increase in yields in about 30% of the trials (Agasimani et al. 1994; Dubey 1997; Vessey 2003). The variations under field conditions are expected due to the effect of various environmental conditions and survival of the inoculant strains in soil. The inoculation of PSB along with Rock phosphate (RP) also resulted in increased availability of P for plant utilization (Jisha and Alagawadi 1996). It was observed that inoculation of mineral phosphate solubilizing bacteria (MPSB) along with 17.5 kg P ha^{-1} as Massourie rock phosphate (MRP) increased dry matter in chickpea and was as effective as single super phosphate (Prabhakar and Saraf 1990). Saraf et al. (1997) showed that PSB inoculation increased seed yield (10.3 q ha^{-1}) of chickpea as compared to control (8.8 q ha^{-1}). Increased grain yield (14%) and uptake of N and P was reported in chickpea by inoculation of PSB along with P fertilizers. The grain and straw yield of chickpea was found to increase with increasing levels of P (0–60 kg P_2O_5 ha^{-1}) which further improved by inoculation of PSB (Sarawgi et al. 1999, 2000). Plant growth-promoting fluorescent pseudomonad isolate PGPR1, which produced siderophore and indole acetic acid, and solubilized TCP under in vitro conditions, significantly enhanced the pod yield (23–26%, respectively), haulm yield and nodule dry weight over the control during 3 years.

Phosphorus deficiency has a negative effect on BNF and the impaired BNF in P-deficient plants is usually explained by an effect of the low P supply on the growth of the host plant, on the growth and functioning of the nodule, or on the growth of both plant and the nodule (Christiansen and Graham 2002). Some particular strategies have been adopted for the adaptation of nodulated legumes to limited P supply, such as the maintenance of concentrations of P in nodules much higher than in other organs (Pereira and Bliss 1987), higher absorption of P from the solution directly by the nodules and bacteroids (Al-Niemi et al. 1998), increased N_2-fixation per unit of nodule mass to compensate for reduced nodulation, (Almeida et al. 2000) and higher accumulation of soluble sugars in nodules than in roots and shoots (Olivera et al. 2004). Araujo et al. (2008) observed an increase in the activities of acid phosphatases and phytases in nodules of common bean genotypes at different levels of P supply indicating that this increase in activities may constitute an adaptive mechanism for N_2-fixing legumes to tolerate P deficiency. Similarly, plants grown at limited P supply can increase the activities of

phosphatases and phytases in roots to hydrolyze organic-P compounds in the soil, thus improving plant P acquisition.

Synergistic effect was observed after coinoculation of N_2-fixing bacteria with PSB. For example, the composite application of *P. putida* and *R. phaseoli* increased P availability to common bean plants and enhanced nodulation of common bean (Grimes and Mount 1984). The seed inoculation with thermo-tolerant PSB, viz. *B. subtilis, B. circulans* and *A. niger* was found to improve nodulation, available P_2O_5 content of soil, root and shoot biomass, straw and grain yield, P and N uptake by mungbean (Gaind and Gaur 1991). High pod yield and P uptake in groundnut due to inoculation of *P. striata* were also recorded (Agasimani et al. 1994). Increased nodulation, yield attributes, seed index and seed yield of rainfed soybean were also reported with combined inoculation of *P. striata* and *B. japonicum* (Dubey 1997). Similarly, a significant increase in nitrogenase activity, plant growth and grain yield of pea was found following dual inoculation of *R. leguminosarum* and PSB (Srivastava et al. 1998).

Attempts to express the mineral phosphate solubilization (MPS) genes in a different host were found to be influenced by the genetic background of the recipient strain, the copy number of the plasmids present and metabolic interactions. Thus, genetic transfer of any isolated gene involved in MPS to improve P-dissolving capacity in PGPR strains, is an interesting approach. An attempt to improve mps in PGPR strains, using a PQQ synthase gene from *E. herbicola* was carried out (Rodriguez et al. 2000). This gene was subcloned in a broad-host range vector pKT230. The recombinant plasmid was expressed in *E. coli* and transferred to PGPR strains of *Burkholderia cepacia* and *P. aeruginosa*, using tri-parental conjugation. Several of the exconjugants showed a larger clearing halo on medium plates containing TCP as the sole P source. This experiment indicated the heterologous expression of this gene in the recombinant strains and improved MPS ability of PGPR.

9.2.1.3 Mineralization of Potassium, Iron and Sulfur Nutrients in the Rhizosphere

Potassium (K) is the third major essential nutrient for plant growth. It plays an essential role in enzyme activation, protein synthesis and photosynthesis. Potassium in soil is present in water-soluble (solution K), exchangeable, non-exchangeable and structural or mineral forms. Of these, water-soluble and exchangeable pools are directly available for plant uptake. At low levels of exchangeable K in certain soils, non-exchangeable K can also contribute significantly to the plant uptake (Memon et al. 1988). India ranks fourth after USA, China and Brazil in terms of total consumption of K-fertilizers. Some microorganisms in the soil are able to solubilize "unavailable" forms of K-bearing minerals, such as micas, illite and orthoclase, by excreting organic acids which either directly dissolves rock K or chelate silicon ions to bring the K into solution (Barker et al. 1998; Bennett et al. 1998). These microorganisms are commonly known as potassium-solubilizing bacteria (KSB) or potassium-dissolving bacteria or silicate-dissolving bacteria whose application is

termed as "biological potassium biofertilizer (BPF)". It was shown that KSB increased K availability in soils and increased mineral uptake by plant (Sheng and Huang 2002; Sheng et al. 2002, 2003). Therefore, application of KSB holds a promise for increasing K availability in soils.

In Egypt, some studies were conducted on potassium-dissolving bacteria which were mainly concentrated on their K releasing capacity along with their effects on growth and K uptake of the treated plants. In a trial conducted by Balabel-Naglaa (1997), there were positive responses of broad bean to inoculation with some species of *Bacillus* (K releasing bacteria). These positive responses were obvious on dry weight of shoot and root, nodule number and dry mass of nodules, nitrogenase activity, N, P, K contents of foliage, number as well as dry weight of pods, seed and straw yields. Hu et al. (2006) isolated two phosphate-and potassium-solubilizing strains, KNP413 and KNP414 from the soil of Tianmu Mountain, Zhejiang Province (China). Both isolates actively dissolved mineral P and K, while strain KNP414 showed higher dissolution capacity even than *Bacillus mucilaginosus* AS1.153, the inoculants of potassium fertilizer widely used in China. In another study, Lian et al. (2008) studied the mechanism for the release of mineralic potassium using a thermophilic fungus *Aspergillus fumigatus*. The thermophilic fungus *A. fumigatus* promoted potassium release by means of at least three likely routes, firstly, by complexing soluble organic ligands, secondly, appealing to the immobile biopolymers such as the insoluble components of secretion and thirdly, involving mechanical forces in association with the direct physical contact between cells and mineral particles.

Iron is yet another essential nutrient and is abundant in soil but most of it is found in the insoluble form, ferric hydroxide. Thus, iron is only available to organisms at concentrations at or below 10^{-18} M in soil solutions at neutral pH. To cope up with its solubility, many microorganisms synthesize extracellular, low molecular weight, high affinity Fe^{3+} chelators commonly referred to as siderophores, in response to iron stress (Neilands 1981; Neilands and Nakamura 1991) that transport iron into bacterial cells. Fuhrmann and Wollum (1989) detected a decrease in the number of taproot nodules and in seedling emergence of soybean and altered nodulation competition among *B. japonicum* strains when coinoculated with *Pseudomonas* spp. Iron availability was implicated as a factor involved in the plant-*B. japonicum*-rhizosphere microflora interactions. Thus, rhizobacteria help plants in absorbing iron from the soil. The metal-chelating agents produced by rhizobacteria also play an important role in the acquisition of heavy metals (Leong 1986). These organic substances scavenge Fe^{3+} and significantly enhance the bioavailability of soil bound iron (Kanazawa et al. 1994) and regulate the availability of iron in the plant rhizosphere (Bar-Ness et al. 1992; Loper and Henkels 1999). The competition for iron in the rhizosphere is controlled by the affinity of the siderophore for iron, and the probable availability of iron to the microorganisms ultimately decides the rhizosphere population structure. The concentration of various types of siderophores, kinetics of exchange and availability of Fe-complexes to microbes as well as plants has been found to control the binding affinity of siderophore (Loper and Henkels 1999). Interestingly, the binding affinity of phytosiderophores for iron

is less than the affinity of microbial siderophores, but plants require a lower iron concentration for normal growth than microbes do (Meyer 2000).

Masalha et al. (2000) reported that plants grown under non-sterile soil systems were better in terms of iron nutrition than those grown under sterile conditions. Their data emphasized the role of microbial community on the iron nutrition of plants. It has been demonstrated that plants grown in metal-contaminated soils are often iron deficient and the production of siderophores by plant growth-promoting bacteria may help plants to obtain sufficient iron (Wallace et al. 1992; Burd et al. 2000). In fact, there is evidence that at least part of the toxic effects of some heavy metals in plants results from an induced iron deficiency and since bacterial siderophores could provide iron to various plants (Bar-Ness et al. 1991; Wang et al. 1993), therefore, siderophores produced by rhizobacteria may reduce nickel toxicity by supplying the plant with iron and hence reduce the severity of nickel toxicity (Bollard 1983; Bingham et al. 1986).

Another plant nutrient, sulfur (S), is the ninth and least abundant essential macronutrient. Its uptake and assimilation is crucial because of the key role played by the S containing aminoacids, methionine and cysteine in maintaining protein structure and because of its role in plant defense (Rasch and Wachter 2005). Sulfur atoms are widely distributed in soil and are found in a wide variety of organic and inorganic forms. These atoms are an integral component of soil humus, plants, microbial biomass and minerals. Scherer (2009) reported that sulfate (SO_4^{2-}), which is a direct source of sulfur for plants, contributed up to 5% of total soil S; generally more than 95% of soils S are organically bound. Sulfur containing minerals include pyrite (FeS_2) which occurs in ingenious rocks, gypsum ($CaSO_4.2H_2O$) and epsonite ($MgSO_4.7H_2O$). Despite its abundance in the earth's crust, S is often present in suboptimal quantities in soil or either in unavailable states. Moreover, the decrease of S input from atmospheric depositions has led to S deficiency of crops over the past two decades on a worldwide scale that reduced yield and affected the quality of harvested products. Especially in Western European countries, incidence of S deficiency has increasingly been reported in oilseed rape, which is an S demanding plant (Fismes et al. 2000). Therefore, more attention should be paid to the optimization of S fertilizer application, in order to cover plant S requirements whilst minimizing environmental impacts.

Sulfur turnover involves both biochemical and biological mineralization (Gharmakher et al. 2008). Biochemical mineralization, which is the release of SO_4^{2-} from the ester sulfate pool through enzymatic hydrolysis, is controlled by S supply, while the biological mineralization is driven by the microbial need for organic C to provide energy. The biological oxidation of elemental S and inorganic S compounds such as H_2S, sulphite and thiosulphate is brought about by chemo-autotrophic bacteria and photosynthetic bacteria. Sulfur-oxidizing bacteria include *Beggiotoa*, *Chromatium, Chlorobium, Thiobacillus*, *Sulfolobus, Thiospira* and *Thiomicrospira*. The species of *Bacillus, Pseudomonas, Arthrobacter* and *Flavobacterium* are also reported to oxidize elemental S or thiosulphate to sulfate. Under anaerobic conditions, sulfate is reduced to H_2S by sulfate-reducing micro-organisms, mostly the bacteria. Many bacteria including species of *Bacillus* and

Pseudomonas are known to reduce S or sulfate to H_2S, but among these *Desulphovibrio desulfuricans* and *Desulfotomaculum* spp. are important.

Nitrogen fixation appears to be affected by S fertilization in faba bean, lucerne, pea and in red clover (DeBoer and Duke 1982; Scherer and Lange 1996; Habtemichial and Singh 2007). An important link between S and N nutrition was found in white clover, lucerne and pea (Zhao et al. 1999; Varin et al. 2009) and sulfur fertilization was found to stimulate N_2 fixation strongly. Scherer et al. (2008) observed that the amount of leghaemoglobin was reduced by S deficiency in peas and alfalfa, when no S was added and nodules devoid of leghaemoglobin were more numerous. Varin et al. (2008) analyzed a set of functional traits in three white clover lines along a gradient of N and S fertilization on a poor soil. Nitrogen was found to be the most limiting factor for the VLF (very low fixation) line. S was the element that modulated the most traits for the nitrogen fixing lines NNU (normal nitrate uptake) and LNU (low nitrate uptake). Nitrogen fertilization was found to inhibit N_2 fixation in clover but N_2 fixation was enhanced when S was added. S fertilization also increased nodule length, as well as the proportion of nodules containing leghaemoglobin. Thus, sensitivity of white clover to S nutrition would be a disadvantage for competition in a situation of sulfur impoverishment.

9.2.2 Synthesis of Auxins, Cytokinins, Gibberlins and Vitamins

Microbial communities of soil and rhizosphere have been found to synthesize auxins, cytokinins, vitamins and gibberellin-like compounds (Arshad and Frankenberger 1991; Derylo and Skorupska 1993; Patten and Glick 1996; Gutierrez-Manero et al. 2001). These compounds increase the rate of seed germination and stimulate the development of root tissues leading to an increase in the capacity of the root system to provide nutrients and water to above ground organs of plants (Arkhipova et al. 2007), and also help the plants to tolerate abiotic stress (Yang et al. 2009). Derylo and Skorupska (1993) reported that stimulation of clover plant growth under gnotobiotic conditions resulted from the secretion of water-soluble B vitamins by fluorescent *Pseudomonas* sp. strain 267. This was demonstrated by enhancement of clover growth by naturally auxotrophic strains of *R. leguminosarum* bv. *trifolii* in the presence of the *Pseudomonas* sp. strain 267 supernatant. The addition of vitamins to the plant medium increased symbiotic N_2-fixation by the clover plants.

Indole acetic acid (IAA) is known as the main auxin in plants and has been implicated in all aspects of plant growth and development (Taele et al. 2006). The exposure of plant roots to exogenous microbially produced IAA can affect plant growth in diverse ways, varying from pathogenesis and growth inhibition to plant growth stimulation (Spaepen et al. 2007). In fact, low levels of IAA released by rhizobacteria has been found to promote primary root elongation, whereas, high levels of IAA stimulated lateral and adventitious root formation (Glick 1995) but inhibited primary root growth (Xie et al. 1996). Thus, plant growth-promoting bacteria can facilitate plant growth by altering the hormonal balance within the

affected plant (Lambrecht et al. 2000; Kamnev 2003). Such relationships of rhizobacteria between different crop species could be cultivar or genotype-specific (Cattelan et al. 1998). For example, the rhizosphere of wheat seedlings harbors a significant proportion of bacteria that produce phytohormone, indole acetic acid (IAA), known to increase root growth (Patten and Glick 2002). Moreover, differential response of inoculation was observed in two genotypes of common bean with *A. brasilense* Sp245 mutant strain having reduced auxin biosynthesis or to addition of increasing concentrations of exogenous auxin (Remans et al. 2008a). Genetic analysis of recombinant inbred lines revealed two quantitative trait loci (QTLs) associated with basal root responsive to auxin in common bean.

Although significant and consistent yield increases of rhizobia-inoculated crops have been attributed to N_2 fixation, plant growth regulators may also be involved (Mayak et al. 1999; Malik and Sindhu 2008). For instance, the rhizobial species are known to produce IAA in vitro (Bandenoch-Jones et al. 1982; Wang et al. 1982; Boiero et al. 2007) and nodulated roots often contained substantially greater auxin concentrations than non-nodulated roots (Dulhart 1967, 1970). Inoculation of soybeans with spontaneous mutants of *Rhizobium japonicum* that overproduced IAA (30-fold more auxin than the wild-type strain) showed a three-fold increase in the number of root nodules (Kaneshiro and Kwolek 1985). Mutants of *B. elkanii* strain deficient in IAA production induced fewer nodules on soybean roots in comparison to the parental strain and the normal numbers of nodules were reestablished following application of exogenous IAA (Fukuhara et al. 1994). IAA derived from *B. elkanii* has been implicated as a causative agent in the swelling of outer cortical cells of soybean roots and was suggested to provide a competitive advantage for nodulation (Yuhanshi et al. 1995). However, enlargement of cortical cells was not observed after inoculation with either IAA-deficient mutants of *B. elkanii* (Yuhanshi et al. 1995) or wild-type *B. japonicum* strains that do not produce IAA (Minamisawa and Fukai 1991). Prinsen et al. (1991) demonstrated that flavonoids released from legume plant roots, which also act as inducers of *Rhizobium* nodulation genes, stimulated the production of IAA, suggesting that nodule morphogenesis could be controlled by the highly specific nodulation signal in combination with phytohormones such as auxins, released by rhizobia.

Coinoculation of legumes with *Rhizobium* and free-living IAA-producing bacteria such as *Azospirillum brasilense* (Yahalom et al. 1990) and several *Bacillus* species (Srinivasan et al. 1996) significantly increased the number of nodules on the host roots and increased nodule fresh weight and nitrogenase activity, compared to inoculation with *Rhizobium* alone. Zhang et al. (1996) reported that *Serratia* stimulated soybean growth through the production of a plant growth-regulating compound, which stimulated overall plant vigor and growth, resulting in subsequent increase in nitrogen fixation. Mayak et al. (1999) showed that an IAA over producing mutant of *P. putida* caused extensive development of adventitious roots on mung bean cuttings. It was suggested that inoculation with these free-living bacteria increase the number of infection sites on roots for attachment and nodulation by *Rhizobium*. In addition, enhanced production of flavonoid-like compounds or phytoalexins in roots of several crop plants by inoculation of *Pseudomonas* sp.

(Parmar and Dadarwal 1999; Goel et al. 2001) could induce the transcription of nodulation (*nod*) genes (Peter and Verma 1990), leading to increase in nodulation. In contrast, similar experiments using mutants of *B. megaterium* with altered IAA production levels had a negative effect on symbiotic parameters (Srinivasan et al. 1996). Mutants of *Pseudomonas* strains altered in IAA production were derived by Tn5 mutagenesis (Malik 2002; Malik and Sindhu 2008). Coinoculation studies of wild-type *Pseudomonas* strains with *Bradyrhizobium* strain S24 and IAA overproducer *Pseudomonas* mutants resulted in more nodules in green gram compared to wild type *Bradyrhizobium* strain at 50 days of growth. Camerini et al. (2008) introduced *iaa*M gene (involved in IAA biosynthesis) from *Pseudomonas savastanoi* and the *tms*2 gene from *A. tumefaciens* into *R. leguminosarum* bv. *viciae* LPR1105. Free-living bacteria harboring the promoter *iaa*M-*tms*2 construct (strain RD20) released 14-fold more IAA in the growth medium than the wild-type parental strain and elicited the development of vetch root nodules containing up to 60-fold more IAA than nodules infected by the wild-type strain LPR1105. The root nodules elicited in vetch by RD20, were heavier and had an enlarged and more active meristem, and showed a significant increase in acetylene reduction activity (ARA).

9.2.3 *Effect of Rhizobacteria on Phytoremediation in Metal Stressed Soil*

Pollution of biosphere by toxic metals has accelerated dramatically since the beginning of the industrial revolution (Kabata-Pendias and Pendias 1989). Heavy metal pollution of soil is a significant environmental problem and has its negative impact on human health and agriculture. The most common heavy metal contaminants are Cd, Cr, Cu, Hg, Pb, and Ni. Heavy metals ions, when present at an elevated level in the environment, are excessively absorbed by roots and translocated to shoot, leading to impaired metabolism and reduced growth (Bingham et al. 1986). In addition, excessive metal concentrations in contaminated soils resulted in decreased soil microbial activity and soil fertility, and yield losses (McGrath et al. 1995). Phytoremediation has been reported to be an effective, in situ, non-intrusive, low-cost, ecofriendly, socially accepted technology to remediate polluted soils (Garbisu et al. 2002). Another alternative is to provide them with an associated plant growth-promoting rhizobacteria, which also is considered an important component of phytoremediation technology (Glick 2003; Jing et al. 2007). Therefore, the use of rhizobacteria to enhance phytoremediation of soil heavy metals pollution has recently received more attention (Weyens et al. 2009).

The functioning of associative plant-bacterial symbioses in heavy-metal-polluted soil can be affected from the side of both the micropartner (plant-associated bacteria) and the host plant (Glick 1995). Chaudri et al. (1992) found that *Rhizobium* populations were reduced at concentrations >7 mg kg^{-1} soil in their Cd treatments. Field studies of metal contaminated soils have similarly

demonstrated that elevated metal loadings can result in decreased microbial community size (Chander and Brookes 1991). Some rhizobacteria can exude a class of rhizobacterial secretion, such as IAA, siderophores, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase which increased bioavailability and facilitated root absorption of heavy metals, such as Fe (Crowley et al. 1991), enhanced tolerance of host plants by improving the P absorption (Liu et al. 2000) and promoted plant growth (Burd et al. 2000; Ellis et al. 2000). Rajkumar et al. (2005) isolated *Pseudomonas* sp. strain RNP4 from tannery waste contaminated soil which tolerated concentrations up to 450 mg Cr^{6+} L^{-1} on a Luria-Bertani (LB) agar medium and reduced a substantial amount of Cr^{6+} to Cr^{3+} in LB liquid medium. The strain also produced substantial amount of IAA, exhibited the production of siderophores and solubilized phosphorus. The strain was found to promote the growth of black gram, Indian mustard and pearl millet in the presence of Cr^{6+}, suggesting the innate capability of the *Pseudomonas* isolate for parallel bioremediation and plant growth promotion. In another study, Safronova et al. (2006) found that pea plants inoculated with root-associated bacteria containing ACC deaminase activity produced longer roots, greater root density and improved nutrient uptake by pea genotypes cultivated in cadmium supplemented soil. Inoculation of pea plants with a poplar endophyte that degraded 2,4-dichlorophenoxyacetic acid (2,4-D) resulted in increased removal of 2,4-D from the soil (Germaine et al. 2006). Moreover, the plants did not show toxic responses and did not accumulate 2,4-D in their tissues.

Liu et al. (2007) demonstrated that inoculation of alfalfa with *Comamonas* sp. strain CNB-1 not only removed 4-chloronitrobenzene (4-CNB) completely within 1 or 2 days from soil but also eliminated the phytotoxicity of 4-CNB to alfalfa plants. Tank and Saraf (2009) selected five plant growth-promoting bacterial strains based on their P solubilization ability, IAA production and biocontrol potentials. These isolates were also able to grow and produced siderophores in presence of heavy metals like Ni, Zn, and Cd. A positive response of bacterial inoculants was observed in chickpea plants towards toxic effect of nickel present in soil at different concentrations (0, 1 and 2 mM) and bacterial inoculants enhanced fresh and dry weight of chickpea plants even at 2 mM nickel concentration. The accumulation of nickel $plant^{-1}$ was just 50% in *Pseudomonas*-inoculated plants as compared to uninoculated plants with 2 mM nickel concentration along with increased biomass. The development of engineered endophytic bacteria that improved the phytoremediation of volatile organic compound trichloroethylene (TCE) was found to protect host plants against the phytotoxicity of TCE and contributed to a significant decrease in TCE evapotranspiration (Barac et al. 2004). Similarly, the genetic modification of the polychlorinated biphenyls (PCB)-degrading bacteria *Pseudomonas fluorescens* F113, to improve its performance in the rhizosphere, could be manipulated by improving symbiotic microorganisms. Thus, the rhizoremediation of PCBs by *P. fluorescens* was improved in which biphenyl degradation is regulated using a system that responds to signal from alfalfa roots (Villacieros et al. 2005).

9.2.4 Rhizobacteria as Biocontrol Agents

The suppression of growth of soil-borne plant pathogens by the use of microorganisms, natural or modified, genes or gene products to reduce the effects of undesirable organisms (pests) is referred to as biocontrol. Rhizobacteria inhibit the growth of various pathogenic bacteria and fungi resulting in suppression of the diseases caused by such pathogens (Weller 1988; Thomashow and Weller 1996). Disease suppression by biocontrol agents involves a sustained manifestation of interactions among the plant, the pathogen, the biocontrol agent, the microbial community on and around the plant, and the physical environment (Pierson and Weller 1994). Strains of *Pseudomonas fluorescens, P. putida, P. aureofaciens, P. cepacia* and *P. aeruginosa* have been found to antagonize the growth of pathogens leading to substantial disease control (Chandra 1997; Weller 2007). Different strains of *Bacillus thruingiensis, B. sphaericus, B. cereus* and *B. subtilis* are also used as biocontrol agents (Asaka and Shoda 1996; Hervas et al. 1997).

Trapero-Casas et al. (1990) reported that coating of chickpea seeds with the *P. fluorescens* (strain Q29z-80) increased the yield which was comparable to those obtained with any of the fungicide seed treatments used to control seed rot and preemergence damping-off disease caused by *P. ultimum* in the field. Hervas et al. (1997) observed that treatment of *B. subtilis,* nonpathogenic *F. oxysporum* and/or *T. harzianum, when* applied alone or in combination, to chickpea cultivars "ICCV 4" and "PV 61" could effectively suppress the disease caused by the highly virulent *F. oxysporum* f. sp. *ciceris*. In comparison with the control, the final disease incidence was reduced by *B. subtilis* (18–25%) or nonpathogenic *F. oxysporum* (18%). The extent of disease suppression was higher and more consistent in cultivar "PV 61" than in "ICCV 4" whether colonized by *B. subtilis,* non pathogenic *F. oxysporum* or *T. harzianum.* Nautiyal (1997) found that among 478 bacteria obtained from roots of chickpea rhizosphere by random selection, 44 rifampicin resistant strains showed biocontrol activity against *F. oxysporum* f. sp. *ciceri*, *R. bataticola* and *Pythium* sp. under in vitro studies. In a greenhouse test, seed bacterization of chickpea with *P. fluorescens* NBRI 1303 increased the germination of seedlings by 25% reduced the number of diseased plants by 45% as compared with non-bacterized controls. Significant growth increases in terms of shoot length, dry weight, and grain yield, averaging 11.6, 17.6, and 22.61%, respectively, above untreated controls were obtained in field trials.

Plant growth-promoting fluorescent pseudomonad isolate PGPR1, which produced siderophore and IAA, and solubilized TCP under in vitro conditions, also suppressed the soil-borne fungal diseases like collar rot of peanut (caused by *A. niger*) in field trials (Dey et al. 2004). Jamali et al. (2004) studied effect of seven antagonistic bacteria on control of Fusarium wilt under green house conditions. Isolates B-120, B-32, B-28 and B-22 were identified as *B. subtilis* and isolates Pf-100, Pf-10 and CHAO were identified as *P. fluorescens*. Results revealed that only isolate B-120 reduced *Fusarium* wilt of chickpea in both seed and soil treatments. Soil treatment of bacteria showed better effects on plant growth than

that of bacterial seed treatment. Statistically significant biocontrol effects were observed when lettuce seedlings were inoculated into naturally *Rhizoctonia solani*-infested lettuce fields with bacterial suspensions of two endophytic strains, *Serratia plymuthica* 3Re4-18 and *P. trivialis* 3Re2-7 with rhizobacterium *P. fluorescens* L13-6-12, 7 days before and five days after planting in the field (Scherwinski et al. 2008). Usually, no general relationship was observed between the ability of a bacterium to inhibit a pathogen under in vitro and in situ disease suppression (Schroth and Hancock 1982; Wong and Baker 1984). Bacterial strains producing the largest zones of inhibition on agar media do not always make the best biocontrol agents. Therefore, some in vitro conditions have been modified to more closely simulate natural conditions (Randhawa and Schaad 1985). Among the numerous examples of biocontrol agents reported for disease control of soil-borne pathogens, only few studies provide mechanistic information for the activities of these agents. Recently, the use of mutants that lack certain in vitro and in situ activities have provided strong evidence for the involvement of specific molecules in biocontrol.

9.2.4.1 Mechanisms Involved in Biocontrol

For effective biocontrol of plant disease, the rhizobacteria must establish and grow in an ecological habitat that includes indigenous pathogenic microorganisms. Thus, root colonization by rhizobacteria appears to be an important factor in biological control and plant growth promotion. In recent years, tremendous progress has been made in characterizing the process of root colonization by biocontrol agents, the biotic and abiotic factors affecting colonization, bacterial traits and genes contributing to pathogen suppression (Benizri et al. 2001; Sindhu et al. 2009b). Rhizobacteria inhibit the growth of phytopathogenic microorganisms by various mechanisms.

Competition for Nutrients and Infection Sites

The rhizosphere microflora directly or indirectly inhibit the invasion of pathogen on plant tissue. Root-inhabiting microorganisms and plant pathogens could compete for space, nutrients or even for binding sites on the root surface. Space and nutrients competition could result in failure of the pathogen to develop critical population densities for disease initiation, whereas, the competition for specific binding sites would reduce the capability of plant pathogen to initiate the infection process. Pseudomonads possess the capacity to catabolize diverse nutrients and have fast generation time in the root zone, and hence, they are logical candidates for competition for nutrients against the slow growing pathogenic fungi and could result in biological control of pathogens (Weller 1985). Elad and Chet (1987) carried out a study to evaluate the antagonistic mechanism of rhizobacteria against damping off disease caused by *Pythium*. The competition for nutrients between

germinating oospores of *Pythium aphanidermatum* and biocontrol rhizobacteria was unique and was correlated significantly with disease suppression.

Interference in Chemotactic Attraction

Crop rotations and tillage management have been shown to influence specific microbial populations (Sturz et al. 1997). Rhizobacteria could spur a root exudation response in plants that is species specific (Merharg and Killham 1995). The close interactions between plants and rhizobacteria encourage the establishment of specific and beneficial rhizosphere, and such associations between different crop species could be cultivar-specific. Thus, certain cultivars of clover can foster the development of rhizo- and endophytic bacteria that favor the growth and development of specific cultivars of potatoes (Sturz and Christie 1998). An additional role of rhizosphere microbes in reducing root disease incidence is in interfering with chemotactic attraction of the pathogen to root receptor sites. Scher et al. (1985) suggested that chemotaxis might be the first step in root colonization. A variety of compounds as components of root exudates may serve as attractants for plant pathogens. Growth of root inhabitants (including mycorrhizal fungi) necessarily reduced both the quantity and diversity of organic compounds diffusing from the root, thereby, diminishing the probability of encounter by a plant pathogen (Davis et al. 1979).

Antibiotic Production

Antibiotic production by rhizobacteria is one of the major mechanisms postulated for antifungal activity to suppress pathogens in the rhizosphere and to promote plant growth. The role of antibiotics in disease suppression has been demonstrated in many biocontrol systems by mutant analyses and biochemical studies using purified antibiotics (Stockwell and Stack 2007). These antimicrobial compounds may act on plant pathogenic fungi by inducing fungistasis, inhibition of spore germination, lysis of fungal mycelia or by exerting fungicidal effects. A large number of antibiotics including phenazine carboxylic acid, diacetyl phloroglucinol, oomycin A, pyocyanine, pyrroles, pyoluteorin, pyrrolnitrin, iturin A, surfactin, etc. are produced by rhizobacteria (Bender et al. 1999; Sindhu et al. 2009b), which help in the suppression of pathogen growth. The first antibiotic clearly implicated in biocontrol by fluorescent pseudomonads was the phenazine derivative that contributed to disease suppression by *Pseudomonas fluorescens* strain 2-79 and *P. chlororaphis* strain 30–84 (formerly *P. aureofaciens*), which were suppressive to the take-all disease of wheat roots caused by *Gaeumannomyces graminis* var. *tritici* (Gurusiddaiah et al. 1986). The antibiotic was found active against several fungi including *G. graminis* var. *tritici*, *R. solani* and *P. aristesporum. Pseudomonas fluorescens* strain CHAO was found to produce a variety of secondary metabolites, i.e., 2,4-diacetyl phloroglucinol, pyoluteorin, hydrogen cyanide, salicylic acid,

pyochelin and pyoverdine, and protected various plants from diseases caused by soil borne pathogenic fungi (Stutz et al. 1986). Anjaiah et al. (2003) found that an isolate of *P. aeruginosa* PNA1, obtained from chickpea rhizosphere, protected the plants from *Fusarium* wilt until maturity in moderately tolerant genotypes of pigeonpea and chickpea. Root colonization of pigeonpea and chickpea, which was measured using a *lac*Z-marked strain of PNA1, showed ten-fold lower root colonization of susceptible genotypes than that of moderately tolerant genotypes, indicating that this plant-bacteria interaction could be important for disease suppression in this plant. Its Tn5 mutants (FM29 and FM13), which were deficient in phenazine production, caused a reduction or loss of wilt disease suppression in vivo. Similarly, *B. cereus* strain UW85 suppressed the diseases caused by the oomycetes. Analysis of *B. cereus* mutants showed a significant quantitative relationship between disease suppressiveness and the production of two antibiotics, zwittermicin A and kanosamine (Silo-Suh et al. 1994; Milner et al. 1996). The purified antibiotics suppressed the disease and inhibited the development of oomycetes by stunting and deforming germ tubes of germinating cysts. *Bacillus subtilis* RB14, which produced antibiotics iturin A and surfactin, was found to suppress damping off disease caused by *Rhizoctonia solani* (Asaka and Shoda 1996).

Production of Siderophores

Iron is an essential element for all living organisms and most of it is found in the insoluble form at neutral pH. To cope up with low solubility of iron, many microorganisms synthesize extracellular Fe^{3+} chelators i.e., siderophores, in response to low iron stress (Neilands and Nakamura 1991) that transport iron into bacterial cells. Plant growth promoting rhizobacteria (PGPR) produced different types of siderophores, which were involved in disease suppression and plant growth promotion (Leong 1986). The various categories of siderophores produced by PGPR include catechol, hydroxamate, pyoverdine, pyochelin, cepabactin, schizokinen and some other types like azotochelin, rhizobactin, anthranilic acid and azotobactin.

Kloepper et al. (1980) were the first to demonstrate the importance of siderophores production in biocontrol of plant pathogens with pseudobactin, a siderophore produced by *Pseudomonas* strain B10. The addition of 1.0 μM ferric chloride to an iron-deficient medium abolished the antagonism under in vitro conditions and the fluorescence by the PGPR were not observed. Studies with various siderophore-negative Tn5 mutants showed that pseudobactin of either pyoverdine and pyochelin type was necessary to achieve wild-type levels of protection against *Pythium*-induced damping off disease (Buysens et al. 1996). Goel et al. (2000) isolated pigment overproducer mutant MRS16M-1 from *Pseudomonas* strain MRS16, that was more inhibitory to the fungal pathogens, whereas, non-producer mutant MRS16M-5 was less inhibitory on nutrient agar medium. Addition of 100 μM ferric chloride to the medium decreased inhibition of fungal growth, suggesting the involvement of siderophores and other antifungal

secondary metabolites. Dileep Kumar et al. (2001) found that both the fluorescent pseudomonads and *Rhizobium* strains exhibited a wide range of antifungal activity against pathogens specific to pea. In a synthetic culture medium, all the plant growth promoting fluorescent pseudomonad strains produced siderophores, which expressed antifungal and antibacterial activity. Seed bacterization with plant growth-promoting strains, alone and together with a *Rhizobium leguminosarum* biovar *viceae* isolate, 361–27 reduced the number of infected peas grown in *Fusarium oxysporum* infested soils. Seed bacterization with siderophore-producing *P. fluorescens* isolates, *viz.* PGPR1, PGPR2 and PGPR4, suppressed the soil-borne fungal diseases like collar rot of peanut caused by *A. niger* and isolate PGPR4 also suppressed stem rot caused by *S. rolfsii* (Dey et al. 2004).

Production of Hydrolytic Enzymes

Some cell wall lysing enzymes produced by rhizobacteria have been found to cause the destruction of pathogens. For example, Chet et al. (1990) cloned the gene encoding chitinase enzyme from *S. marcescens* and transferred it into *E. coli*. The partially purified chitinase caused extensive bursting of the hyphal tips. This chitinase preparation was effective in reducing disease incidence caused by *R. solani* under greenhouse conditions. In other study, Chet et al. (1993) isolated three different chitinase genes from *Serratia*, *Aeromonas*, and *Trichoderma*. The cloned genes were expressed in *E. coli* and subsequently introduced into *R. meliloti*, *P. putida*, and *Trichoderma* strains resulting in increased chitinolytic activity of transformants against *Sclerotium rolfsii* and *R. solani*. Recombinant strains of *R. meliloti* were constructed which carried *chi*A genes to produce chitinase. The recombinant strain expressed chitinase during symbiosis in alfalfa roots (Sitrit et al. 1993). Khot et al. (1996) reported that certain isolates of *Pseudomonas* and *Bacillus* produced chitinase, β-1,3 glucanase (laminarinase) and siderophores. Seed inoculation of these bacteria or application of cell free extract on seed resulted in 48.6 and 31.6% reduction of the wilt incidence of chickpea under field conditions in a wilt sick nursery. *Pseudomonas* strains isolated from the rhizosphere of chickpea and green gram were also found to produce chitinases and cellulases in culture-free supernatants and inhibited growth of *P. aphanidermatum* and *R. solani* on potato dextrose agar medium plates (Sindhu and Dadarwal 2001).

Production of Secondary Metabolites

Among other metabolites, hydrogen cyanide (HCN) is produced by many rhizosphere bacteria and has been demonstrated to play a role in biological control of pathogens (Voisard et al. 1989). HCN over-producing bacterial strains resulted in small but statistically significant increase in the suppression of symptoms caused by *Mycophaerella graminicola* and *Puccinia recondita* f. sp. *tritici* on wheat seedling

leaves. *Pseudomonas aeruginosa* strain zag2 was reported to produce pyocyanin, siderophore and hydrogen cyanide (Hassanein et al. 2009). The minimum inhibitory concentration of the extracted pigmented compound against *Candida albicans* was 40.69 μg ml^{-1} and the antifungal activity of the compound was remarkable at 100°C for 20 min. The toxic volatile compound HCN produced by the bacteria was found to reduce the growth of both *F. oxysporum* and *Helminthosporium* sp. whereas *A. niger* was not affected.

The fungal pathogens also cause the plant to synthesize stress ethylene (van Loon et al. 2006) and much of the damage sustained by plants infected by phytopathogens occurs as a result of the response of the plant to the increased levels of ethylene (van Loon 1984). It is well known that exogenous ethylene often increases the severity of fungal infection, while some ethylene synthesis inhibitors significantly decrease the severity of a fungal infection (Elad 1990; Robinson et al. 2001). A number of PGPR, which stimulated root growth of different plant species were found to contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolysed the ethylene precursor ACC to ammonia and α-ketobutyrate, and as a result, decreased ethylene biosynthesis by plants (Glick et al. 1994; Hall et al. 1996; Belimov et al. 2001). The ACC deaminase-containing biocontrol bacterial strains were also found more effective than biocontrol strains that did not possess this enzyme (Wang et al. 2000).

Induction of Systemic Resistance

Some biocontrol agents induced a sustained change in the plant and increased its tolerance to infection against fungal and bacterial pathogens (Maurhofer et al. 1998), a phenomenon known as induced systemic resistance (ISR). Various non-pathogenic rhizobacteria have the ability to induce a state of systemic resistance in plants, which provided protection against a broad spectrum of phytopathogenic organisms including fungi, bacteria and viruses (Bakker et al. 2007). Induced resistance brought about by prior inoculation of the host by a pathogen, avirulent or incompatible forms of a pathogen, or heat killed pathogens has been attributed to induce physiological response of the host plant against subsequent inoculation by the virulent pathogens (Hoffland et al. 1996). Induced systemic resistance in plants has been demonstrated in over 25 crops, including legumes, cereal crops, cucurbits, solanaceous plants and trees against a wide spectrum of pathogens. The mechanism of ISR has also been studied in plant growth-promoting *Bacillus* spp. (Kloepper et al. 2004). Bacterial production of the volatile 2,3-butanediol triggered the expression of *Bacillus*-mediated ISR in *Arabidopsis*. The signaling pathway that is activated in this case depended on ethylene but was independent of salicylic acid and jasmonic acid signaling (Ryu et al. 2004). Whether or not biocontrol agents suppress disease by inducing resistance, it is essential that ISR and biocontrol strategies be compatible because future agricultural practices are likely to require the integration of multiple pest control strategies.

9.2.5 Selection of Rhizobacteria with Multiple Plant Growth-Promoting Traits

The use of beneficial soil microorganisms as agricultural inputs for improved crop production requires selection of rhizosphere-competent microorganisms with plant growth-promoting attributes. The selection of PGPR strains depends largely on their growth promoting activities, such as, production of IAA and siderophores, P solubilization and inhibition of pathogenic microorganisms. However, the presence of one or all of these traits does not qualify them to be a PGPR for one particular crop or spectrum of crops. For example, Cattelan et al. (1999) reported one rhizobacterial isolate which did not share any of the PGPR traits tested in vitro except antagonism to *Sclerotium rolfsii* and *Sclerotinia sclerotium*, but it promoted soybean growth. This indicates that besides such growth promoting traits, there are unexplained mechanisms, which also influence the growth of plants and requires the close proximity of PGPR to the roots of plants. Hence, there is no clear separation of growth promotion in plants and biological control induced by bacterial inoculants (Lugtenberg et al. 1991; Bloemberg and Lugtenberg 2001). Bacterial strains selected initially for in vitro antibiosis as part of evaluating biological control activity frequently demonstrated growth promotion in the absence of target pathogen (Sindhu et al. 1999; Goel et al. 2002). Similarly, PGPR selected initially for growth promotion in the absence of pathogens, may demonstrate biological control activity when challenged with the pathogens, presumably by controlling deleterious microorganisms or non-target pathogens.

Direct growth promotion occurs when a rhizobacterium produces metabolites that directly promote plant growth without interactions with native microflora (Kloepper et al. 1991). Dileep Kumar and Dube (1992) reported that fluorescent siderophore-producing *Pseudomonas* strain RBT13, originally isolated from the tomato roots, enhanced seed germination of chickpea and soybean, and resulted in increased root and shoot weight as well as yield of the crops. Sindhu et al. (2002b) reported plant growth promoting effects of fluorescent *Pseudomonas* sp. on coinoculation with *Mesorhizobium* sp. *Cicer* strain under sterile and "wilt sick" soil conditions in chickpea. The coinoculation resulted in enhanced nodulation by *Mesorhizobium* sp. and increased shoot dry weight by 3.92–4.20 times in comparison to uninoculated controls. Under indirect growth promotion mechanism, the production of antibiotics, siderophores and HCN by microorganisms decreased the population and activities of pathogens or deleterious microorganisms and thereby, increased the plant growth (Pierson and Weller 1994).

Nine different isolates of PGPR (*Pseudomonas* sp.) were selected from a pool of 233 rhizobacterial isolates obtained from the peanut rhizosphere based on ACC-deaminase activity (Dey et al. 2004). Four of these isolates, *viz*. PGPR1, PGPR2, PGPR4 and PGPR7 produced siderophore and IAA. In addition, *P. fluorescens* PGPR1 also possessed the properties like, P-solubilization, ammonification and inhibited *A. niger* and *A. flavus* under in vitro conditions. In addition to the traits exhibited by PGPR1, the strain PGPR4 showed strong in vitro inhibition to

S. rolfsii. In field trials on peanut, plant growth-promoting fluorescent pseudomonad isolates, *viz*. PGPR1, PGPR2 and PGPR4, significantly enhanced the pod yield (23–26, 24–28, and 18–24%, respectively), haulm yield and nodule dry weight over the control in 3 years. Inoculation with plant growth-promoting *P. fluorescens* isolates, *viz*. PGPR1, PGPR2 and PGPR4, was found to suppress the soil-borne fungal diseases like collar rot of peanut caused by *A. niger* and isolate PGPR4 also suppressed stem rot caused by *S. rolfsii*. Hynes et al. (2008) screened 563 bacteria originating from the roots of pea, lentil and chickpea for the suppression of legume fungal pathogens and for plant growth promotion. Screening of bacteria showed that 76% isolates produced siderophore, 5% isolates showed ACC deaminase activity and 7% isolates were capable of indole production. Twenty-six isolates (5%) suppressed the growth of *Pythium* species strain p88–p3, 7% suppressed the growth of *Fusarium avenaceum* and 9% suppressed the growth of *R. solani* CKP7. Four isolates promoted the growth of lentil and one isolate promoted the growth of pea. Fatty acid profile analysis and 16S rRNA sequencing of the isolates showed that 39–42% were the members of Pseudomonadaceae and 36–42% of the Enterobacteriaceae families.

In search of efficient PGPR strains, 72 bacterial isolates were obtained from different rhizospheric soil and plant root nodules (Ahmad et al. 2008). Of these, more than 80% of the isolates belonging to genera *Azotobacter*, *Pseudomonas,* and *Mesorhizobium* produced IAA, whereas, only 20% of the *Bacillus* was IAA producer. Solubilization of P was commonly detected in the isolates of *Bacillus* (80%) followed by *Azotobacter* (74%), *Pseudomonas* (56%) and *Mesorhizobium* (17%). All tested isolates produced ammonia. Siderophore production and antifungal activity of these isolates except *Mesorhizobium* were exhibited by 10–13% isolates. HCN production was more common trait of *Pseudomonas* (89%) and *Bacillus* (50%). *Pseudomonas* Ps5 and *Bacillus* B1 isolates showed broad-spectrum antifungal activity against *Aspergillus*, *Fusarium* and *Rhizoctonia bataticola*.

9.3 Biotic and Abiotic Factors Affecting Rhizosphere Colonization

It is well established that root colonization by biocontrol agent and beneficial microorganisms is a prerequisite to suppress the plant disease and to enhance plant growth. Root colonization by introduced bacteria could be improved by increasing the population size, distribution or survival of bacteria, along with manipulation of soil factors that may positively or negatively affect colonization. Bacterial traits such as growth rate, cell surface properties, motility (Boelens et al. 1994), chemotaxis to root exudates, production of secondary metabolites and tolerance to stressed environment (e.g., dehydration and temperature) also contributes to rhizosphere competence. Plant characteristics, like root structure, age and plant genotype as well as physico-chemical properties of soil, application of pesticides etc. were

found to affect rhizosphere colonization by the beneficial rhizobacteria. Use of green fluorescent protein (*gfp*) and in situ monitoring based on confocal laser scanning microscope (CLSM) could be used to understand the rhizosphere competence and root colonization (Johri et al. 2003). Using this technique, it was found that the *Pseudomonas* (biocontrol strains) colonized the seed and root surface at the same position, as did the pathogenic fungi that they controlled (Bloemberg et al. 2000). Another promising option considered important for understanding colonization is to screen mutants directly. Mutants of *Pseudomonas* strains of both phenotypes have been identified and analysis of these mutants indicated that prototrophy for amino acids and vitamins, rapid growth rate, utilization of organic acids and lipopolysaccharide properties contributed to colonization ability. Modification of genes involved in the biocontrol activity of biological control agents also played a key role in improving the potential rhizosphere competence as well as antifungal activity of biological control agents (Carroll et al. 1995). Moreover, biocontrol activity of *P. fluorescens* carrying PCA coding mini-Tn5 vector was enhanced by introducing *phz*H gene from *Pseudomonas chlororaphis* PCL1391 (Timmis-Wilson et al. 2000).

9.4 Development of Bacterial Inoculants and Constraints in Their Use

Rhizobium and *Bradyrhizobium* inoculants have been marketed with success for over a century. Releases of these nodule-forming microorganisms into soils have been successful. Inoculation with such inoculants has resulted in their establishment into soil and onto plant roots to a level sufficiently higher for the intended purpose. However, the desired impact of biofertilizer application under field conditions has been variable and inoculation of legume plants with commercial inoculant strains often fails to improve crop productivity (van Elsas and Heijnen 1990; Akkermans 1994). The problem is of the survival of inoculant diazotrophic bacteria under field conditions. For each introduction, abiotic soil factors such as texture, pH, temperature, moisture content and substrate availability need critical assessment since these factors largely determine the survival and activity of the introduced microorganisms (van Veen et al. 1997). In addition, the response of the inoculant to the prevailing soil conditions also depends on its genetic and physiological constitution (Brockwell et al. 1995). The use of genetic markers like resistance to antibiotics or introduction of metabolic markers from other bacterial species could help in tracing the introduced strains, whether it is rhizobia, cyanobacteria, azotobacters or azospirilla (Wilson et al. 1995). Another important reason for the inconsistency observed due to inoculation of PGPR could be the coating of seeds by low number of rhizobial cells. Higher or lower dosage of PGPR may have a detrimental effect on nodulation and growth of plant as demonstrated by Plazinski and Rolfe (1985). On the commercial front, approximately 20 bacterial biocontrol products based on *Pseudomonas*, *Bacillus*, *Streptomyces* and *Agrobacterium* strains have been

marketed. The discovery of many traits and genes involved in the beneficial effects of PGPRs has resulted in a better understanding of the performance of bioinoculants in the field.

Some of these strains may provide effective control of diseases in certain soils, in certain geographic regions or on particular crops**.** Generally, microorganisms isolated from the rhizosphere of a specific crop are better adapted to that crop and may provide better control of disease than organisms originally isolated from other species (Cook 1993). Despite the extensive research where biological agents have been used to control plant diseases, there have been limited commercial success. Many biological agents do not perform better in the field due to the complexity and variability of physical, chemical, microbiological and environmental factors in the field. Therefore, applications of a mixture of biocontrol agents may be a more ecologically sound approach because it may result in better colonization and enhance the level and consistency of disease control by providing multiple mechanisms of action, a more stable rhizosphere community and effectiveness over a wide range of environmental conditions occurring throughout the growing season. In addition, the genetic diversity of these strains may be tapped by combining them in mixed inoculants. Certain mixtures of fluorescent pseudomonads suppressed disease more effectively than did single-strain inoculants (Pierson and Weller 1994; Duffy et al. 1996).

Spadro and Cullino (2005) concluded that the use of genetically modified microorganisms could play an important role in crop production and protection. Genetic manipulation could result in new biocontrol strains with increased production of toxic compounds or lytic enzymes, improved space or nutrient competence, wider host range or enhanced tolerance to abiotic stress (Glick and Bashan 1997). Thus, biocontrol performance of soil pseudomonads may be improved by the introduction of antibiotic biosynthetic genes (Maurhofer et al. 1992; Haas and Keel 2003). Recombinant DNA strains with greatly increased diacetyl phloroglucinol (DAPG) and phenazine-1-carboxamide (PCN) antibiotics production have been constructed (Mavrodi et al. 1998, 2001). The production of DAPG and PCN could be placed under the control of strong promoters or of exudate-induced or rhizosphere-induced promoters (Mavrodi et al. 2006). Genes and enzymes involved in the biocontrol mechanism could also be applied directly or transferred to crops.

From the perspective of developing nations, these are exciting strategies that may help to increase yield while reducing the inputs and environmental problems. However, most of the microbial biodiversity in soil remains unexplored and much work remains to be done to first identify and then characterize microorganisms that could be used in such applications. Furthermore, such approaches require a detailed knowledge of the molecular signaling that takes place between plants and microbes to drive expression of desirable traits and to suppress unwanted effects in a controlled manner. Future strategies are required to clone genes involved in the production of antibiotics, siderophores and other metabolites, and to transfer these cloned genes into the rhizobacterial strains having good colonization potential along with other beneficial characteristics such as N_2 fixation, P-solubilization and/or hormone production. Exploiting plants and microbes by using such an

integrated approach requires a coordinated strategy to understand the degree and complexity of plant-microbe interactions employing modern "genomics/proteomics" technologies. The generation of complete genomic sequences for plant-associated bacteria, including pathogens and symbionts is already increasing our knowledge of these organisms. The increasing amount of genomic data available for the model plant species and their associated microorganisms, will assist in determining the most suitable beneficial bacterial strains for inoculation. In the near future, the molecular tools adopted in manipulation of bacterial traits are likely to improve the availability of nutrients, efficiency of phytoremediation and enhancement of biocontrol activity that will consequently improve the crop productivity and also protect the food chain by reducing levels of agrochemicals in food crops.

9.5 Conclusion

Although striking advances have been made in understanding the molecular and biochemical mechanism regulating N_2 fixation, P solubilization and hormone production, this has yet to be translated into applied environments. To overcome the problem of establishment of inoculated microbes, the beneficial bacteria intended for inoculation should be selected from local ecological niches and reinoculated into the same environment to ensure the desired benefits. The effects of soil and environmental factors on the physiology and ecology of introduced microorganisms are still poorly understood. Research is therefore, needed to understand the in situ physiology of inoculant cells and strategies must be developed as to how such microbes could be manipulated for desired performance. For example, the use of reporter genes inserted either randomly or directly into the bacterial genome may allow the specific detection and possible enhancement of in situ gene expression in inoculant cells.

The complex interactions among the PGPR, the pathogen, the plant and the environment are responsible for the variability observed in disease suppression and plant growth promotion. However, genetic manipulation of PGPR has the potential to construct significantly better strains with improved biocontrol efficacy (Trevors et al. 1990; Chet et al. 1993). Further, the efficacy of biocontrol bacteria can be improved by developing better cultural practices and delivery systems that favor their establishment in the rhizosphere. From the application point of view, consortia of ecologically diverse strains for N_2 fixation, P-solubilization, root growth promotion among others, should be practiced instead of single strain. In near future, both traditional and biotechnological approaches could be employed to increase rates of N_2 fixation, P solubilization, hormone production and increase in efficiency of biocontrol activity along with bioremediation of contaminants, leading to increase in crop yield under sustainable agricultural crop production system.

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Chapter 10
Mycorrhizosphere Interactions for Legume Improvement

Rosario Azcón and José-Miguel Barea

Abstract Legumes, plant species of great agronomical and ecological interest, are known to establish beneficial symbiotic relationships with two types of soil-borne microorganisms: N_2-fixing bacteria and arbuscular mycorrhizal fungi. Additionally, the legume rhizosphere harbors other associative beneficial microorganisms such as plant growth promoting rhizobacteria (PGPR). These microorganisms interact among themselves, and with legume roots, to develop the multifunctional legume mycorrhizosphere, a scenario of diverse activities relevant for legume productivity either in sustainable agriculture or in the maintenance of natural plant communities. This Chapter highlights strategic and applied research conducted so far, which have allowed a comprehensive understanding of the formation and functioning of the legume mycorrhizosphere. Manipulation of the microbial activities allows tailoring efficient mycorrhizosphere systems for improving legume productivity. The technology for the production of efficient rhizobial, free-living PGPR, and AM-fungal inoculants, nowadays commercially available, is likely to support sustainable and environmentally friendly low-input agrotechnological practices. The possibilities to use these bioproducts to help a sustainable development of legumes in either agrosystems or natural ecosystems are discussed.

10.1 Introduction

The production of healthy crops and the self-sustainability of the ecosystems are largely dependent on soil quality (Altieri 1994). And hence, maintaining the quality and fertility of soil is a key issue not only for optimizing the stability and

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productivity of either agro-ecosystems or natural ecosystems but also to prevent erosion and to minimize negative cultural and environmental stresses (Buscot 2005; Chaudhary et al. 2009). It is well known that (1) soil quality/fertility is determined by interactions of the chemical, physical, and biological soil components (2) the variation in soil fertility is based on the diverse genetic and functional groups of extensive soil microbial populations, and (3) the activities of microbial communities affect critical soil functions (Barea et al. 2005b; Mallik and Williams 2008; Avis et al. 2008). Among other functions, soil microorganisms are involved in the biogeochemical cycling of nutrients and matter and the maintenance of plant health and soil quality (Barea et al. 2005a). These activities are particularly relevant at the root–soil interface microhabitats, known as the rhizosphere, where microorganisms interact with plant roots and soil constituents. Formation, development, and significance of the rhizosphere have been widely reviewed (Barea et al. 2002b, 2005b; Richardson et al. 2009; Faure et al. 2009; Jones et al. 2009; Lambers et al. 2009; Hartmann et al. 2009; Dessaux et al. 2010).

Microbial interactions in plant rhizosphere play important roles in the overall development of legumes. Actually, legumes, plant species of great agronomical and ecological interest, are able to establish beneficial symbiotic relationships with two types of soil-borne microorganisms: N_2-fixing bacteria and mycorrhizal fungi. Like most of the major plant families, legume plants also form associations with arbuscular mycorrhizal (AM) fungi (Barea et al. 2004), the most universal mycorrhizal type (Smith and Read 2008). Nodulated and mycorrhizal legumes has to be the normal in sustainable agriculture and in natural ecosystems because both N_2-fixing bacteria and AM fungi naturally protagonize activities fundamental to legume nutrition and health. In turn, legumes impact the fundamental soil properties, including the development/performance of N_2-fixing bacteria and AM fungi (Lupwayi and Kennedy 2007). Since legume–rhizobium symbiosis is discussed by others in this book, emphasis is placed on mycorrhizas in this chapter.

Mycorrhizas are symbiotic, generally mutualistic and balanced, associations established between certain soil fungi and most vascular plants where both partners exchange nutrients and energy (Brundrett 2002). Basically, the host plant receives mineral nutrients via the fungal mycelium (mycotrophism), while the heterotrophic fungus obtains carbon compounds from the host's photosynthates. It is universally accepted that mycorrhizal symbioses, which can be found in almost all ecosystems worldwide, are fundamental to improve plant fitness and soil quality through key ecological processes (Smith and Read 2008). The mycorrhizal fungi colonize the root cortex and develop an extraradical mycelium, which overgrows the soil surrounding plant roots. This hyphal net is a structure specialized for the acquisition of mineral nutrients from the soil, particularly those whose ionic forms have poor mobility or are present in low concentration in the soil solution, as is the case with P (Barea 1991). This mycorrhizal function provides the plant with an adaptive strategy for P acquisition in soils with low P availability, which is an important

nutrient for legumes because these species require P for N_2-fixation (Postgate 1998; Vance 2001).

Apart from these microbial symbioses, legumes, like many other plant species, live in association with a great array of soil saprophytic microorganisms inhabiting rhizosphere (Barea et al. 2005b). Both symbionts and saprobes interact in the rhizosphere (Finlay 2008; Jaderlund et al. 2008; Kiers and Denison 2008; Adesemoye and Kloepper 2009), and some of the resultant interactions are fundamental for sustainable legume developments (Barea et al. 2008). Particularly, after mycorrhiza establishment, rhizosphere microorganisms interact with mycorrhizal structures to generate the so-called mycorrhizosphere, a key issue for legume productivity improvement (Barea et al. 2005c), as will be explained in the later section. This chapter critically reviews the related literatures focusing on (1) the types of microorganisms and processes involved in the establishment and functioning of the mycorrhizosphere, (2) the impact of the mycorrhizosphere activities on legume productivity, and (3) the possibilities to tailor an efficient mycorrhizosphere to be used as a biotechnological tool to improve legumes in either agrosystems or natural ecosystems.

10.2 Microorganisms and Processes Involved in the Establishment and Functioning of the Mycorrhizosphere

Many microbial groups live and perform important functions in the ecosystem (Giri et al. 2005; Buée et al. 2009). However, most studies on rhizosphere microbiology, especially those describing cooperative plant–microbial interactions, have focused their attention only on bacteria and fungi (Barea et al. 2004; de Boer et al. 2005). Accordingly, the two types of microorganisms are discussed in the following section.

10.2.1 Beneficial Rhizosphere Bacteria and Fungi in Agro- and Ecosystems

The prokaryotic bacteria and the eukaryotic fungi have a great variety of trophic/living habits whose saprophytic or symbiotic relationship with the plant could be either detrimental (pathogens) or beneficial (mutualists). The beneficial saprophyte microbes promote plant growth and health acting as (1) decomposer of organic substances (detritus), (2) plant growth-promoting rhizobacteria (PGPR), or (3) antagonists of plant pathogens. Beneficial plant mutualistic symbionts include the N_2-fixing bacteria and the multifunctional AM-fungi (Barea et al. 2005c).

10.2.1.1 Saprophytic Beneficial Rhizosphere Bacteria and Fungi

The term rhizobacteria refers to those rhizosphere bacteria that are able to colonize the root environments (Kloepper et al. 1991). Beneficial root colonizing rhizosphere bacteria, the PGPR, however, must have the ability to colonize root, and be able to survive and multiply in microhabitat associated with the root surface, in competition with native microbiota, at least for the time needed to express their beneficial plant activities (Kloepper 1994). Novel techniques to study the colonization pattern, bacterial characterization, and molecular determinants of the root colonization have been described (Gamalero et al. 2004; Richardson et al. 2009; Dessaux et al. 2010). The PGPR are known to participate in many important ecosystem processes, such as the biological control of plant pathogens, nutrient cycling, and/or seedling growth (Adesemoye et al. 2009; Zahir et al. 2004; Lucy et al. 2004; Barea et al. 2004; Lucas-Garcia et al. 2004). Numerous PGPR have been identified as biocontrol agents and used to reduce losses to crops caused by plant pathogens (de Boer et al. 2003; Chin-A-Woeng et al. 2003; Avis et al. 2008). Biological control of soil-borne diseases is known to result from (1) the reduction in the saprophytic growth of the pathogens followed by reduction in the frequency of the root infections through microbial antagonism, and/or (2) the stimulation of "induced systemic resistance (ISR)" in the host-plants (van Loon et al. 1998). Some microorganisms, however, can benefit plants by more than one mechanism. For example, *Trichoderma* species controls fungal pathogens by acting both as antagonist and by inducing localized and systemic responses (Harman et al. 2004). The processes involved in nutrient cycling by PGPR include nitrogen-fixation and phosphate solubilization besides releasing other nutrients in soil (Zaidi et al. 2009; Richardson et al. 2009; Marschner 2008).

Microbial N_2-fixation is the first step in cycling N to the biosphere from the atmosphere, a key input of N to plant productivity (Vance 2001). It is a well established fact that members of the prokaryotic bacteria are the only organisms able to fix N_2 as they are the only organisms possessing the key enzyme nitrogenase, which specifically reduces atmospheric N to ammonia in the symbiotic root nodules (Leigh 2002; Markmann and Parniske 2009). Furthermore, the PGPR are also known to mediate processes involved in P cycling. In this context, it has been shown that many rhizobacteria (and rhizofungi) are able to solubilize sparingly soluble phosphates (Khan et al. 2007, 2010) largely by releasing chelating organic acids (Farhat et al. 2009; Xiao et al. 2009; Marschner 2008). Phosphate-solubilizing bacteria (PSB) selected from existing PGPR populations have been assayed, but their effectiveness in the soil–plant system is variable (Barea et al. 2007; Zaidi et al. 2009). One of the reasons besides other factors accounting for such variation in the P-solubilizing activity of PSB could be the refixation of P applied exogenously by the soil's constituents before they reach to the root surface. However, if the phosphate ions, as released by the PSB, are taken up by a mycorrhizal mycelium, this would result in a synergistic microbial interaction that in turn improves P acquisition by the plant. This mycorrhizosphere activity is discussed in Sect. 10.4.

10.2.1.2 Beneficial Mutualistic Symbionts: N_2-Fixing Bacteria and Arbuscular Mycorrhizal Fungi

The bacteria able to fix N_2 in symbiosis with legume plants belonging to diverse genera (Willems 2007) are collectively termed as "rhizobia." How these bacteria interact with legume roots leading to the formation of N_2-fixing nodules, the signaling processes involved, the evolutionary history, and particularly, the molecular aspects determinants of host specificity in the rhizobial–legume symbiosis are described elsewhere in this book and will not be explained here. Other bacteria (actinomycetes), belonging to the genus *Frankia,* form nodules on the root of the so-called "actinorrhizal" species, plants having a great ecological importance (Vessey et al. 2004). The other major groups of mutualistic microbial symbionts are the fungi, which establish the arbuscular mycorrhizal associations with the roots of most plant species (Smith and Read 2008). The AM fungi are obligate microbial symbionts, which are not able to complete their life cycle without colonizing a host plant. They are ubiquitous soil-borne microbial fungi, whose origin and divergence dates back to more than 450 million years (Redecker et al. 2000a). The AM fungi were formerly included in the order Glomales, Zygomycota (Redecker et al. 2000b), but they have recently been moved to a new phylum Glomeromycota (Schüβler et al. 2001), as it is currently accepted (Rosendahl 2008; Helgason and Fitter 2009; Gamper et al. 2010).

Earlier studies on diversity of AM fungal communities were based largely on the morphological characterization of their large multinucleate spores. However, more recently, the ribosomal DNA sequence analysis has been used to determine the diversity of natural AM populations (Santos-González et al. 2007; Hempel et al. 2007; Öpik et al. 2008a,b; Toljander et al. 2008; Alguacil et al. 2009; Rosendahl et al. 2009). A lack of relationship between genetic diversity and functional diversity has been often described (Munkvold et al. 2004; Croll et al. 2008; Ehinger et al. 2009). However, fingerprinting techniques, using gel electrophoresis of PCR-amplified rDNA fragments, are being applied to analyze AM fungal species composition in spore, root, or soil (Cornejo et al. 2004; Santos-González et al. 2007; Hempel et al. 2007; Öpik et al. 2008a,b; Sonjak et al. 2009). Despite the advancement in molecular techniques, the identification approaches employed for AM fungi based on morphological characteristics are still valid and used and are considered complementary to the molecular methods (Oehl et al. 2009; Morton 2009). Recent advances in the genetic and genomics of the AM fungi have been reviewed (Gianinazzi-Pearson et al. 2004; Parniske 2004; Azcón-Aguilar et al. 2009). In this regard, the complete genome of the model AM fungus *Glomus intraradices* has been determined (Martin et al. 2008)

10.2.2 Arbuscular Mycorrhiza

There are two main types of mycorrhiza, ecto- and endomycorrhiza, which have considerable differences in their structure and physiological relationships with

symbionts (Smith and Read 2008). In ectomycorrhizas, the fungus develops a sheath or mantle around the feeder roots. The mycelium penetrates the root and develops between the cortical cells forming the so-called "Hartig net" that constitutes the site of nutrient exchange between partners. About 3% of higher plants, mainly forest trees in the Fagaceae, Betulaceae, Pinaceae, *Eucalyptus,* and some woody legumes, form ectomycorrhiza. The fungi involved are mostly Basidiomycetes and Ascomycetes. In endomycorrhizas, the fungi colonize the root cortex both intercellularly and intracellularly. Some endomycorrhizal types are restricted to species in the Ericaceae ("ericoid" mycorrhiza) or Orchidaceae ("orchid" mycorrhiza), while the common arbuscular mycorrhizal (AM) type is widely distributed throughout the plant kingdom. The widespread and ubiquitous AM symbiosis is characterized by the tree-like symbiotic structures, termed "arbuscules," which the fungus develops within the root cortical cells, and where most of the nutrient exchange between the fungus and the plant is thought to occur. An intermediate mycorrhizal type, the ectendomycorrhiza, is formed by plants in families other than the Ericaceae, but in the Ericales, and in the Monotropaceae and Cistaceae. In these mycorrhizal associations, the fungi form both a sheath and intracellular penetrations (Smith and Read 2008). The obligate character of the AM fungi are such that specific methodological approaches are needed to investigate the processes involved in the formation and functioning of the symbiosis (Lambais 2006; Balestrini and Lanfranco 2006; Reinhardt 2007; Martin 2008; Garcia-Garrido et al. 2009; Gianinazzi-Pearson et al. 2009; Facelli et al. 2009; Gryndler et al. 2009; Smith et al. 2009). The AM fungi contribute to nutrient, particularly P, acquisition and supply to plants by linking the geochemical and biotic portions of the soil ecosystem, thereby affecting rates and patterns of nutrient cycling in both agricultural and natural ecosystems (Jeffries and Barea 2001). In addition, the AM fungi are able to tap other nutrients (Barea et al. 2005a), especially N, either from inorganic (Tobar et al. 1994a,b) or organic (Leigh et al. 2009) sources.

The extraradical mycelium of AM fungi is profusely branched and provides a very efficient nutrient-absorbing system beyond the Pi-depletion zone surrounding the plant roots, thereby reducing the distance that Pi must diffuse through the soil prior to its interception. Actually, the AM fungal mycelium can spread through the soil over considerably longer distances (usually several cm) than root hairs (Finlay 2008). The ability of the AM hyphae to grow beyond the root Pi-depletion zone and deliver the intercepted Pi to the plant is thought to be the reason why AM associations increase Pi accumulation and plant growth in soils with low P availability (Smith and Read 2008). The AM symbiosis not only influence nutrient cycling in soil–plant systems but also improves plant health through increased protection against environmental stresses including biotic (e.g., pathogen attack) or abiotic (e.g., drought, salinity, heavy metals, organic pollutants), and enhancing soil structure through the formation of the aggregates necessary for good soil tilth (Rillig and Mummey 2006; Turnau et al. 2006; Pozo and Azcón-Aguilar 2007; Ruíz-Lozano et al. 2008; Barea et al. 2008; Finlay 2008; Smith and Read 2008; Varma 2008; Ferrol and Pérez-Tienda 2009).

10.2.3 Interactions Between AM and Rhizosphere Microorganisms to Establish a Functional Mycorrhizosphere

Rhizosphere microorganisms can either interfere with or benefit mycorrhiza establishment (Gryndler 2000; Pivato et al. 2009). A particular interest has been about the so-called "mycorrhiza-helper-bacteria" (MHB), a term that was coined by Garbaye (1994) and later updated by Frey-Klett et al. (2007) for those bacteria which stimulate mycorrhizal mycelial growth and/or enhance mycorrhizal formation. This applies both to Ectomycorrhiza (Frey-Klett et al. 2005) and to AM associations (Azcón-Aguilar and Barea 1992; Barea et al. 2004; Sabannavar and Lakshman 2008). Conversely, the establishment of PGPR inoculants in the rhizosphere can also be affected by AM fungal coinoculation (Barea et al. 2005b; Artursson et al. 2006; Jaderlund et al. 2008; Mallik and Williams 2008).

The establishment of the AM fungus in the root cortex is known to change many key aspects of plant physiology. These include the mineral nutrient composition in plant tissues, the hormonal balance and the patterns of C allocation. Therefore, the AM symbiotic status changes the chemical composition of root exudates, while the development of an AM soil mycelium, which can act as a carbon source for microbial communities (Barea et al. 2002a,b). AM-induced changes in plant physiology affect the microbial populations, both quantitatively and qualitatively, in either the rhizosphere and/or the rhizoplane. In addition, there are specific modifications in the environment surrounding the AM mycelium itself, the *mycorrhizosphere* (Linderman 1988; Andrade et al. 1997; Gryndler 2000). Therefore, the rhizosphere of a mycorrhizal plant, generically termed as the mycorrhizosphere, can have features that differ from those of a nonmycorrhizal plant (Finlay 2008). The mycorrhizosphere functions known to improve plant growth and health, and soil quality are depicted in Fig. 10.1.

10.3 Interactions Between AM Fungi and Rhizobial Bacteria to Improve Legume Productivity

Janse (1896) was the first to report the coexistence of endophytic bacteria and fungi colonizing legume roots. The fungal "root infection" was later on recognized as a "mycorrhizal" development (Jones 1924). Thereafter, Asai (1944) concluded that nodulation by rhizobial bacteria appear to be dependent on mycorrhiza formation by the common host legume. Subsequently, both the widespread presence of the AM symbiosis in nodulated legumes and the impact of AM fungi in improving nodulation and N_2-fixation were recognized (Barea and Azcón-Aguilar 1983; Hayman 1986; Mosse 1986; Zaidi et al. 2003). Despite a positive effect of AM fungi on nodule formation and function, some reports on mycorrhiza–legume interaction are contradictory. For example, Franzini et al. (2009) found in an experiment that the tested AM

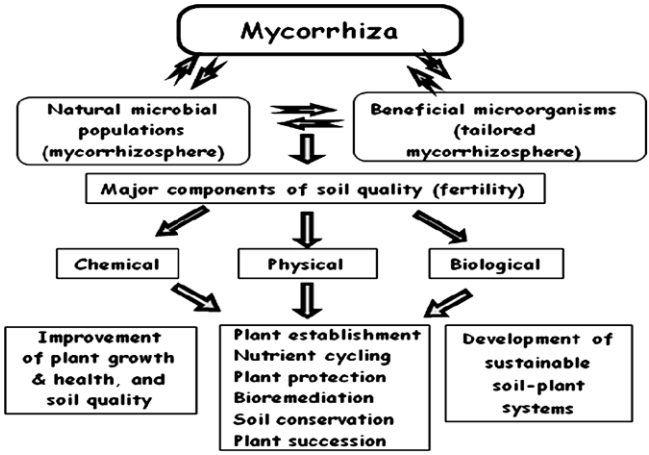

Fig. 10.1 Mycorrhizosphere functions

fungi did not improve nodule formation and function. Between the pioneering work by Asai (1944) and recent publications (Bisht et al. 2009), many papers have reported on the conceptual approaches and experimental developments with regard to the formation and functioning of the tripartite symbiosis. The relevant information will be summarized in this section under the following heads (1) fundamental aspect of the formation and functioning of the tripartite symbiosis in legumes, (2) strategy as to how the performance of legumes could be improved, and (3) strategic studies related to the role of legumes in the revegetation of degraded ecosystems.

10.3.1 Fundamental Aspect of the Formation and Functioning of the Tripartite Symbiosis in Legumes

Rhizobial bacteria and AM fungi are known to interact among themselves and with their common legume host roots, either at the colonization stages or at the symbiotic functional level (Azcón 1987; Barea and Azcón-Aguilar 1983). In this context, numerous studies have been carried out in recent times, which will briefly be summarized under the following heads (1) genetic and molecular relationships of AM fungi and rhizobia, (2) physiological interactions related to the formation and functioning of the tripartite symbiosis, and (3) use of ^{15}N to ascertain the AM role on N_2 fixation by legume–rhizobia associations.

10.3.1.1 Genetic and Molecular Relationships of AM Fungi and Rhizobia

Developmental genetics and evolution timing analysis of microbe–plant symbioses, including both mutualistic, either N_2-fixing or mycorrhizal, and pathogenic associations, have revealed a common developmental program for all of these compatible

microbe–plant associations (Markmann et al. 2008; Provorov and Vorobyov 2009b). As the rhizobia–legume symbiosis evolved much later than the AM symbiosis (Douglas 2008; Martínez-Romero 2009; Provorov and Vorobyov 2009a; Zhukov et al. 2009), the cellular and molecular events occurring during legume nodulation may have evolved from those already established in the AM symbiosis (Hirsch and Kapulnik 1998; Parniske 2008; Zhukov et al. 2009). However, the information generated from molecular tools suggests that some plant genes can modulate both types of legume symbiosis (Parniske 2004; Gianinazzi-Pearson et al. 2009). For example, the use of mycorrhiza-defective legume mutants (Myc^-) has provided relevant information, which helped in understanding the common cellular and genetic programs involved in the legume root symbioses (Gianinazzi-Pearson and Brechenmacher 2004; Gianinazzi-Pearson et al. 2009). The use of Myc^- legumes has also contributed to a better understanding of the signaling processes involved in the formation of microbe–legume symbioses, and hence, it has been suggested that both AM formation and nodulation share a common signal transduction pathway (Parniske 2004).

10.3.1.2 Physiological Interactions Related to the Formation and Functioning of the Dual Symbiosis

A great number of reports have focused on the physiological and biochemical basis of AM fungal × rhizobia interactions. The information, reviewed by Barea et al. (1992), established that the main cause of such beneficial interactions is the supply of P by the AM fungi to satisfy the high P demand of nodule formation. The AM fungi has also been shown to have a general influence on plant nutrition, but more localized effects of AM fungi are reported either at the root, nodule, or bacteroid levels. In a study, it was reported that nodules in fact had two to three times more P than the root (Mosse 1986), which was revealed in a time-course experiment that corroborated that the nodules and their rhizobial bacteroids have a "special demand" for P and call first for this nutrient with respect to other plant organs (Asimi et al. 1980). Furthermore, the tripartite symbiosis has been investigated both at physiological and structural levels, with results indicating that the effects depend on the particular endophyte combination (Ruíz-Lozano and Azcón 1993) and/or the legume genotype (Monzón and Azcón 1996). However, during symbiosis under natural conditions, AM fungi and rhizobia do not seem to compete for infection sites and colonize the root almost simultaneously (Bethlenfalvay et al. 1985). Accordingly, when host photosynthesis is limited, AM fungi usually show a competitive advantage for carbohydrates over the rhizobia (Brown and Bethlenfalvay 1988; Ruíz-Lozano and Azcón 1994), but under normal situations, the photosynthetic capacity of plants exceeds the C demand of the tripartite symbiosis (Ha and Gray 2008). The energy cost of the tripartite symbiosis as investigated by Ames and Bethlenfalvay (1987) suggests that the CO_2 fixation rate expressed as g C g^{-1} shoot dry matter h^{-1} increased in symbiotic plants. This is in fact a mechanism that enhances photosynthesis to compensate for the C cost of the symbioses. These

results were further corroborated (Mortimer et al. 2008) in experiments, which added new insights into the topic. These authors found that the nodular growth was suppressed during the early development of AM colonization under low P conditions. However, once AM colonization was established, and the efficiency of P nutrition increased, nodule development and host growth were improved, and concomitantly, N_2-fixation enhanced. This indicates that the AM fungi were the dominant symbiont for host C in the tripartite symbiosis, due to its rapid development. The subsequent AM role in supplying P benefited both host legume and nodules performance.

Other effect of AM fungi × rhizobia coinoculation on physiological aspects of symbiotic developments in legumes were investigated with regard to the bio-safety use of genetically modified (GM) rhizobia inoculants in agriculture. Particularly, the effects on AM formation and function of a wild type (WT) *Rhizobium meliloti* strain with those of its GM derivative were compared in time-course greenhouse experiments. It was found that either rhizobial strains coinoculated with the representative AM fungus *Glomus mosseae* increased the number of AM colonization units and the nutrient acquisition ability in AM alfalfa plants (Barea et al. 1996). Indeed, these response to variables were higher in plants inoculated with the GM rhizobial strain than in those inoculated with the WT one (Tobar et al. 1996; Galleguillos et al. 2000). The use of histochemical staining methods as succinate dehidrogenase (SDH) enzyme marker evidenced that both the WT and its GM derivative *R. meliloti* improved the physiological/biochemical activity of the AM fungus *G. mosseae* colonizing alfalfa (*Medicago sativa* L.) roots (Vázquez et al. 2000), and the nitrate reductase activity, protein content, etc. (Vázquez et al. 2002).

10.3.1.3 The Use of ^{15}N to Ascertain the AM Role on N_2-Fixation by Legumes

The addition of a small amount of ^{15}N-enriched inorganic fertilizer and an appropriate "non-fixing" reference crop is the basis for a direct method, which allows us to distinguish the relative contribution of the three N sources for a fixing crop, i.e., soil, atmosphere, and fertilizer (Danso 1988). Consequently, ^{15}N-based methodologies have been used to ascertain and quantify the amount of N that is actually fixed by legume–rhizobia consortia in a particular situation. Particularly, these methods have been applied to measure the contribution of the AM symbiosis to the process in greenhouse and field studies (Azcón et al. 1988, 1991; Barea et al. 2002c). A lower $^{15}N/^{14}N$ ratio in the shoots of rhizobia-inoculated AM plants with respect to those achieved by the same rhizobial strain in nonmycorrhizal plants was found. This indicated an enhancement of the N_2 fixation rates (an increase in ^{14}N from the atmosphere), as induced by the AM activity. The information about the possible role of AM fungi in legume symbiotic performance based on the use of ^{15}N isotope dilution technology has been reviewed (Barea et al. 2005a; Chalk et al. 2006). In addition, the isotopic techniques have been also used to measure N-transfer in mixed cropping where legumes are usually involved (Zapata et al. 1987). Since the AM mycelia can link different

plant species growing nearby, and help overlap the pool of available nutrients for the intercropped plant species, the N released into the overlapping mycorrhizospheres by legume root exudation, or by nodule decay, can result in nitrogen available for nonfixing plants (Haystead et al. 1988).

10.3.2 Strategic Studies Related to Legume Performance in Agriculture

Only a few studies involving AM fungi–rhizobia interactions were carried out under field conditions in the past, most of them conducted by us. In one of these studies, Azcón-Aguilar et al. (1979) reported for the first time that the dual inoculation improved the growth and nutrition of *M. sativa* grown in normal cultivation systems using arable soil (Fig. 10.2). Later on, a ^{15}N-based technique was applied to estimate N_2 fixation by the forage legume *Hedysarum coronarium,* and to ascertain the role of AM inoculation in plant N nutrition throughout a growing season under field conditions. The absence of the specific rhizobia for the forage legume in the test soil allowed the use of ^{15}N methodology with the same legume as reference "non-fixing" crop (Barea et al. 1987). The AM fungal inoculation enhanced dry matter yield, N concentration, and total N yield. The use of ^{15}N allowed us to distinguish the effect of AM fungi on N acquisition where mycorrhizal fungi enhanced both the amount of N derived from soil and from fixation, as compared with phosphate-added or control plants. This indicated that AM fungi acted both by a P-mediated mechanism to improve N_2 fixation and by enhancing N uptake by the legume from soil. The isotope ^{15}N was used also to measure both the N_2-fixation by white clover and N-transfer from clover to perennial ryegrass. Pure and mixed stands of these pasture plants were established in a field soil (Barea et al. 1989). The total N, P, and dry matter yields in the grass/clover mixture were greater than in monocultures. A lower ^{15}N enrichment of the grass growing together compared to those growing alone suggested N-transfer. The AM colonization of the grass in the

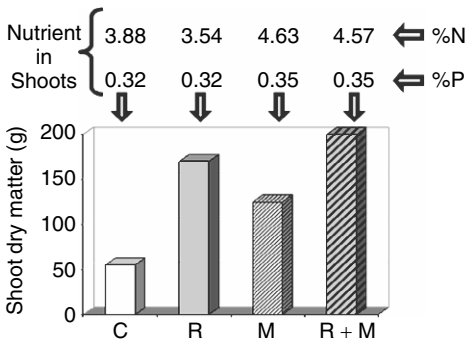

Fig. 10.2 Field inoculation of *Medicago sativa* with *Rhizobium* (R) and Mycorrhizal fungi (M)

mixed grass/clover sward was significantly enhanced, as compared with that of the grass in pure stands. Since the AM hyphae are known to be involved in NH_4^+ uptake, translocation, and transfer to the host plants, this may partly explain the improvement of pasture productivity when grass and mycotrophic legumes are grown together.

Apart from these pioneering reports, a number of experiments aimed at evaluating the role of AM fungi in improving N_2-fixation, either in controlled or in real field conditions, have been carried out during the last two decades. The accumulating data suggest a beneficial impact of AM fungi on legume symbiotic improvement in agriculture, particularly under low soil P levels (Barea et al. 2005c; Chalk et al. 2006). However, it is important to identify and select the appropriate rhizobial strain/AM fungus combination in order to optimize the benefit of the "tripartite symbiosis" on legume productivity (Azcón et al. 1991; Ruíz-Lozano and Azcón 1993; Ahmad 1995). In any case, the results depend largely on the fertility level of the soils. For instance, recent findings indicate that when the assailable P levels under field conditions were high, the tripartite symbiosis was not effective and hence did not promote N_2 fixation, either by soybean in Canada (Antunes et al. 2006) or *Calliandra calothyrsusen* grown in Senegal (Lesueur and Sarr 2008). In contrast, when the available P and N contents in the test soils were low, the appropriate management of legume by consortia of microbial symbionts improved soil fertility/productivity and consequently the overall performance of legumes. This has also been reported for forage legumes in Spain (Azcón 1993), soybean plants in Nigeria (Babajide et al. 2009), with an agro-forestry system, including tropical tree legumes, in Brazil (Pagano et al. 2008) or with common bean-based production system in Turkey (Uyanoz et al. 2007). Multitrophic interactions involving other microorganism, such as PGPR, have been analyzed in different studies, which describe how these bacteria enhance the beneficial effects of the legume microsymbionts (Azcón 1993; Bisht et al. 2009; Rinu and Pandey 2009). Those multitrophic interactions involving phosphate-solubilizing microorganisms are discussed later in this chapter (Sect. 10.4), and those helping the plant to support environmental/cultural stresses are discussed in Sect. 10.5.

10.3.3 Strategic Studies Related to the Role of Legumes in the Revegetation of Degraded Ecosystems

The information reviewed in this section will highlight the interactions of AM fungi and rhizobia adopted for restoration (by revegetation) of areas suffering an evident disturbance of their plant cover. The analyzed studies describe only field experiments, most of them concerning with Mediterranean desertification-threatened ecosystems. Mycorrhizosphere interactions to improve legume development in soils subjected to environmental/cultural stresses (heavy metal contamination, drought, salinity, pathogen attack, etc.) will be discussed in Sect. 10.5.

As a result of degradation/desertification processes, disturbance of natural plant communities is often accompanied, or preceded by loss of physical, chemical, and biological soil properties, such as soil structure, plant nutrient availability, organic matter content, microbial activity, etc. (Jeffries and Barea 2001). Physicochemical soil properties are fundamental for soil quality in particular with respect to soil structure, especially aggregate stability and organic matter accumulation, being one of the most influential factors (Miller and Jastrow 2000; Buscot 2005). Degradation of physical, chemical, and biological soil properties limits reestablishment of the natural plant cover. In particular, desertification causes disturbance of plant–microbe symbioses, which are a critical ecological factor affecting plant growth in degraded ecosystems (Francis and Thornes 1990). This is the reason why in revegetation programs the recovery of the natural ability of AM fungi and rhizobial bacteria is fundamental to initiate an integral restoration of a degraded area (Jeffries and Barea 2001). In this regard, some experiments aimed at investigating the application of this restoration strategy have been carried out (Barea et al. 2005a). In one of these studies, Herrera et al. (1993) conducted a 4-year field revegetation trial in a semiarid region of Spain, using a number of woody species, commonly used in revegetation programs in Mediterranean ecosystems. These included two native shrubs (*Anthyllis cytisoides* and *Spartium junceum*) and four tree legumes (*Acacia caven, Medicago arborea, Prosopis chilensis,* and *Robinia pseudoacacia*). Plant species and microsymbionts were screened to identify appropriate combinations, and a simple procedure to produce plantlets with an optimized mycorrhizal and nodulated status was developed. Results indicate that (1) only the native shrub legumes were able to establish under the local environmental conditions and (2) inoculation with rhizobia and AMF improved plant survival and biomass development. Since these two shrub legumes are found in the natural plant community, a reclamation strategy was proposed to revegetate desertified areas using these ecosystem-adapted shrub legumes. This strategy, which involves the artificial acceleration of natural revegetation, could be accomplished by replanting randomly spaced groups of these shrubs according to the natural pattern and structure of the undisturbed ecosystem (Francis and Thornes 1990). Particularly, *A. cytisoides*, a drought-tolerant legume species, very dependent on AM to achieve optimal development in natural conditions (López-Sánchez et al. 1992), was selected for these aims.

Using legume, Requena et al. (2001) carried out a long-term field experiment to ascertain the impact of inoculation with indigenous nocrosymbionts as a part of a reclamation strategy. A representative area within a desertified semiarid Mediterranean ecosystem in southeast Spain was chosen. The existing natural vegetation was a degraded shrubland where *A. cytisoides* was the dominant species (Requena et al. 1997). *Anthyllis* seedlings inoculated with an indigenous rhizobial and AM fungal inocula were transplanted to field plots in a 5-year trial. The tailored mycorrhizosphere not only enhanced establishment of the target legume but also increased soil fertility and quality. This included enhanced seedling survival rates, growth, P-acquisition, N-fixation, and N-transfer from N-fixing to associated non-fixing species in the natural succession. The improvement in the physicochemical

properties in the soil around the *Anthyllis* plants was shown by the increased levels of N, organic matter, and number of hydrostable soil aggregates. The role of the AM fungi, cooperation with other microbes, in the formation of water-stable soil aggregates (Rillig and Mummey 2006) is relevant here. The glomalin-related proteins, glycoproteins, produced by the external hyphae of AM fungi was found to be involved (Miller and Jastrow 2000; Bedini et al. 2009). The increase in N content in the rhizosphere of the legume can be accounted for by the supply of N-richer root exudates due to an improvement in nodulation and N-fixing capacity resulting from inoculation with both microsymbionts. The dually inoculated shrub legumes were a source of AM fungal inoculum for the surrounding area and in improving N nutrition for non-N-fixing vegetation. Figure 10.3 summarizes the role of AM symbiosis at improving nodulation and N_2-fixation in legumes and helping "N-transfer" from the rhizosphere of a N_2-fixing legume to a nonfixing plant growing nearby.

The tree legume *M. arborea* was positively benefited by coinoculation with AM fungi, *R. meliloti* strains, and PGPR (Galleguillos et al. 2000), suggesting that the mixtures of microbial symbionts could serve as a successful biotechnological alternative to aid the recovery of desertified ecosystems in semiarid areas. In follow up studies, a representative legume species from Mediterranean ecoystems, *Retama sphaerocarpa*, was selected as a target species for revegetation programs of degraded land in semiarid areas. Inoculation with native AM fungi improved plant establishment (Caravaca et al. 2003b), enzymatic activities related with C, N, and P cycling, and soil aggregation (Alguacil et al. 2005). In addition, the application of composted urban residues resulted in a complement of the AM effects to benefit such enzymatic activities and aggregate stability in the tailored mycorrhizosphere of transplanted *R. sphaerocarpa* (Caravaca et al. 2003a, c). Similar complementary effects were found by other organic amendments such as sewage sludge (Alguacil et al. 2004; Caravaca et al. 2005a, b) or composted dry olive residues

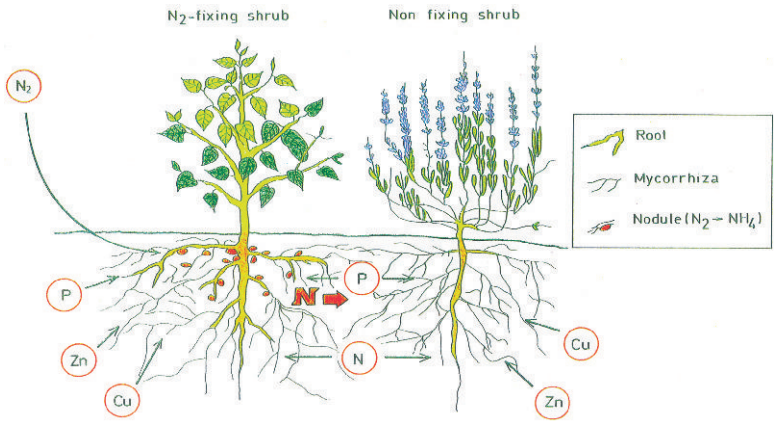

Fig. 10.3 Arbuscular mycorrhiza and nutrient acquisition (and cycling) in plant communities including legumes and nonlegumes plants (drawing by Esperanza Campos)

(Caravaca et al. 2006). The impact of AM inoculation in combination with organic amendments was further tested for *Dorycium pentaphyllum*, an autochthonous legume from semiarid areas in Southeast Spain, which is being used for restoration purposes. The tailored AM-seedlings were transplanted to a degraded semiarid area in Southeast Spain. Several types of *Aspergillus niger*-treated organic amendments and rock phosphate additions were also applied. These treatments, which included either sugar beet residues (Caravaca et al. 2004a) or dry olive cake residues (Medina et al. 2004; Alguacil et al. 2008), produced beneficial effects on physical, chemical, and biological properties of soils in the mycorrhizosphere soil of the transplanted target legume. Apart from the field experiments carried out in Mediterranean ecosystems, Bhatia et al. (1998) demonstrated that dual inoculation with rhizobia and AM fungi helped the establishment and biomass production of the woody legume (*Prosopis* sp.) in wasteland in India. More recently, Siviero et al. (2008) and Schiavo et al. (2009) demonstrated the successful effects of dual inoculation with AM fungi and rhizobia on the performance of representative tree legumes in Brazil. These and other associated data thus suggests that the management of appropriate microsymbionts can help legumes to promote the stabilization of a self-sustaining ecosystem. The mycorrhizal shrub/tree legumes act as a "fertility islands" (Caravaca et al. 2005a), which could serve as sources of symbiont inocula for the surrounding area and to improve N nutrition for the non N_2-fixing vegetation in stressed ecosystems.

10.4 Interactions Between AM Fungi and Phosphate-Solubilizing Microbes to Improve the Use of Natural Phosphate Sources and/or Agro-Industrial Residues by Legumes

The interactions between AM fungi and phosphate-solubilizing-microorganisms (PSM) are important for P acquisition by the legume plants. Therefore, the information generated from several experiments investigating this mycorrhizosphere activity merit some detailed consideration here.

Two general types of microbiologically mediated processes involved in P cycling have been described for increasing the Pi availability in soils (1) solubilization of unavailable P-sources in soils and (2) the uptake of solubilized Pi by plants (Kucey et al. 1989; Richardson 2001). The solubilization/mineralization of unavailable P compounds is carried out by diverse saprophytic bacteria and fungi (Marschner 2008; George and Richardson 2008; Richardson et al. 2009; Khan et al. 2010). Furthermore, since the external mycelium of the AM fungi acts as a bridge between roots and the surrounding soil microhabitats, AM establishment is the main mechanism involved in the uptake of solubilized Pi by plants (Barea 1991). On the other hand, phosphate-solubilizing bacteria (PSB) have also been tested for their ability to improve plant P nutrition (Kucey et al. 1989; Zaidi et al. 2009). The Pi made

available by PSB acting on sparingly-soluble P sources, however, may not reach to the root surface due to limited diffusion of this ion in soil solution. However, it was proposed that if the P is solubilized by PSB, AM fungi can tap these phosphatic ions and translocate it to plants suggesting a microbial interaction, which could improve P supply to the host plants synergistically or additively, as reported by Barea (1991). This hypothesis has been tested and found effective later on by several workers (Barea et al. 2005a, 2007; Wani et al. 2007; Zaidi et al. 2003), which involved the application of poorly reactive rock phosphate (RP) and the use of ^{32}P-tracer methodologies. Upon adding a small amount of ^{32}P to label the exchangeable soil P pool, the isotopic composition, or "specific activity" (SA = ^{32}P/^{31}P quotient), was determined in plant tissues (Zapata and Axmann 1995). These studies found that dual inoculation reduced the SA of the host plant, indicating that these plants acquired P from sources, either endogenous or from added RP, which were not directly available to noninoculated or singly inoculated plants (Toro et al. 1997). Particularly relevant for this Chapter were those experiments using legumes as host plant (Toro et al. 1998). To validate these results, a series of experiments were carried out either in a glasshouse or in the field (Barea et al. 2002c) using rhizobium inoculated legume plants to investigate the interactive effects of PSB and AM-fungi on P capture, cycling, and supply either from naturally existing P-sources or from added RP. A collateral objective was to investigate the agronomic effectiveness of the target microbial interactions on nodulation and legume performance under field conditions. For the greenhouse experiment, the exchangeable soil P pool was labeled with ^{32}P. Both RP addition and microbial inoculation improved biomass production and P accumulation in the test plants, with dual microbial inoculation being the most effective treatment. Independently of RP addition, AM and PSB coinoculated plants showed a lower SA than the noninoculated controls. This confirmed that these tailored legume mycorrhizosphere could acquire soil P from sources unavailable to noninoculated plants. Possibly, the PSB were effective in releasing ^{31}P from sparingly soluble sources, either from the soil components or from the added RP. This release of Pi would constitute a part of the total ^{31}P pool from which the AM mycelium acquired P and transferred it to the plants. Such microbial activities could result in the lower SA in dually inoculated plants. The use of ^{15}N allowed the corroboration of a positive effect of P-supply from the tailored microbial treatments on N_2-fixation by the test inoculated legume. Results from the field trial suggested that interactions between AM fungi, rhizobia, and PSB can have a cooperative fundamental role in P- and N-cycling in a tailored legume mycorrhizosphere.

The use of P-solubilizing fungi in combination with AM-fungi to improve legume mycorrhizosphere performance has been also investigated (Vassilev et al. 2002). For example, the mixtures of agrowastes (sugar beet), P-solubilizing fungus *A. niger*, and RP gives way to a fermentation product that was later investigated in a series of experiments. The host plant (*Trifolium repens*) was inoculated with an AM fungus and grown on a P-deficient soil. It was shown that product improved plant growth and P acquisition and that AM inoculation further favored the effectiveness of the fermentation product. As shown by the isotopic ^{32}P dilution technique, a lowering in

the SA was evidenced indicating that plants benefited from P solubilized from RP by the microbial activities. Nodulation and N_2-fixation were also improved by the tailored legume mycorrhizosphere. Recently, Vassilev et al. (2007) reported the effect of four agroindustrial wastes (sugar beet, olive cake, olive mill wastewaters, and dry olive cake) as substrates for microbial (*A. niger*) solubilization of RP. Amendments resulting from all these fermented products improved plant growth and P acquisition, which were further enhanced by AM inoculation. These fermentation products were applied to a degraded and P-limited soil for restoration purposes in combination with AM fungal inoculation (Medina et al. 2005). These biotechnological products improved plant growth, soil structure, and soil biochemical characteristics. The significance of treated agrowastes in interaction with PGPR and AM fungi will be further discussed in relation with the effect of legume mycorrhizosphere in phytoremediation of soils contaminated with heavy metals.

The capacity of rhizobia strains for phosphate solubilization (Rivas et al. 2007; Alikhani et al. 2007) was tested for improving legume nutrition in interaction with AM fungi and/or PGPR (Zaidi et al. 2003; Zarei et al. 2006; Matias et al. 2009). Synergistic improvements in legumes were suggested to be due to P-solubilizing activity of the tested bacterial strains.

10.5 Mycorrhizosphere Interactions to Improve Legumes in Soils Suffering from Environmental/Cultural Stresses

The quality and sustainability (stability and productivity) of either agroecosystems or natural ecosystems is usually endangered by different cultural or environmental stresses (Buscot 2005). These stresses are known to affect AM fungal diversity and activity (Chaudhary et al. 2009). However, tailored mycorrhizosphere can help plants to grow better by offsetting the negative impact of stress situations (Barea et al. 2005b). The effect of mycorrhizosphere interactions to help legume performance in soils suffering from environmental/cultural stresses will be considered here for three types of stresses: (1) contamination with heavy metals (HMs), (2) presence of osmotic stresses (drought, salinity, extreme temperatures), and (3) the attack of plant microbial pathogens.

The influence of climatic change on AM formation and function has been the subject of other studies (Staddon et al. 2003; Vargas et al. 2010), but only a few experiments using legumes as test plant will be discussed here. Particularly, the temperature component of the climatic change seems more influential than its CO_2 component (Gavito et al. 2003).

10.5.1 Phytoremediation of Soil Contaminated with Heavy Metals

The use of plants for the remediation (phytoremediation) of soils contaminated with heavy metals, radionuclides, or polycyclic aromatic hydrocarbons has been

benefited by the application of AM fungi (Leyval et al. 2002). Depending on the type of pollutant, different AM-assisted strategies of phytoremediation, such as phytostabilization, phytodegradation, and phytoextraction, have been investigated (Leyval et al. 1997). Most phytoremediation assays involving legume mycorrhizosphere concern heavy metals (Turnau et al. 2006; Jasper 2007; Maki et al. 2008; Teng et al. 2008; Azcón et al. 2009a). These studies mostly concentrated on Zn, Cu, Cd, Pb, or Ni (see below for references), but contamination with other metals, like arsenic, have also been attempted (Dong et al. 2008). Most of the reports on this topic concluded that AM colonized plants translocate less HMs to their shoots than the corresponding nonmycorrhizal plants, as shown for herbaceous (Díaz et al. 1996; Redon et al. 2009) or tree (Lin et al. 2007) legumes. These findings suggest that the role of AM-fungi in phytoremediation is mainly based on the immobilization (phytostabilization) of HMs in soil (Leyval et al. 2002; Turnau et al. 2006). Furthermore, both rhizobacteria and AM-fungi have been found to interact synergistically to benefit phytoremediation; however, the selection of HM-adapted microbial components to produce a tailored mycorrhizobacteria is necessary (Biró et al. 1998). This was investigated in a series of phytoremediation experiments using legumes (*T. repens*) as host plants (Vivas et al. 2003a, b, c, d, e, 2005a, b, 2006a, b, c). An agricultural soil from Nagyhörcsök Experimental Station (Hungary), which was contaminated in 1991 with suspensions of 13 microelement salts applied separately (Biró et al. 1998), was the target soil. Microorganisms isolated from this HM-contaminated soil ("autochthonous metal-adapted AM fungi and/or bacteria") were compared to microorganisms in the same taxa from culture collections, which were nonadapted to the tested HMs. The main achievements resulting from these experiments were (1) a number of efficient bacteria and the AM-fungi were isolated and identified by 16S rDNA or 18S rDNA, (2) the target bacteria were able to accumulate large amounts of metals, (3) coinoculation with a HM-adapted autochthonous bacteria and AM fungi increased biomass, N and P content as compared to noninoculated plants, and also enhanced the establishment of symbiotic structures (nodule number and AM colonization), which were negatively affected as the level of HM in soil increased, (4) dual inoculation lowered HM concentrations in *Trifolium* plants, inferring a phytostabilization-based activity; however, as the total HM content in plant shoots was higher in dually inoculated plants, due to the effect on biomass accumulation, a possible phytoextraction activity was suggested, (5) inoculated HM-adapted bacteria increased dehydrogenase, phosphatase and β-gluconase activities, and auxin production, in the mycorrhizosphere, indicating an enhancement of microbial activities related to plant development (Vivas et al. 2005a, b, 2006a, b, c; Azcón et al. 2009a). In yet other study, when agrowasted residues were added to the mycorrhizosphere system, additive/synergistic effects were evidenced (Medina et al. 2005, 2006). Particularly, the antioxidant activities involved in detoxifying the toxicity of heavy metals to plants were found to be increased (Azcón et al. 2009b).

The physiological/biochemical mechanisms by which the tested bacterial isolates enhanced phytoremediation activity in AM plants include: (1) improved rooting, and AM formation and functioning, (2) enhanced microbial activity in

the mycorrhizosphere, and (3) accumulation of metals in the root–soil environment, thus avoiding their transfer to the trophic chain, or to aquifers. In conclusion, even though a clear effect of mycorrhizosphere cooperative interactions was obvious on "phytostabilization," a significant effect on "phytoextraction" was also shown. Therefore, whatever be the mechanisms, a tailored mycorrhizosphere, by using selected HM-adapted microorganisms, can apparently be engineered to improve plant tolerance to HMs and to benefit bioremediation of HM-contaminated soils. The molecular mechanisms involved in HM tolerance in AM inoculated plants have been recently discussed (González-Guerrero et al. 2009).

10.5.2 Plant Performance in Soils Exposed to Osmotic Stresses

Since AM colonization can help plants to cope with drought and salinity stresses (Augé 2001; Ruíz-Lozano 2003), the role and the mechanisms involved in the AM symbiosis to help plant performance under osmotic stress conditions has been the subject of many studies (Ruíz-Lozano et al. 2008; Ruíz-Lozano and Aroca 2008). In this context, the pioneering work of Ruíz-Lozano and Azcón (1993) and Azcón et al. (1988) showed that AM inoculation improved nodulation and N_2 fixation at low levels of water potential, and the negative effects of salinity on nodulation and N_2 fixation could be compensated by AM inoculation. More recent experiments have corroborated a positive effect of the interactions between AM fungi and nodulating rhizobia under drought conditions (Goicoechea et al. 2000; Ruiz-Lozano et al. 2001; Echeverria et al. 2008). Particularly, it was found that AM inoculation protected soybeans plants against the detrimental effects of drought to prevent the premature nodule senescence induced by drought stress (Ruiz-Lozano et al. 2001). The alleviation of the oxidative damage exerted by AM inoculation seems to be one of the mechanisms involved in these protective AM-activities (Porcel et al. 2003; Porcel and Ruíz-Lozano 2004). In addition, indigenous drought-tolerant AM fungi are known to improve nutrient acquisition, gas exchange, nitrate reductase (Caravaca et al. 2004a,b,c), water transport, and root development (Marulanda et al. 2006) in the shrub legume *R. sphaerocarpa* under dry conditions. The presence of diverse plasma membrane-localized water channels (PIP aquaporins) in plants, which appears to be involved in water (or other solute) transport (Yamada and Bohnert 2000), has prompted other workers to elucidate further the role of AM fungi under osmotic stress (Ruíz-Lozano et al. 2008). Accordingly, experiments using legume have investigated aquaporin gene expression and its involvement in drought stress tolerance in AM-plants. As an example, research by Porcel et al. (2006) concluded that AM inoculated soybean plants respond to drought stress by downregulating the expression of the PIP genes studies. This could be a mechanism to decrease membrane water permeability to allow cellular water conservation. In any case, PIP gene expression in AM plants (*Phaseolus vulgaris*) depended on the particular conditions of the

different stress tested: drought, cold, and salinity (Aroca et al. 2007). The signaling molecule H_2O_2 seems to be involved in the PIP aquaporin regulation in *P. vulgaris* plants (Benabdellah et al. 2009).

10.5.3 Soil-Borne Pathogen Infested Soils

The establishment of AM fungi in plant roots has been shown to reduce damage caused by soil-borne plant pathogens leading to an enhancement in plant resistance/tolerance in mycorrhizal plants. In any case, the effectiveness of AM in biocontrol depends on the AM fungus involved, as well as the substrate and the host plant (Barea et al. 2005b; Pozo and Azcón-Aguilar 2007; Pozo et al. 2009). Different mechanisms have been suggested for the biocontrol activity of AM-fungi (Barea et al. 2005b). One mechanism involves microbial changes that results as the mycorrhizosphere develops, which is based on the shifts and resulting microbial equilibria that could help plant health. Activation of plant defense mechanisms, which can develop systemic resistance reactions, including protection against foliar pathogens, have been also reported (Pozo and Azcón-Aguilar 2007; Pozo et al. 2009).

10.6 Biotechnological Developments for Integrated Management of Legume Improvement

An increasing demand for low-input agriculture has resulted in greater interest in the manipulation and use of some soil microorganisms because of their beneficial impacts on plant growth and health and soil quality. It is expected that an appropriate management of beneficial soil microbes can reduce the use of chemicals and energy in agriculture leading to a more economical and sustainable production, while minimizing environmental degradation. These strategies are becoming more attractive as the use of chemicals for fumigation and disease control is progressively discouraged and fertilizers have become more and more expensive (Atkinson 2009). Consequently, some biotechnological inputs have been proposed concerning mycorrhizosphere technology, a fact of special interest for legume growers. These agrobiotechnological approaches include the use of microbial inoculants. For legumes, the target microbes are obviously the AM-fungi and rhizobia, but other selected PGPR can also be included and tested under pot/field conditions (Barea et al. 2002c; Zaidi and Khan 2007; Wani et al. 2007), as summarized in Table 10.1. While the technology for the production of inexpensive rhizobial and free-living PGPR is commercially available, the production of inocula and the development of inoculation techniques have limited the manipulation of AM-fungi. The difficulty to culture AM fungi in absence of

Table 10.1 Interactions of arbuscular mycorrhiza with soil microorganisms in a tailored mycorrhizosphere

Types of microorganisms	Interaction effects	References
N_2-fixing rhizobia	Increased N availability	Barea et al. (2005c)
Phosphate solubilizers	Increased P availability	Barea et al. (2007), Zaidi and Khan (2007)
Plant hormone producers	Rooting and establishment of seedlings	Artursson et al. (2006)
Antagonists	Control of plant pathogens	Barea et al. (2005b)
Specific rhizobacteria	Remediation of contaminated soil	Turnau et al. (2006)
Microorganisms involved in soil aggregation	Improvement of physical soil quality	Barea et al. (2005a)

their host plant, obligate symbionts, is a major obstacle to produce AM inoculants (Baar 2008). Despite these problems, several companies worldwide are producing plant-based AM inoculum products, which are now commercially available (Gianinazzi and Vosátka 2004; Vosátka et al. 2008). Selection of the appropriate AM fungi is however a key step (Estaún et al. 2002), while specific procedures are required to multiply AM-fungi and to produce high quality inocula (von Alten et al. 2002). Recent developments in AM-inoculum production systems rang from nursery plots (Koltai et al. 2008; Cuenca et al. 2008) to in vitro monoxenic root organ cultures (Bago and Cano 2005). The resulting materials (spores, hyphae, root fragments, etc.) are added to different carriers, resulting in a wide range of formulations, including encapsulation, to be applied at an agronomical scale using different application methods (Cuenca et al. 2008; Vosátka et al. 2008), including hydroseeding (Estaún et al. 2007). It is indeed a matter of discussion whether "generic products" containing several AM fungi, potentially suitable for a range of applications, are more appropriate for the market than those with precise formulations and AM fungi specifically tuned to particular end-uses (Smith and Read 2008).

Inoculation at broad scale in highly developed farming systems have, however, many constrains. To overcome this, management of indigenous populations is currently a viable option (Brito et al. 2008). However, at a relatively small-scale (nursery production), AM inoculation is feasible and advantageous. Inoculation of seedlings is potentially a good method for establishing selected fungi in roots before potting on or planting-out into the field. Inoculation is appropriate where transplanting is part of the normal production system, as is the case with horticulture, including plantation crops. Management strategies for inoculum build-up include the use of pastures, sequential cropping, or intercropping. Biodynamic and organic farm management results in higher per cent colonization of roots of pasture and annual crops than conventional management. In particular, the use of AM fungi in horticulture, in association with other beneficial microorganisms, appears as an effective way for improving fertilizer use and for minimizing losses due to disease (Baar 2008; Brito et al. 2008).

10.7 Conclusion and Future Perspective

Legumes are plant species of great agricultural/environmental importance known to establish beneficial symbiotic relationships with N_2-fixing bacteria and AM fungi. Associated to this tripartite symbiosis live many saprophytic microorganisms, which develop a peculiar mycorrhizosphere systems. Managing the microbial symbiont and saprobe, including PGPR, involved in legume mycorrhizosphere will have a great relevance to improve legume productivity either in sustainable agriculture or in the maintenance of natural plant communities. Current developments in the ecology, physiology, biochemistry, molecular biology, and biotechnology of the microbe–plant relationships have given new insights into the understanding of the formation and functioning of legume mycorrhizosphere. However, further research is still needed to better understand the tripartite symbiosis and consequently rationalized agricultural/forestry applications. While the technology for the production of rhizobia and free-living PGPR is commercially available, the production of inocula and the development of inoculation techniques have restricted the manipulation of AM fungi. However, biotechnological approaches now available, allows the production of efficient AM-fungal inoculants. Therefore, an appropriate management of selected AM fungi, rhizobia and PGPR is currently a viable alternative for agriculture, horticulture, and revegetation of degraded ecosystems. The application of selected microbial inoculants are likely to become even more important in future due to the agroecological threats of agrochemicals, which urgently requires to be reduced, and even avoided, to increase food quality, sustainable food production and environmental protection. Therefore, popularizing and improving the use of tailored mycorrhizospheres in legume plants is a major challenge for both the scientists and industry. To achieve such objective, it is important to explore the natural diversity of rhizobial, PGPR, and AM fungal populations in the mycorrhizosphere of legumes, and to exploit selected microbes as a source of inocula for providing sustained and balanced nutrients to legumes. This is though difficult, but particularly relevant for developing countries.

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Chapter 11
Role of Phosphate-Solubilizing Bacteria in Legume Improvement

Almas Zaidi, Munees Ahemad, Mohammad Oves, Ees Ahmad, and Mohammad Saghir Khan

Abstract Microbial communities inhabiting soil or rhizosphere play important roles in growth and development of plants. Of these, phosphate-solubilizing bacteria (PSB) play fundamental roles in biogeochemical phosphorus cycling in agroecosystems. Phosphate-solubilizing microbes transform the insoluble phosphorus to soluble forms by acidification, chelation, exchange reactions, and polymeric substances formation. The use of phosphate-solubilizing microbes in agronomic practices helps not only to offset the high cost of phosphatic fertilizers but also to mobilize insoluble phosphorus in the fertilizers and soils to which they are applied. And hence, application of such naturally occurring organisms possessing multiple growth-promoting activities hold greater promise for increasing the productivity of crops including legumes. Another agronomically promising organism is rhizobia which are known exclusively for its ability to form symbiosis with legumes and enrich nitrogen pool of soil, can also facilitate plant growth by synthesizing plant growth regulators and solubilizing insoluble phosphorus besides providing nitrogen to plants. In addition, under low nitrogen fertilizer inputs, availability of phosphorus is a major factor restricting the rate of N_2-fixation in legumes. The combined inoculation of N_2-fixers, PSB, and mycorrhizal fungi could be more effective than single organism for providing a more balanced nutrition for legume plants under conditions of reduced nutrient inputs. In this chapter, the strategic and applied research conducted so far to understand as to how PSB along with other symbionts enhance nutrient availability to legumes and concomitantly improve yield are reviewed and discussed. The application of synergically interacting yet phylogenetically diverse microbes is likely to help sustaining the legume productivity in different agricultural production systems.

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11.1 Introduction

An increasing need to produce food for the ever expanding world population is putting tremendous pressure on cultivable lands around the world. And hence, to meet out such challenges, a continuous expansion of food-producing ecosystems into less fertile areas is required. In every production system, crops must be provided with major nutrients for better growth and substantial yields (Rengel 2008). In this context, chemical fertilizers have excessively been used in agriculture worldwide to provide nutrients to support plant growth and consequently to boost crop productivity. Undoubtedly, chemical fertilizers have offered benefits to modern cropping systems, but the overuse of it has resulted in deteriorating the health of agricultural soils leading to both lower production yield and lower use efficiency of fertilizers. Scientists are therefore, currently interested in developing alternative technology to minimize the dependence on chemical fertilizers to encourage the use of biofertilizers on a large scale in agronomic practices. As a result of which, use of biological fertilizers including microbes has received greater attention. Application of such beneficial microbes alone or along with fertilizers as providers of nutrients presents an economically and environmentally promising strategy and can aid in replenishing and maintaining long-term soil fertility by providing good soil biological activity; by suppressing pathogenic soil organisms; by stimulating microbial activity in the rhizosphere; and to improve plant health (Zayed and Abdel-Motaal 2005a, b; Biswas and Narayanasamy 2006; Ouahmane et al. 2007). Of the various plant nutrients, even though P is abundant in soil, its availability is limited in plants due to fixation by other soil elements such as insoluble phosphates of iron, aluminium, and calcium (Khan et al. 2007). As a result of this, the plant-available P fraction and the concentration in the soil solution may be insufficient to satisfy plant requirements (Khan et al. 2010). Since deficiency of P (the second most important plant nutrient after N) is an important chemical factor restricting plant growth, chemical phosphatic fertilizers are widely used to achieve optimum yields (Del Campillo et al. 1999; Shenoy and Kalagudi 2005). Soluble forms of P fertilizer after application are, however, easily and rapidly precipitated as insoluble forms and become inaccessible to plants (Goldstein 1986; Takahashi and Anwar 2007). For example, the P deficiency can severely limit plant growth and productivity (Fernández et al., 2007), in particular in legumes, where both the plants and their symbiotic bacteria are affected, and this may have a deleterious effect on nodule formation, development, and function (Robson et al. 1981).

Providing P to plants through biological means is an eco-friendly and viable alternative. Among heterogeneously distributed soil microflora, a group of microorganisms commonly referred to as phosphate-solubilizing microorganisms (PSM) including bacteria (PSB), fungi (PSF), and actinomycetes have been found active in conversion of insoluble P to soluble forms and making it accessible to plants (Khan et al. 2009a; Jorquera et al. 2009; Khan et al. 2007). Among different plant growth-promoting rhizobacteria (PGPR) able to solubilize insoluble P (Zaidi et al 2009), the conversion of organic (Abd-Alla 1994) and inorganic P (Antoun et al. 1998;

Alikhani et al. 2006; Rivas et al. 2006; Daimon et al. 2006; Sridevi and Mallaiah 2009) by rhizobia to available P has dual advantages; besides P solubilization, they can provide other essential nutrient, for example, N to plants and also have the ability to improve legume growth synergistically with other PGPR and/arbuscular mycorrhizal fungi (Zaidi and Khan 2007; Wani et al 2007a; Zaidi et al 2003). Accordingly, it is reported that the phosphate-solubilizing bacteria (PSB) when applied with PGPR could reduce P fertilizer application by 50% without any significant reduction in crop yields (Jilani et al. 2007; Yazdani et al. 2009) suggesting that PSB as inoculant/biofertilizers hold greater promise for sustaining crop production with optimized P fertilization. However, under certain real soil situations, the use of PSB to augment crop productivity has been limited largely due to the variability and inconsistency of results observed under laboratory, greenhouse, and field trials. Such variation in the performance of PSB has been attributed to many factors including nutrient status of soils, plant genotypes, root exudates etc. Despite all these factors, increase in crop yields following PSB applications in the growth chambers and field trials have been observed (Deepa et al. 2010; Ahemad and Khan 2009; Wani et al. 2007b). The major focus of this chapter is to describe how a single P-solubilizing microorganism endowed solely with P-solubilizing activity or multiple growth-promoting activities improve legume productivity in different agro-ecosystems.

11.2 Phosphate Solubilization and Growth Regulators

Phosphorus, a major plant nutrient is required for various metabolic processes such as cell division and development, energy transport, signal transduction, macromolecular biosynthesis, photosynthesis, and respiration (Khan et al. 2009b; Ahemad et al. 2009; Shenoy and Kalagudi 2005). Phosphorus is present in soils both in organic and inorganic forms accounting for about 0.05% of soil content on average; however, only 0.1% of the total P is available to plants (Zou et al. 1992), of which organic forms, as found in humus and other organic materials including decayed plant, animal, and microbial tissues, are an important reservoir of immobilized P accounting for about 20–80% of total soil P (Richardson 1994). Phosphorus in labile organic compounds can be enzymatically mineralized by PSB like *Pseudomonas*, *Enterobacter*, and *Pantoea* (Jorquera et al. 2009) as available inorganic P or it can be immobilized as part of the soil organic matter (Mckenzie and Roberts 1990). The process of mineralization or immobilization (Fig. 11.1) is carried out by microorganisms and is highly influenced by soil moisture and temperature. Mineralization and immobilization are most rapid in warm, well-drained soils (Busman et al. 2002). On the other hand, P release from insoluble P reported for several microorganisms that are isolated from conventional soils or stressed environment (Kundu et al. 2009; Vyas et al. 2009; Wu et al. 2009; Chang and Yang 2009; Sharan et al. 2008; Wani et al. 2007b) besides other factors (Fig. 11.2) has been attributed

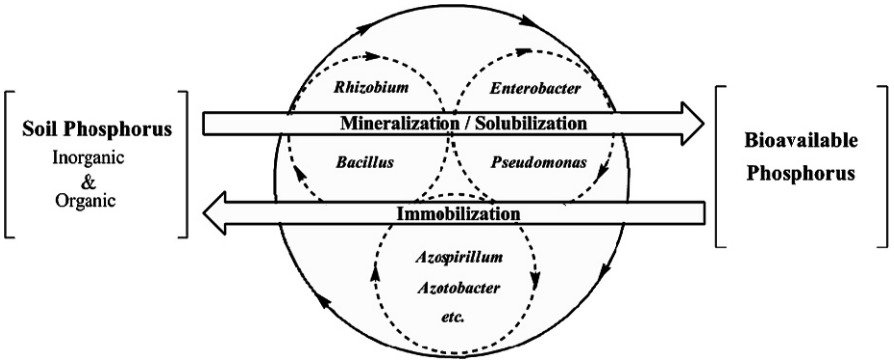

Fig. 11.1 Schematic diagram of soil phosphorus mobilization and immobilization by bacteria (adapted from Khan et al., 2009a)

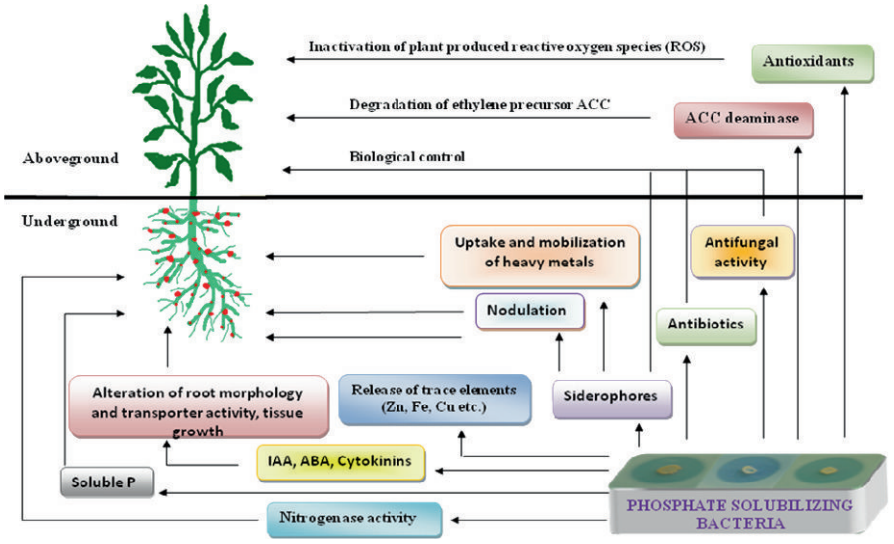

Fig. 11.2 Mechanism of PSB mediated growth promotion of legume plants

mainly to the production of organic acids (Table 11.1) secreted by PSB (Vyas and Gulati 2009; Intorne et al. 2009; Farhat et al. 2009; Xiao et al. 2009; Yi et al. 2008; Maliha et al. 2004) and their chelation capacity (Chen et al 2006; Delvasto et al. 2006; Kim et al 1998). Direct periplasmic oxidation of glucose to gluconic acid is considered as the metabolic basis of inorganic P solubilization by many Gram-negative bacteria as a competitive strategy to transform the readily available C sources into less readily utilizable products by other microorganisms (Goldstein 1995).

Table 11.1 Organic acids involved in P solubilization and produced by phosphate-solubilizing bacteria

Phosphate-solubilizing bacterial communities	Organic acids	References
Stenotrophomonas maltophilia YC	Gluconic acid	Xiao et al. (2009)
Serratia marcescens strain CTM 50650	Gluconic acid	Farhat et al. (2009)
Gluconacetobacter diazotrophicus	Gluconic	Intorne et al. (2009)
Pantoea agglomerans strain MMB051	Gluconic acid	Sulbarán et al (2009)
Pseudomonas fluorescens, P. poae, P. trivialis, and *Pseudomonas* spp.	Gluconic acid, oxalic acid, 2-ketogluconic acid, lactic acid, succinic acid, formic acid, citric acid, and malic acid	Vyas and Gulati (2009)
Pseudomonas corrugate (NRRL B-30409)	Gluconic acid 2-ketogluconic acid	Trivedi and Sa (2008)
Pseudomonas striata	Gluconic acid, tartaric acid, citric acid, maleic acid, succinic acid, glyoxalic acid	Vikram et al. (2007)
Burkholderia, *Serratia*, *Ralstonia,* and *Pantoea*	Gluconic acid	Pérez et al. (2007)
Bacillus, Rhodococcus, *Arthrobacter*, *Serratia*, *Chryseobacterium*, *Delftia*, *Gordonia, Phyllobacterium*, *Arthrobacter ureafaciens*, *Phyllobacterium myrsinacearum*, *Rhodococcus erythropolis*, *Delftia* sp.	Citric acid, gluconic acid, lactic acid, succinic acid, propionic acid	Chen et al. (2006)
Enterobacter intermedium	2-ketogluconic	Hoon et al. (2003)
Bacillus amyloliquefaciens, *B. licheniformis*, *B. atrophaeus*, *Penibacillus macerans*, *Vibrio proteolyticus*, *xanthobacter agilis*, *Enterobacter aerogenes*, *E. taylorae*, *E. asburiae*, *Kluyvera cryocrescens*, *Pseudomonas aerogenes*, *Chryseomonas luteola*	Lactic acid, itaconic acid, isovaleric acid, isobutyric acid, acetic acid	Vazquez et al. (2000)

Improving soil fertility by releasing bound P by microbial inoculants is an important aspect for achieving optimum crop yields. In this regard, the beneficial effects of the inoculation with PSB, used either alone (Poonguzhali et al. 2008, Chen et al. 2008) or in combination with other rhizospheric microbes on various plants including legumes have been reported (Mishra et al. 2009; Vikram and Hamzehzarghani 2008; Wani et al. 2007c; Zaidi and Khan 2006). Phosphate-solubilizing microbes besides enriching soil P pool and consequently providing it to the plants, augment the growth of plants by other mechanisms (Fig. 11.3) like stimulating the efficiency of BNF, enhancing the availability of other trace elements (such as iron, zinc), and by synthesizing important plant growth-promoting

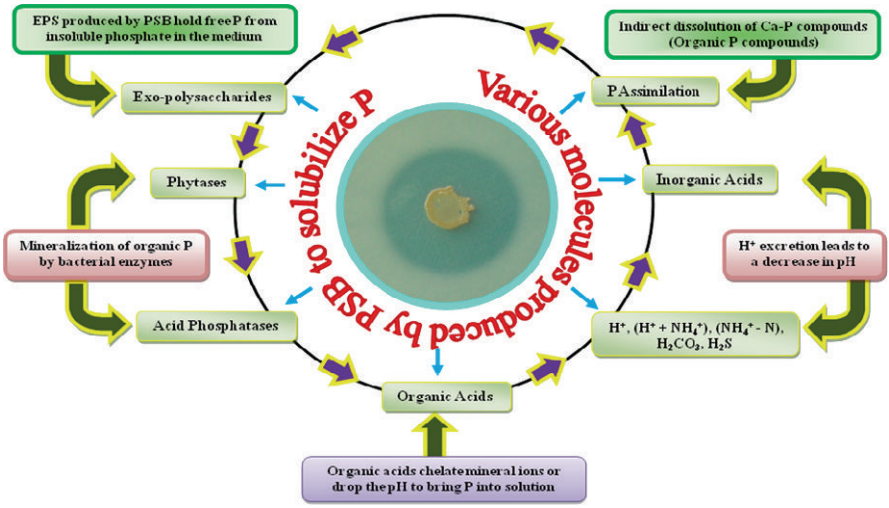

Fig. 11.3 Various organic and inorganic substances produced by PSB responsible for phosphate solubilization in soils

substances (Taurian et al. 2009; Mittal et al. 2008; Wani et al. 2008a) including siderophores (Mahalakshmi and Reetha 2009; Tank and Saraf 2009; Wani et al. 2008b; Jiang et al. 2008; Vassilev et al. 2006), antibiotics (Lipping et al. 2008; Dilantha et al. 2006), and providing protection to plants against soil borne pathogens (biocontrol) (El-Mehalawy, 2009; Singh et al. 2008; Khan et al. 2002). Moreover, application of these bioproducts in bioremediation of disturbed soils is another interesting strategy of PSB that could help to grow plants in derelict soils (Vassileva et al. 2010; Ahemad and Khan 2009). Various plant growth regulators released by PSB are briefly summarized in Table 11.2.

11.3 Phosphate-Solubilizing Bacteria and Legume Improvement

Inoculation of PSB when used either alone or as mixtures in soils, has been shown to improve the overall performance of crop plants including legumes around the world (Wani et al. 2007a; Shaharoona et al. 2008) and hence, PSB could be developed as inoculants/biofertilizer for raising the productivity of agronomic crops in different agro-ecological niches. An attempt is made in the following section to evaluate the impact of PSB, when used either singly or in synergism with other PGPR, on the performance of legumes grown in different agro-ecosystems.

Table 11.2 Growth promoting substances released by phosphate-solubilizing bacteria

Phosphate-solubilizing bacteria	Plant growth-promoting traits	References
Enterobacter aerogenes sp. (NII-0907 and NII-0929), *E. cloacae* subsp. *cloacae* sp. (NII-0931) , *E. asburiae* sp. (NII-0934)	IAA, HCN	Deepa et al (2010)
Pseudomonas aeruginosa PS1	IAA, siderophore, HCN, ammonia	Ahemad and Khan (2009)
Pantoea spp.	Siderophores, IAA, biocontrol (antibiosis)	Taurian et al. (2009)
Pseudomonas sp.	IAA, siderophores, HCN, and biocontrol potential	Tank and Saraf (2009)
Pseudomonas aeruginosa PS1	IAA, siderophores, HCN, EPS	Ahemad and Khan (2009)
Acinetobacter rhizosphaerae	IAA, siderophores, ACC deaminase	Gulati et al. (2009)
Pseudomonas sp.	ACC deaminase, IAA, siderophore	Poonguzhali et al. (2008)
Bacillus subtilis	IAA, siderophore, antifungal activity	Singh et al. (2008)
Serratia marcescens	IAA, siderophore, HCN	Selvakumar et al. (2008)
Pseudomonas fluorescens	ACC deaminase	Shaharoona et al. (2008)
Acinetobacter sp., *Pseudomonas* sp.	ACC deaminase, IAA, antifungal activity, N_2- fixation	Indiragandhi et al. (2008)
Enterobacter sp.	ACC deaminase, IAA, siderophore	Kumar et al. (2008)
Burkholderia	ACC deaminase, IAA, siderophore, heavy metal solubilization	Jiang et al. (2008)
Pseudomonas jessenii	ACC deaminase, IAA, siderophore, heavy metal solubilization	Rajkumar and Freitas (2008)
Pseudomonas aeruginosa	ACC deaminase, IAA, siderophore	Ganesan (2008)
Pseudomonas sp.	ACC deaminase, IAA, siderophore, heavy metal solubilization	Rajkumar and Freitas (2008)
Azotobacter sp., *Mesorhizobium* sp., *Pseudomonas* sp., *Bacillus* sp.	IAA, siderophore, antifungal activity, ammonia production, HCN	Ahmad et al. (2008)
Fluorescent Pseudomonas	IAA, siderophores, HCN, antifungal activity	Shweta et al. (2008)
Pseudomonas vancouverensis.	IAA, HCN, siderophore, antifungal activity	Mishra et al. (2008)
Bacillus spp.	IAA, siderophores, ammonia production, HCN, chromium reduction, metal solubilization	Wani et al. (2007a, b)
Pseudomonas PSB5, *Bacillus* PSB9	IAA and siderophores	Wani et al. (2007c)
Klebsiella oxytoca	IAA, nitrogenase activity	Jha and Kumar (2007)
Bacillus subtilis	IAA	Zaidi et al. (2006)
Pseudomonas sp., *Bacillus* sp.	IAA, siderophore	Rajkumar et al. (2006)
Pseudomonas putida	Antifungal activity, siderophore, HCN	Pandey et al. (2006)

(*continued*)

Table 11.2 (continued)

Phosphate-solubilizing bacteria	Plant growth-promoting traits	References
Pseudomonas fluorescens PRS_9, *Pseudomonas fluorescens* GRS_1	IAA, siderophores	Gupta et al. (2005)
Pseudomonas fluorescens	IAA, siderophores, antifungal activity, ACC deaminase	Dey et al. (2004)
Azospirillum brasilense, *Azospirillum amazonense*	IAA, nitrogenase activity, antibiotic resistance	Thakuria et al. (2004)
Rhizobium strain PS1, *Pseudomonas* strain PS2, *Proteous* strain PS3	IAA, siderophores, phorate degrader, antifungal activity	Bano and musarrat (2003)

11.3.1 Effect of PSB as Monoinoculant on Legumes

Problems associated with intensive farming practices and spiraling costs of N and P fertilizers have renewed interest in development and application of biofertilizers to offset both costs and environmental risks. Accordingly, the inoculation of a good solubilizer of Fe and P, *Sinorhizobium meliloti 3DOh13*, used for alfalfa (*Medicago sativa*); *Bradyrhizobium japonicum TIIIB* used for soybean (*Glycine max*) and two PSB; and *Pseudomonas putida* (*SP21* and *SP22*) used for both alfalfa and soybean have shown a significant effect on these crops (Rosas et al. 2006). Similarly, the soybean plants inoculated with *Burkholderia* sp. (PER2F) had the highest aerial height and an appropriate N/P ratio but inoculation with *Enterobacter* sp., and *Bradyrhizobium* sp. did not increase P uptake by plants (Fernández et al. 2007). They suggested from this finding that PSB inoculation does not necessarily improve P nutrition in soybean, nor was there any relationship between P availability in the soil plate assay and P content in the shoot of soybean raised in greenhouse. Moreover, P-solubilizing fluorescent pseudomonads (PS1 and PS2) isolated from the rhizosphere of groundnut (*Arachis hypogaea*) when used as inoculants for groundnut, enhanced germination up to 15 and 30% with subsequent increase in grain yield by 66 and 77%, respectively. Conversely, when the pathogen (*M. phaseolina)* alone was tested, a 57% decrease in yield was recorded. This study thus revealed the potential of the two pseudomonads that acted not only as biocontrol agents against *M. phaseolina* but also as a good growth promoter for groundnut (Shweta et al. 2008). In other study, Dey et al. (2004) assessed the growth-promoting potentials of most promising P-solubilizing bacterium *Pseudomonas fluorescens* PGPR1 isolated from the peanut rhizosphere, using peanut as test crops grown for 3 years both in pots and field trials. The seed inoculation with isolate PGPR1 resulted in a significantly higher pod yields than control in pots, during rainy and postrainy seasons. The contents of N and P in soil, shoot, and kernel were also enhanced significantly in treatments inoculated with this P-solubilizing isolate in

pots during both seasons. In the field trials, isolate PGPR1 enhanced pod yields by 23–26%, haulm yield and nodule dry weight over control. Other attributes like root length, pod number, 100-kernel mass, shelling out-turn, and nodule numbers were also enhanced. In addition, the seed bacterization with *P. fluorescens* PGPR1 suppressed the soil-borne fungal diseases like collar rot of peanut caused by *A. niger*. The growth-promoting activity of this isolate was attributed to the synthesis of IAA, ACC-deaminase and siderophore, and anti-fungal activity against *A. niger* and *A. flavus* besides its P-solubilizing activity under in vitro conditions. In a follow up study, Rathi et al. (2008) evaluated the P-solubilizing bacterial isolates (4GRP, 25MRP, 27MRP, 28MRP, 33MRP, and 34MRP) recovered from the rhizosphere of greengram (*Vigna radiata*) and mustard (*Brassica compestris*) on greengram and mustard in a pot experiment. Under pot house conditions, a maximum increase in plant dry biomass of both crops was recorded with 25MRP with URP followed by 33MRP with URP. In mustard, maximum P uptake was observed for 25MRP with URP (284%) followed by 4GRP with URP (143%) at 60 days after sowing. In greengram, maximum P uptake was observed in 25MRP with URP (224%) followed by 33MRP with URP (182%) at 60 days after sowing. In a similar study, Gulati et al. (2009) reported a significant increase in the growth of pea (*Pisum sativum*), chickpea (*Cicer arietinum*), maize (*Zea mays*), and barley (*Hordeum vulgare*) under both controlled conditions and field testing following P-solubilizing *Acinetobacter rhizosphaerae*. The strain besides solubilizing inorganic and organic P produced auxin, ACC deaminase, ammonia, and siderophore. The rifampicin mutant of this strain effectively colonized the pea rhizosphere without adversely affecting the resident microbial populations.

Furthermore, a P-solubilizing strain of *Mesorhizobium mediterraneum* (PECA21) substantially increased the growth and P content in chickpea plants when grown in soil amended with or without TCP in a growth chamber experiment (Peix et al. 2001). The strain PECA21 could mobilize P efficiently and increased the P contents by 100%. Also, the dry matter accumulation, N, K, Ca, and Mg content were dramatically increased in inoculated plants, grown in soil treated with insoluble P. These results, therefore, suggested that the inoculation of soil with rhizobia should be considered not only for its N_2-fixing potential but also for its ability to solubilize P. Likewise, inoculation of greengram seeds with PS bacteria revealed a highest nodule number, nodule dry weight, shoot dry matter and total dry matter, and P-content and P uptake compared to RP and single super phosphate (SSP) control. However, plant growth-promoting ability of microbial communities varied considerably (Vikram and Hamzehzarghani 2008). Similarly, Gull et al. (2004) reported that chickpea growth, shoot P and N concentrations, nodulation efficiency, and nitrogenase activity were significantly enhanced in the presence of P-solubilizing bacterial strains isolated from rhizosphere, roots, and nodules of chickpea. Phosphate-solubilizing strains, CPS-2, CPS-3, and Ca-18 had the maximum positive effect on shoot length, shoot dry weight, and nodulation of chickpea plants.

11.3.2 Synergistic Effect of Phosphate-Solubilizing Bacteria with Other PGPR/AM-Fungi

PSB while inhabiting the rhizosphere can form an intimate relationship with other PGPR and could improve additively or synergistically the overall performance of legumes in different soils. For example, in a study, Guiñazú et al. (2010) evaluated the effect of single or mixed inoculation of symbiotic rhizobium *S. meliloti* B399 and P-solubilizing bacterium *Bacillus* sp. M7c upon nodulation and BNF of alfalfa plant. A beneficial effect of both isolates on alfalfa growth was observed in coinoculation assays. *Pseudomonas* sp. FM7d caused a significant increase in dry matters of plant organs (root and shoot), length and surface area of roots, number, and symbiotic properties of alfalfa plants. The plants coinoculated with *S. meliloti* B399 and *Bacillus* sp. M7c showed significant increase in the measured parameters suggesting that strains *Pseudomonas* sp. FM7d and *Bacillus* sp. M7c could be considered for developing composite inoculants. In a similar study, presowing inoculation of mungbean seeds with different inoculants *(Rhizobium*, PGPR, and PSB) alone or in combination, significantly increased the nodulation and grain yield over uninoculated control. Nodulation and grain yield was highest when seeds were inoculated together with *Rhizobium*, PGPR, and PSB followed by *Rhizobium* coinoculated with PGPR and *Rhizobium* alone, in all the three *kharif* seasons. The pooled analysis also gave significantly highest number of nodules/plant (21/plant), dry weight of nodules/plant (87.66 mg) and grain yield (12.94 q/ha) following combined inoculation of *Rhizobium*, PGPR, and PSB. The increase in yield (12.14 q/ha) was at par with *Rhizobium* used with PGPR (Bansal 2009).

In field experiments conducted during the winter seasons of 2005–2006 and 2006–2007 at Sekhampur, West Bengal, India, Dhananjoy and Bandyopadhyay (2009) evaluated the performance of chickpea (cv. Mahamaya-2) with variable rates of P (0, 13.1, 26.2, and 39.3 kg/ha) and bio-fertilizers (no seed inoculation, phosphobacterin *[Pseudomonas striata]* and coinoculation of *Rhizobium* with phosphobacterin) in entisol under rainfed conditions. P and biofertilizers application influenced significantly the growth attributes, nodulation, leghaemoglobin content, nitrogenase activity, yield components, seed yields, harvest index, and P uptake of chickpea. The highest seed yield (1,085 kg/ha) was obtained with 39.3 kg P/ha, producing 40.7, 27.4, and 4% increase over control (without P), 13.1 kg and 26.2 kg P/ha, respectively. Seed inoculation with *Rhizobium* and phosphobacterin was significantly superior over uninoculated or inoculated solely with phosphobacterin. Combined application of P (26.2 kg/ha) with mixture of *Rhizobium* and phosphobacterin further enhanced the biological and chemical properties of chickpeas compared to other levels of P used with biofertilizer. A similar study was conducted to investigate seed inoculation of chickpea with *Rhizobium*, N_2-fixing *Bacillus subtilis* (OSU-142), and P-solubilizing *B. megaterium* (M-3) in controlled environment and in field conditions in 2003 and 2004 in Erzurum (29° 55′ N and 41° 16′ E with an altitude of 1,950 m), Turkey. In the controlled environment and in the field trials, single, dual, and triple inoculations with *Rhizobium*, OSU-142, and

M-3 significantly increased plant height, shoot, root, and nodule dry weight, N%, chlorophyll content, pod number, seed yield, total biomass yield, and seed protein content compared with the control treatment, equal to or higher than N, P, and NP treatments. In the field environment, all the combined treatments containing *Rhizobium* were better for nodulation than the use of *Rhizobium* alone. However, nodulation by native soil *Rhizobium* population was increased in single and dual inoculations of OSU-142 and M-3. Increase in the seed yield under different inoculation treatments ranged between 18% (*Rhizobium*) and 31% (*Rhizobium* with OSU-142 and M-3) over the control, whereas N, P, and NP applications corresponded to increases of 27%, 11%, and 33%, respectively. In general, the increase in seed and total biomass yields were more pronounced in dual and triple inoculations. In conclusion, seed inoculation with *Rhizobium*, *B. subtilis*, and *B. megaterium*, especially dual and triple combinations, may substitute costly NP fertilizers in chickpea production even in cold highland areas such as in Erzurum (Elkoca et al. 2008). Furthermore, Toro et al. (2008) in a trial assessed the interactive effects of multiple microbial inoculations and rock phosphate (RP) on N and P acquisition by alfalfa plants using ^{15}N and ^{32}P isotopes. The microbial inocula included a wild type (WT) *R. meliloti* strain, its genetically modified (GM) derivative, which had an enhanced competitiveness, the arbuscular mycorrhizal (AM) fungus *Glomus mosseae* (Nicol. and Gerd) Gerd and Trappe, and a P-solubilizing bacterium (*Enterobacter* sp.). The Inoculated organisms established well inside root tissues and/or in the alfalfa rhizosphere. Of these, GM *Rhizobium* strain did not interfere with AM colonization. Even though, the inoculated P-solubilizing bacterium established in the alfalfa rhizosphere, but the level of establishment was lower where the natural population of P-solubilizing bacterium was stimulated by AM inoculation and RP application. The stimulation of these indigenous bacteria was also greater in the rhizosphere of alfalfa nodulated by the GM *Rhizobium*. Improvements in N and P accumulation in alfalfa corroborate beneficial effects of the improved GM *Rhizobium* on AM performance, in RP-amended plants. Inoculation with *Enterobacter,* however, did not improve the AM effect on N or P accumulation in the RP-added soil, but it did in the non-RP-amended controls. In addition, ^{15}N:^{14}N ratio in plant shoots indicated enhanced N_2 fixation rates in *Rhizobium*-inoculated AM-plants, compared to those obtained by the same *Rhizobium* strain in nonmycorrhizal plants. Regardless of the *Rhizobium* strain and of whether or not RP was added, AM-inoculated plants showed a lower specific activity (^{32}P:^{31}P) than did their comparable nonmycorrhizal controls, suggesting that the plant was using otherwise unavailable P sources. The P-solubilizing, AM-associated, microbiota could in fact release P ions, either from the added RP or from the indigenous "less-available" P. Additionally, the proportion of plant P derived either from the labeled soil P (labile P pool) or from RP was similar for AM inoculated and nonmycorrhizal controls (without *Enterobacter* inoculation) for each *Rhizobium* strain, but the total P uptake, regardless of the P source, was far higher in AM-plants, which could probably be due to P activity of *Enterobacter*. Furthermore, during the course of a project carried out in two regions of Spain, Castilla y León and Andalucía, while searching suitable microbial inoculants (biofertilizers) for staple grain-legumes,

identified an efficient rhizobia nodulating chickpea (termed as C-2/2) and a potential P-solubilizing bacterial strain (termed as PS06) (Valverde et al. 2006). The 16S rDNA sequence analysis established them as the *M. ciceri* and *Pseudomonas jessenii*, respectively. Subsequently, the effects of sole and composite inoculations of phylogenetically different organisms were tested under both greenhouse and field experiments using chickpea (ecotype ILC-482) as a test legume. The sole application of *M. ciceri* C-2/2 under greenhouse conditions had the highest accumulation of dry matters in aerial organ while the single inoculation of *P. jessenii* PS06 increased the dry matter production by 14% in inoculated chickpea relative to the uninoculated control plants; however, the increase in dry matter production by P-solubilizing bacterium did not correlate with P contents of shoot. The mixture of the two organisms (C-2/2 with PS06) on the contrary resulted in a decrease in shoot dry weight while comparing the effect of single inoculation of C-2/2. In comparison, the plants inoculated with *M. ciceri* C-2/2 either alone or in combination and grown under field conditions had higher symbiotic activity (nodule fresh weight and nodule numbers) and shoot N content compared to other treatments. Inoculation with *P. jessenii* PS06 however, did not have any significant effect on plant growth. The co-culture treatment showed the highest positive effect on nodulation and synergistically increased the seed yield by 52% compared to the uninoculated chickpea plants. These data, thus, suggest that *P. jessenii* PS06 since acted synergistically with *M. ciceri* C-2/2 and hence, could be of great practical importance in raising the productivity of chickpeas. Similarly, when *M. ciceri* and P-solubilizing *Pseudomonas* or *Bacillus* were used together in sandy clay loam soils, increased nodulation, dry biomass of the plants, grain yield, and P and N uptake by chickpea plants (Wani et al. 2007a, b).

The beneficial microbes involved in P solubilization as well as better scavenging of soluble P can also enhance plant growth by improving the efficiency of BNF, accelerating the availability of other trace elements and by production of phytohormones in addition to providing P to plants (Wani et al. 2007a). Accordingly, increase in yield of various legumes has been observed following seed or soil inoculation with N_2-fixing organisms and PSB (Wani et al. 2007b) or PSB when used with nodule bacteria and/AM-fungus (Khan and Zaidi 2007; Zaidi and Khan 2006; Zaidi et al. 2003). Based on the various trials on the coinoculation effects of P-solubilizing and N_2-fixing bacteria, it has been suggested that about 50% of P fertilizer requirement could be saved by the combined inoculation of N_2^- fixer (e.g., *Rhizobium)* with P-solubilizers (e.g., *Bacillus*) in legumes as reported for groundnut (Natarajan and Subrammaian, 1995). Similarly, a field experiment with greengram was conducted by Chesti and Ali (2007) for two consecutive kharif seasons during 2004 and 2005 to study the effect of graded doses of P along with organic and P-solubilizing bacteria on yield of greengram, availability of nutrients, and transformation of P. The results revealed that the grain yield was significantly increased with P application. Highest grain yield was recorded with the inoculation of P-solubilizing bacteria in combination with P (at 30 kg P_2O_5 ha^{-1}). There was a buildup of available N, P, and K in soil in integrated nutrient management. The amount of P recovered in Fe–P, Al–P, and Ca–P form increased significantly with

the application of inorganic fertilizers and their combined use with organic and biofertilizers.

The other major group of microbial plant mutualistic symbionts is the mycorrhizal fungi that forms a functional symbiosis with the roots of most plant species. Mycorrhizal symbioses can be found in almost all ecosystems worldwide to improve plant fitness and soil quality by increasing the plant uptake of P and N by absorbing phosphate, ammonium, and nitrate from soil and also assists plant host in uptake of the relatively immobile trace elements such as zinc, copper, and iron. In addition, mycorrhizal symbiosis improves plant health through increased protection against biotic and abiotic stresses and soil structure through aggregate formation (Garg and Chande 2010; Lingua et al. 2008; Turnau et al. 2006; Barea et al. 2005a, b; Jeffries et al. 2003). Thus, when mycorrhizal fungi are used together with P-solubilizing bacteria, increase in overall performance of legumes is obvious (Zaidi et al. 2003). For example, Mehdi et al. (2006) in a study evaluated the responses of lentil (*Lens culinaris* cv. "Ziba") to coinoculation with AM-fungi and some indigenous rhizobial strains varying in P-solubilizing ability in a calcareous soil with high pH and low amounts of available P and N. The results showed that the effects of AM-fungi (*Glomus mosseae* and *G. intraradices*), rhizobial strains *(R. leguminosarum* bv. viciae and a mixed rhizobial inoculant with an effective P-solubilizing activity *M. ciceri)*, and P fertilizers (superphosphate and phosphate rock) were highly significant for all the characteristics like the dry matter of shoots, plus seeds, their P and N contents, and percent of root colonized by AM fungus. The rhizobial strain with P-solubilizing ability showed a more beneficial effect on plant growth and nutrient uptake than the strain without this ability, although both strains had similar effectiveness for N_2-fixation in symbiosis with lentil. Synergistic relationships were observed between AM-fungi and some rhizobial strains that related to the compatible pairing of these two microsymbionts. The P-uptake efficiency was increased when P fertilizers were applied along with AM-fungi and/or P-solubilizing rhizobial strains. Likewise, Zaidi and Khan (2006) and Zaidi et al. (2004) while evaluating the single or combined effects of N_2-fixing [(*Bradyrhizobium* sp. (vigna)], P-solubilizing bacteria (*Bacillus subtilis*/*P. striata*), P-solubilizing fungus (*Aspergillus awamori and Penicillium variable*), and AM-fungus (*G. fasciculatum*) on the biological and chemical properties of greengram plants grown in P-deficient soils observed that the triple inoculation of AM-fungus, *Bradyrhizobium* sp. (vigna), and *B. subtilis*/*P. striata* significantly increased dry matter yield, chlorophyll content in foliage, and N and P uptake of plants, which in turn resulted in substantial increase in seed yield (24%) relative to the uninoculated plants. Moreover, the symbiotic properties (nodule occupancy) of inoculated plants as determined by indirect enzyme-linked immunosorbent assay (ELISA) increased by 77% (*Bradyrhizobium* with *A. awamori*) and 96% (*Bradyrhizobium* used with *G. fasciculatum* and *B. subtilis*) at flowering, which decreased considerably at the pod-fill stage. However, a negative effect occurred on all the considered parameters when *P. variable* was added to the combination of *Bradyrhizobium* sp. (vigna) and *G. fasciculatum*. In addition, the available P status of the soil improved by the addition of *P. striata* with *Bradyrhizobium* sp. (vigna) and

AM-fungus. The N content of the soil, however, did not show appreciable changes after the inoculation. The population of PSM in some treatments, percentage root infection, and spore density of the AM fungus in the soil increased between 35 and 50 days of plant growth. The present findings showed that rhizospheric microorganisms can interact positively and promote plant growth synergistically leading to improved grain yield and quality (for detail see Chap. 17).

11.4 Conclusion

Nitrogen and phosphorus are the two essential nutrients for plant growth and development. The extensive use of chemical fertilizers to provide these nutrients in agriculture is currently under debate due to environmental concern and questions are raised regarding the consumers health. Recent advancements in the field of biofertilizers offer an opportunity to environmental friendly sustainable agricultural practices to reduce dependence on chemical fertilizers and thereby decrease adverse environmental effects. PSB in association with nitrogen fixers and arbuscular mycorrhizal fungi can lead to increase in legume growth through a range of mechanisms, which could be of great practical value in sustainable, low-input agricultural cropping systems that rely on biological processes to maintain soil fertility and plant health. Although there are numerous reports highlighting interactions among phosphate-solubilizers, nitrogen fixers, and mycorrhizal fungi, the underlying mechanisms behind these associations are in general not conclusive. Moreover, the development of effective microbial inoculants for raising the productivity of legumes remains a major scientific challenge. And hence, functional properties of interacting microbes together with the development of suitable microbial pairing still require further experimental confirmation in order to achieve optimum benefits of such natural resources. Future research should, therefore, strive hard toward an improved understanding of the functional mechanisms behind such microbial interactions, so that compatible organisms could be identified and applied as effective inoculants within sustainable legume production systems.

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Chapter 12
Legume Responses to Arbuscular Mycorrhizal Fungi Inoculation in Sustainable Agriculture

Diriba Muleta

Abstract Currently, the sustainability of ecosystems is in danger due to both application of varied degrading agents and intensive exploitation of tropical forests. During the last decades, inventories of the soil's productive capacity indicate severe degradation and loss of arable lands. The situation is highly exacerbated in economically disadvantaged countries. The ever increasing human populations prompt extensive usage of agrochemicals to attain optimum yields. The use of such chemicals leads to losses in soil fertility, and hence, requires an alternative to boost crop productivity while sustaining ecological quality. Globally, there is widespread interest in the use of legumes due to their multifaceted functions. It is a well established fact that legumes are essential components in natural and managed terrestrial ecosystems. The arbuscular mycorrhizal fungi (AMF) are universal and ubiquitous rhizosphere microflora forging symbiosis with plethora of plant species and acting as biofertilizers, bioprotectants, and biodegraders. The arbuscular mycorrhizal-legume symbiosis is suggested to be the ideal solution to the improvement of soil fertility and the rehabilitation of arid lands. The voluminous literature has revealed that AMF improve the overall growth of leguminous plants growing under diverse agroecological zones. Furthermore, the tripartite symbiosis between legume–mycorrhizal–rhizobium has shown superior improvements in legumes. In this chapter, attention is paid to association of AMF with leguminous plants and effect of composite inoculation of legume plants with mycorrhizal fungi and rhizobia under different growth conditions. Furthermore, mycorrhizal dependency of legumes, effects of arbuscular mycorrhizal fungi on productivity of legumes with special emphasis on alleviation of environmental stresses, and rehabilitation of desertified and/or degraded habitats is described.

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12.1 Introduction

The intense exploitation of tropical forests has led to the degradation of once stable ecosystems. The depletion of cultivated soils in some countries and nutrient seepage from cultivated lands in others, coupled with the ever increasing demand for foods, fibers, and fuel, makes it pertinent to improve the nutrient use efficiency of crops. There have been changes in abiotic and biotic soil properties, which hamper the re-establishment of proper vegetation cover (Miller 1987). Increased pressure for food production in turn has led to the development of intensive agricultural systems that involves the use of significant quantities of agrochemicals (Hooker and Black 1995). There is now substantial evidence of the environmental costs of this high-input strategy, which has forced agricultural systems to be modified in order to make them more sustainable (van der Vossen 2005). Recognition of the intertwined existence of plants and symbiotic fungi opens up the new possibility of a more sustainable agricultural production system.

Legumes form symbioses that are not formed by most other plants, including *Arabidopsis*. Legume roots are invaded and colonized by rhizobia (Spaink 1996) and also with mycorrhizal fungi (Harrison 1999). Legumes have been grown for the food, feed, forage, fiber, industrial and medicinal compounds, flowers, and other end uses. Leguminous plants are also highly suitable for agroforestry system, the area that receives due attention for sustainable agriculture. Agroforestry, a land-use system and technology in which trees are deliberately planted on the same unit of land with agricultural crop and/or animals, has been recognized as one of the most promising strategy for rehabilitating the already degraded areas. Agroforestry has certain advantages, such as being used to ameliorate chemical and physical properties of soils, reduce soil erosion, improve weed control, and increase availability of fuel wood and/or fodder (Young 1997). In addition, agroforestry practices may also be important in maintaining the mycorrhizal inoculum potential in soils (Cardoso et al. 2003; Muleta et al. 2008). However, nutrient-acquisition symbioses between plants and soil microbes are important to plant evolution and ecosystem function (Simms and Taylor 2002). Beneficial plant–microbe interactions in the rhizosphere are primary determinants of plant health and soil fertility (Jeffries et al. 2003). Arbuscular mycorrhizas, which forms symbioses with majority of plants, influence plant community development, nutrient uptake, water relations, and above-ground productivity (Gosling et al. 2006; van der Heijden et al. 2008). Arbuscular mycorrhizas also act as bioprotectants against pathogens and toxic stresses (van der Heijden et al. 2008). However, in order to maximize their benefits, it is essential to ensure that management practices include minimum tillage, reduced use of inappropriate fertilizer, adopt appropriate crop rotations with minimal fallow, and rationalized pesticide use. As AMF evolved long before legumes, it has been assumed that all legumes have the potential to form symbiosis with AMF (*Lupinus* is the only known legume genus in which this ability is absent; Oba 2001; Sprent and James 2007). For legumes, AM-fungi have fundamental effects on the ecophysiology, on the biota of the surrounding soil and on associated non-legumes

(Bethlenfalvay and Newton 1991). For example, Barea and Azcon-Aguilar (1983) have demonstrated that AMF are known to be one of the most efficient ecological factors in improving growth and N content in legumes.

Arbuscular mycorrhizal fungi and rhizobia play a key role in natural ecosystems and influence plant productivity, plant nutrition, and plant resistance (Demir and Akkopru 2007). The majority of legumes form symbiotic associations with both phosphorus-acquiring AMF and nitrogen (N)-fixing rhizobia (Sprent 2001; Lodwig et al. 2003). The N_2 fixing activity of rhizobia is enhanced in the rhizosphere of mycorrhizal plants, where synergistic interactions of such organisms with AMF have been demonstrated (Barea et al. 2002; van der Heijden et al. 1998, 2006). In pot experiments as well as field trials, coinoculation with both symbionts resulted in higher plant biomass and better N and P acquisition, although these effects were also dependent on the specific symbiont combination (Azcón-Aguilar and Barea 1981; Rao et al. 1986; Azcón et al. 1991; Requena et al. 2001; Xavier and Germida 2002). Similarly, the tripartite symbiosis of legume–mycorrhizal–rhizobium has conclusively shown improvements in overall growth of leguminous plants (Jia et al. 2004; Babajide et al. 2008; Wu et al. 2009). For some plant species, the association with mycorrhizal fungi is indispensable. However, the degree of dependence varies with plant species, particularly the root morphology, and conditions of soil and climate (Plenchette et al. 2005; Ghosh and Verma 2006). Moreover, several studies have indicated the remarkable roles of AMF in amelioration of various types of stresses (abiotic/biotic) in leguminous plants (Vivas et al. 2003; Rabie and Almadini 2005; Khan 2006; Aysan and Demir 2009; Shokri and Maadi 2009). Mycorrhizal legumes are also well known for rehabilitation of badly degraded lands and/or desertified habitats emphasizing the ecological significance of this special association (Zaharan 1999; Requena et al. 2001; Valdenegro et al. 2001; Quatrini et al. 2003).

Under conditions of low N and P availability, which occur in many tropical soils, the AMF mediated transfer of nutrients has been reported from the host plant to another plant. Hyphae of mycorrhizas may spread from one infected plant and enter the roots of one or more other plants (Heap and Newman 1980). It has been shown that assimilates may be transported from one plant to another through AM hyphal connections. In a study, transfer of ^{14}C photosynthate from one plant to another was found primarily through AM hyphae rather than leakage from the roots of the donor plants (Reid and Woods 1969; Brownlee et al. 1983; Francis and Read 1984). Similar results were obtained in a ^{32}P experiment, where hyphal linkage between plants was the dominant factor for transferring P (Chiariello et al. 1982). More specifically, different experimental results have verified the transfer of fixed N from legume mycorrhizal plants to nearby/adjacent non-leguminous plants via active hyphal connections (Vankessel et al. 1985; Hamel et al. 1991; Snoeck et al. 2000; Rogers et al. 2001; Li et al. 2009). The AMF, however, differs in their capacity to supply plant nutrients such as P (van der Heijden et al. 2003; Ghosh and Verma 2006) suggesting mass production of the suitable strains for sustainable inoculum development. Although the technology for the production of rhizobial and free-living PGPR is commercially available, the production of AM-fungi inocula and the

development of inoculation techniques have limited the manipulation of AM-fungi. An appropriate management of selected AM-fungi is now available for exploiting the benefits of these microorganisms in agriculture, horticulture, and in revegetation of degraded ecosystems (Barea et al. 2005). And large quantities of AMF inoculum can be produced by pot culture technique (Nopamornbodi et al. 1988). The traditional and most widely used approach has been to grow the fungus with suitable host plants in solid growth medium individually or in combination on the solid growth media (Tiwari and Adholeya 2002). However, current biotechnology practices now allow the production of efficient AM-fungal inoculants to mass propagate them for large scale production systems (Gianinazzi and Vosátka 2004).

12.2 Mycorrhizal Association with Legumes

Arbuscular mycorrhizal fungi are known to form close association with an array of members of family leguminosae (Pagano et al. 2007; Valsalakumar et al. 2007; Molla and Solaiman 2009). In this context, Muleta et al. (2007, 2008) have reported more AMF spore counts under *Acacia abyssinica*, *Albizia gummifera,* and *Millettia ferruginea* shade trees than under non-leguminous shade trees in both natural coffee forest and in soils of smallholder agroforestry coffee system in southwestern Ethiopia. Similar observations have also been reported elsewhere under canopies of legume plants (He et al. 2004), and Colozzi and Cardoso (2000) have demonstrated that legume intercropping cultivation increased spores concentration of AMF in the soil. Various types of shade trees in forests (Wubet et al. 2003, 2004), including medicinal and N_2-fixing species, have also been found to be associated with AMF. Valsalakumar et al. (2007) in a field study identified the AM-fungi associated with greengram [*Phaseolus aureus* Roxb. (=*Vigna radiate* var. *radiata*)]. The findings show that *Glomus mosseae*, *G. microcarpum*, *Gigaspora margarita*, and *Scutellospora* sp. colonized the greengram, and *G. mosseae* was the most abundant AM fungal associate (81%) followed by, *G. microcarpum* (24%) and *G. margarita* (24%), and *Scutellospora* sp. (5%). The range of distribution varied from a single species of AM fungus to three species belonging to two genera in one sample. Similarly, Bakarr and Janos (1996) examined the fine roots of 27 tree species for mycorrhizas, which occurred in natural forest, a forestry plantation and a reforestation site in Sierra Leone, West Africa. Twenty-three species had AMF, of which seven were ectomycorrhiza, colonizing six legume species belonging to Caesalpinioideae. Three species of Australian *Acacia* used widely in reforestation in Sierra Leone had AMF.

The effects of AMF, P addition, and their interaction on the growth and P uptake of three facultative mycotrophic legume trees (*Anadenanthera peregrina, Enterolobium contortisiliquum,* and *Plathymenia reticulata*) were investigated (Pagano et al. 2007). Phosphorus fertilization improved growth of all legume tree species. In turn, P enhanced the positive effects of AMF on the test species. Tissue nutrient concentrations showed slight variation among species and

were influenced by both AMF inoculation and P. Plants inoculated with higher doses of KH_2PO_4 showed more vigorous seedlings. Results suggest that in low fertility soils *A. peregrina, E. contortisiliquum,* and *P. reticulata* seedlings should be inoculated with AMF to enhance plant growth. More recently, Molla and Solaiman (2009) have investigated the association of AMF using ten different leguminous crops grown in five agro-ecological zones (AEZs) of Bangladesh. Of these, grasspea, lentil, chickpea, mungbean, cowpea, blackgram, gardenpea, and soybean were highly mycotrophic. A poor mycorrhizal association was found in bushbean and groundnut, while AM colonization and spore population differed among different legumes in different AEZs. However, the AM fungal structures such as hyphae, arbuscules, and vesicles in the root system of the test legume varied. Among AM fungi, *Glomus* was the most common while *Sclerocystis* was the least prevalent genus in the studied rhizosphere soil samples. The application of AMF in soils has shown a tremendous improvement in growth and yields of diverse legumes raised under both greenhouse and field conditions. For instance, inoculation with AMF improved growth of chick-pea (*Cicer arielinum* L.) and doubled P uptake at low and intermediate levels of P in a pot experiment on sterilized low-P calcareous soil (Weber et al. 1992). In another greenhouse experiment, the influence of two tropical isolates of AM-fungi, *G. fasciculatum* and *G. mosseae*, on the nutrient uptake and growth of *Sesbania grandiflora* was determined (Habte and Aziz 1985). Inoculation of sterile soil with the fungi significantly improved growth and nutrient uptake by *S. grandiflora*, but the response of the legume was markedly better when soil was inoculated with *G. fasciculatum* than when it was inoculated with *G. mosseae*. Nutrient uptake and growth of *S. grandiflora* in nonsterile soil was also stimulated by inoculation, but the legume did not respond differently to the two endophytes. In a follow up study, Ndiaye et al. (2009) evaluated the effects of different indigenous AMF on the mobilization of P from Senegalese natural rock phosphate (NRP) for growth of *Gliricidia sepium* and *Sesbania sesban* seedlings. Levels of tested NRP were compatible with high AMF proliferation but changed the pattern of root colonization, which varied in accordance with plant cultivar and fungal species. The NRP and AM inoculation facilitated growth parameters and shoot mineral mass of *G. sepium* and *S. sesban* after 4 months of cultivation. More than 200% of weight gains in *S. sesban* were recorded with all AMF when used with 600 or 800 mg NRP. For *Gliricidia*, only *G. aggregatum* in the presence of high NRP levels showed similar effects. In contrast, *G. fasciculatum* enhanced the height of *Sesbania* by twofolds when grown in the presence of 400, 600, and 800 mg NRP. Generally, the impact of composite application of AMF and NRP on nutritional content was more obvious for *Sesbania* than for *Gliricidia* seedlings. It is interesting that certain legume tribes that cannot form nodules may be colonized by AMF. For example, Cárdenas et al. (2006) investigated early responses to Nod factors and mycorrhizal colonization in a non-nodulating *Phaseolus vulgaris* mutant. The results indicate that even though *P. vulgaris* non-nodulating mutant (NN-mutant) is deficient in early nodulin gene expression, when inoculated with *Rhizobium etli*, it can be effectively colonized by AM-fungus,

G. intraradices. Sometimes Nod-mutants of other legumes fail to establish a mycorrhizal symbiosis (Bradbury et al. 1991) indicating that common elements of the infection process may exist in both associations.

12.3 Composite Inoculation of Legume Plants with Mycorrhizal Fungi and Rhizobia

The majority of legumes have the capacity to engage in a dual symbiotic interaction with N_2-fixing rhizobia and P acquiring AM-fungi (Hazarika et al. 2000; Sprent 2001; Lodwig et al. 2003; Navazio et al. 2007). Arbuscular mycorrhizal fungi and rhizobia together play a key role in natural ecosystems and influence plant productivity, nutrition, resistance, and plant community structure (Cleveland et al. 1999; van der Heijden et al. 2006; Demir and Akkopru 2007). The activities of N_2 fixing bacteria improving the bioavailability of N and P are enhanced in the rhizosphere of mycorrhizal plants following synergistic interactions between the two groups of microorganisms (Barea et al. 2002; Zaidi et al. 2003). The authors further suggested that the inoculation of such phytobeneficial microbes profoundly improved the overall performance of legumes indicating the importance of the tripartite symbiosis between legumes-mycorrhiza and rhizobia in a given ecosystem. Recent studies have demonstrated that the two symbioses share some components of their developmental programs (Harrison 2005; Oldroyd et al. 2005; Navazio et al. 2007). Synergistic effect of dual colonization of roots with AMF and *Rhizobium* on growth, nutrient uptake, and N_2 fixation in many legume plants have been reported (Xavier and Germida 2002; Stancheva et al. 2008) and discussed in the following section.

12.3.1 Dual Inoculation with Mycorrhizal Fungi and Rhizobia Under Greenhouse Conditions

Response of *Leucanea leucocephala* to inoculation with *G. fasciculatum* and/or *Rhizobium* was studied in a P deficient unsterile soil (Manjunath et al. 1984). The findings show that *G. fasciculatum* inoculation alone improved nodulation by native rhizobia and sole application of *Rhizobium* increased colonization of roots by native mycorrhizal fungi. However, when AM-fungi and *Rhizobium* were used together, it improved nodulation, mycorrhizal colonization, dry weight, and N and P contents of the plants further when compared with single inoculation of each organism. In a similar study, Eom et al. (1994) evaluated two wild legume plants, *Glycine soja* and *Cassia mimosoides* var.*nomame*, and a cultivated plant, soybean inoculated with *Scutellospora heterogama*. The AMF colonized wild legume plants showed greater growth compared with soybean, whereas the soybean demonstrated more nodulation than AM colonized *Cassia mimosoides* plants. Babajide et al. (2008) in a

greenhouse experiment determined the effect of different rhizobial and mycorrhizal species (*Glomus clarum)* on growth, nodulation, and biomass yield of soybean grown under low fertile eroded soil condition. Plant growth and biomass yield were significantly enhanced by AM-fungus in both sterile and nonsterile soils when compared with the control. However, combined inoculation of mycorrhiza with any of the rhizobial strains further improved plant growth and biomass production. The effect of composite inoculation of mycorrhiza with *Rhizobium* R25B was more pronounced, which substantially increased the plant height (68.8 cm), stem circumference (2.94 cm), number of leaves (39.0), shoot dry weight (16.1 g), and root dry weight (4.6 g), relative to control values of 33.2, 0.60 cm, 15, 4.4, and 1.6 g, respectively. Nodulation was equally enhanced by mycorrhizal and rhizobial inoculations under sterile and nonsterile soils. The percentage mycorrhizal root colonization ranged from 4 to 42%, and root colonization was highest for mycorrhiza inoculated plants grown in sterile soil. These findings suggested that dual inoculation of mycorrhiza and *Rhizobium* may be beneficial to soybean production in the tropics, where nutrients particularly available P and total N are very low. Ahmad (1995) while assessing the impact of mixed inoculation on three local cultivars (Miss Kelly, Portland Red, Round Red) of red kidney bean *(Phaseolus vulgaris*, L.) with four strains of *Rhizobium phaseoli* (B36, B17, T2, and CIAT652) and three species of AM-fungi (*Glomus pallidum, G. aggregatum*, and *Sclerocystis microcarpa*) in sterilized and nonsterilized soil observed a profound increase in symbiotic efficiency, plant growth, and N and P of kidney bean. The rhizobial strains B36 and B17 co-inoculated with *G. pallidum* or *G. aggregatum* increased the growth of Miss Kelly and Portland Red, while rhizobial strain T2 paired with any of the three AM-fungi was found as the best compatible pairing for the Round Red kidney beans. It was suggested that even though dual inoculation significantly improved the growth of the bean plants, the best performing combination of AM fungus and rhizobia requires further trials so that it is recommended for legume promotion in different geographical regions. Similarly, Jia et al. (2004)) investigated the effects of the interactions between the microbial symbionts (*Rhizobium* and AMF) on N and P accumulation by broad bean (*Vicia faba*), and how increased N and P content influence biomass production, leaf area, and net photosynthetic rate. The AM-fungus was found to promote biomass production and photosynthetic rates by increasing P/N accumulation, and an increase in P was consistently correlated with an increase in N accumulation and N productivity, expressed in terms of biomass and leaf area. Photosynthetic N use efficiency, irrespective of the inorganic source of N (e.g., NO^{3-} or N_2), was enhanced by increased P supply due to AMF colonization. However, the presence of *Rhizobium* significantly declined AMF colonization irrespective of N supply; without *Rhizobium*, AMF colonization was higher in low N treatments. Presence or absence of AMF did not have a significant effect on nodule mass but high N with or without AMF led to a significant decline in nodule biomass. Furthermore, plants with the *Rhizobium* and AMF had higher photosynthetic rates per unit leaf area.

Geneva et al. (2006) reported that the dual inoculation of pea plants with *Glomus mosseae* or *G. intraradices* and *Rhizobium leguminosarum* bv. viceae, strain D 293

significantly increased the plant biomass, photosynthetic rate, nodulation, and N_2 fixing activity in comparison with single inoculation of *R. leguminosarum* bv. *viceae* strain D 293. In addition, the co-inoculation significantly increased the total P content in plant tissues, acid phosphatase activity, and percentage of root colonization. Among all microbial pairing, the mixture of R. *leguminosarum* and *G. mosseae* was most effective at low P level while *G. intraradices* inoculated with *R. leguminosarum* was most effective at higher P level, as also reported for lentil (*Lens culinaris* cv. Laird) (Xavier and Germida 2002). Recently, Wu et al (2009) in a pot experiment determined the single and combined effects of *G. mosseae* and *Rhizobium* on *Medicago sativa* grown on three types of coal mine substrates, namely, a mixture of coal wastes and sands (CS), coal wastes and fly ash (CF), and fly ash (FA). When *Rhizobium* was used alone, it did not result in any growth response but sole application of *G. mosseae* had a significant effect on plant growth. Inoculation of G. *mosseae* also increased the survival rate of *M. sativa* in CS substrate. When *G. mosseae* inoculated *M. sativa* plant was grown with CF and FA substrates, the dry matter accumulation in tested plants was 1.8 and 5.1 times higher than those without inoculation. However, when *M. Sativa* was inoculated by *G. mosseae* and *Rhizobium* together and grown in CS and CF substrates, the N, P, and K uptake and legume growth increased substantially, suggesting a synergistic effect of the two phylogenetically distinct organisms, which could be exploited for revegetation of coal mine substrates.

In other greenhouse trial, Mehdi et al. (2006) reported that the effects of AM fungi (*G. mosseae* and *G. intraradices*), rhizobial (*R. leguminosarum* bv. viciae) strains, and P (superphosphate and phosphate rock) fertilizers considerably increased the dry biomass of shoots and seeds, P and N contents (shoots and seeds) of lentil cv. "Ziba" plants and percent of root colonized by AM fungus. The rhizobial strain possessing P-solubilizing ability showed a more beneficial effect on plant growth and nutrient uptake than the strain without this activity, although both strains had similar N_2-fixing efficiency. Moreover, the P-uptake efficiency was increased when P fertilizers were applied along with AM-fungi and/or P-solubilizing rhizobial strains emphasizing the remarkable importance of dual inoculation in the improvement of plant growth responses as also reported by Zarei et al. (2006) for rhizobium-mycorrhizas inoculated lentil plants. Furthermore, Meghvansi et al. (2008) observed the comparative efficacy of 3 AMF combined with cultivar specific *Bradyrhizobium japonicum* (CSBJ) in soybean under greenhouse conditions. Soybean seeds of four cultivars (JS 335, JS 71-05, NRC 2, and NRC 7) were inoculated with *G. intraradices*, *Acaulospora tuberculata,* and *Gigaspora gigantea* and CSBJ isolates, individually or in combination, and were grown in pots using autoclaved alluvial soil of a non-legume cultivated field of Ajmer (Rajasthan). Their findings indicate that amongst the single inoculations of 3 AMF, *G. intraradices* produced the largest increases in nodulation, plant growth, and seed yield followed by *A. tuberculata* and *G. gigantea* indicating that plant acted selectively on AMF symbiosis. The dual inoculation of AMF with *B. japonicum* CSBJ further improved these parameters demonstrating synergism between the two microsymbionts. Among all the dual treatments, *G. intraradices* along with

B. *japonicum* showed the greatest increase (115%), in seed weight per plant suggesting a strong selective synergistic relationship between AMF and *B. japonicum*. The cv. JS 335 exhibited maximum positive response toward inoculation. The variations in efficacy of different treatments with soybean cultivars, however, indicated the specificity of the inoculants. These results provide a basis for selection of an appropriate combination of specific AMF and *Bradyrhizobium*, which could further be utilized for identifying the symbiotic effectiveness and competitive ability of microsymbionts under field conditions. Likewise, in a pot trial setup to evaluate the response of alfalfa to *G. intraradices* and *Sinorhizobium meliloti* strain 1021, Stancheva et al. (2008) reported that the dual inoculation of alfalfa plants with *G. intraradices* and strain 1021 significantly increased the percent of root colonization and acid phosphatase activities in the root tissue and in soil in comparison with a single inoculation of *G. intraradices*. Co-inoculation also significantly increased the plant biomass, total P and N content in plant tissues. Under conditions of dual inoculation, high nitrogenase activity was established, especially at the floral budding stage compared with the single inoculation of strain 1021.

12.3.2 Performance of Inoculated Legumes in Field Environment

Field investigations were conducted to study the effects of AM inoculation and triple superphosphate fertilization on nodulation, dry matter yield, and tissue N and P contents of *Bradyrhizobium*-inoculated soybean and lablab bean (Mahdi and Atabani 1992). Inoculation of both legumes with any of four AMF enhanced nodulation, dry matter yield, and plant N and P contents more than did triple superphosphate. *Gigaspora margarita* and *G. mosseae* were superior to *Gigaspora calospora* and *Acaulospora* species and resulted in more extensive root infection, especially in soyabean. The integration of N_2 fixing trees into stable agroforestry systems in the tropics is being tested because of their ability to produce higher biomass N and P yields, when symbiotically associated with rhizobia and AMF (Marques et al. 2001; Kayode and Franco 2002). Accordingly, in a field trial, Marques et al. (2001) evaluated the effect of dual inoculation of N_2-fixer (*Rhizobium* spp.) and AMF on the growth of *Centrolobium tomentosum* Guill. ex Benth, a native legume tree of the Brazilian Atlantic Forest. Complete fertilization was compared with inoculation treatments of selected rhizobia strains BHICB-Ab1 or BHICB-Ab3 associated or not to AM fungi. The dual inoculation increased the height and growth of plants treated with rhizobia alone. Plants inoculated with strain BHICB-Ab1 and AMF increased the dry matter by 56% over uninoculated control, and N accumulation was greater than those observed for BHICB-Ab3 inoculated plants. Strain BHICB-Ab1 formed a synergetic relationship with mycorrhizal fungi as the combined inoculation enhanced plant height and dry weight more than single inoculation, while the growth of BHICB-Ab3 plants was not modified by AMF inoculation. Arbuscular mycorrhizal fungi also improved plants

survival and possibly favored the nodule occupation by rhizobial strains when compared with the non-mycorrhizal plants. Similarly, *Acacia mangium* inoculated with rhizobial strains (BR 3609 and BR 3617) and three AM-fungi, *Glomus clarium*, *Gigaspora margarita,* and *Scutellospora heterogama* grew better than seeds planted without rhizobia and AMF inoculants (Kayode and Franco 2002). They observed that *S. heterogama* facilitated the growth better in both fallow and degraded soils. Seeds inoculated with rhizobia strains and AMF, however, produced more nodules and had higher AMF infection rates than seeds inoculated with rhizobia or AMF inoculants alone (Marques et al. 2001; Kayode and Franco 2002). In other study, Singh et al. (1991) evaluated the effect of live yeast cells (*Saccharomyces cerevisiae*) on nodulation and dry biomass of shoot and roots of legumes such as *Leucaena leucocephala, Glycine max, Cajanus cajan, Phaseolus mungo, Phaseolus aureus,* and *Vigna unguiculata* in the presence of both AMF and *Rhizobium* strains. The results indicate that inoculation with live yeast cells remarkably enhanced the measured plant parameters. Root infection (native AMF) and the formation of vesicles, arbuscules, and spores were also increased with yeast inoculation. The increase in the parameters, however, varied with legumes and the type of yeast culture.

Although voluminous literature reports show superiority of plant performances under dual inoculation, sometimes the usual synergism was found to be less effective. For example, Nambiar and Anjaiah (1989), in a field experiment, reported that the effects of AMF on competition among inoculated bradyrhizobia were less evident, but inoculation with *Bradyrhizobium* strains increased root colonization by AMF and certain AMF/*Bradyrhizobium* inoculum strain combinations produced higher nodule numbers. Plants grown without *Bradyrhizobium* and AMF, but supplied with ammonium nitrate (300 g ml^{-1}) and potassium phosphate (16 g ml^{-1}), produced higher dry-matter yields than those inoculated with both symbionts in the pot experiment. Inoculation with either symbiont in the field, however, did not result in higher pod yields at harvest. In a similar trial, Camila and Lazara (2004) tested response to mineral fertilization and inoculation with rhizobia and/or AMF of the *Anadenanthera colubrina*, *Mimosa bimucronata*, and *Parapiptadenia rigida* (Leguminosae-Mimosoideae) native trees from Brazilian riparian forests, in nursery conditions. The findings showed that AMF inoculations did not enhance the mycorrhizal colonization, and P uptake was not sufficient to sustain good growth of plants. The level of P added affected negatively the AMF colonization in *A. colubrina* and *M. bimucronata*, but not in *P. rigida*. The absence of mineral N limited growth of *A. colubrina* and *P. rigida*, but in *M. bimucronata* the deficiency of N was corrected by biological nitrogen fixation(BNF). However, N mineral applied inhibited nodulation, although spontaneous nodulation occurred in *A. colubrina* and *M. bimucronata*. Rhizobia inoculation enhanced the number of nodules, nitrogenase activity, and leghemoglobin content of these two species. Thus, the extent of rhizobial and mycorrhizal symbiosis in these species under nursery conditions affected growth and consequently the post-planting success.

Evidence is also available that improved formation of AM can inhibit nodulation, possibly due to inter-endophyte incompatibility of competition (Behlenfalvay

et al. 1985). On the contrary, Pacovsky et al. (1986) revealed that even though nodule numbers may not significantly be increased by AM colonization, yet the size and N fixing activity may be increased. However, there is a report that suggests that symbiotic N_2 fixation is clearly accelerated in legume following AMF inoculation, but the response of *Rhizobium* symbiosis may vary according to the strains of the AM fungus involved (Linderman and Paulitz 1990). These and other associated data thus indicate that the *Rhizobium*-AMF partnership nearly always exists, but may not necessarily be optimal with the best combination of smybionts for the host legumes.

12.4 Mycorrhizal Dependency of Legumes

For some plant species, the association with mycorrhizal fungi is indispensable. The degree of dependence, however, varies with plant species, particularly the root morphology, and conditions of soil and climate (Hayman 1986). Plants with thick roots poorly branched and with few root hairs are usually more dependent on mycorrhizas for normal growth and development. These species include onions, grapes, citrus, cassava, coffee, and tropical legumes. When the level of soil fertility and humidity are increased, the dependence on the mycorrhizal condition decreases to a point where the plant becomes immune to colonization (Sharma et al. 1996; Khaliel et al. 1999). Furthermore, mycorrhizal dependencies of leguminous plants grown in stressed situations have also been well documented (Sharma et al. 1996; Plenchette et al. 2005; Ghosh and Verma 2006).

Growth and mineral uptake of 24 tropical forage legumes and grasses were compared under glasshouse conditions in a sterile low P oxisol, one part inoculated and the other not inoculated with mycorrhizal fungi (Duponnois et al. 2001). Shoot and root dry weights and total uptake of P, N, K, Ca, and Mg of the entire test plants were significantly increased by mycorrhizal inoculation. On the one hand, mycorrhizal inoculation, with few exceptions, decreased the root/shoot ratio. Non-mycorrhizal plants, conversely, had lower quantities of mineral elements than mycorrhizal plants. However, plant species did not show any correlation between percentage mycorrhizal infection and growth. A great variation in dependence on mycorrhiza was observed among forage species. Total uptake of all elements by non-mycorrhizal legumes and uptake of P, N, and K by non-mycorrhizal grasses correlated inversely with mycorrhizal dependency. Mycorrhizal plants of all species used significantly greater quantities of soil P than the non-mycorrhizal plants, and utilization of soil P by non-mycorrhizal plants was correlated inversely with mycorrhizal dependency. As the production of grain and herbaceous legumes is often limited by low levels of available P in most savanna soils, the potential for managing AMF by selecting lines or accessions dependent on AMF as a strategy to improve plant P nutrition and productivity is required (Plenchette et al. 2005; Ghosh and Verma 2006). Accordingly, Nwoko and Sanginga (1999) evaluated the interactions between AMF and *Bradyrhizobia* sp. and their effects on growth

and mycorrhizal colonization of ten recent selections of promiscuous soybean breeding lines and two herbaceous legumes (*Lablab purpureus* and *Mucuna pruriens*). Mycorrhizal colonization differed among promiscuous soybean lines (ranging from 16 to 33%) and was on average 20% for mucuna and lablab. Three groups of plants were identified according to mycorrhizal dependency (MD): (1) the highly dependent plants with MD >30% (e.g., soybean line 1,039 and mucuna); (2) the intermediate group, with MD between 10 and 30% (e.g., soybean line 1,576 and lablab); and (3) the majority of soybean lines (five lines out of ten) that were not mycorrhizal dependent. This great variability in MD and response to P application among promiscuous soybean and herbaceous legumes offers a potential for the selection of plant germplasm able to grow in P deficient soil. Similar results have also been reported for different species of woody leguminous trees. For instance, Ghosh and Verma (2006) evaluated the effects of three AMF species (*Glomus occultum, G. aggregatum*, and *G. mosseae*) inoculations on growth responses of *Acacia mangium* in lateritic soil. All inoculations significantly enhanced growth with respect to shoot height, root diameter, leaf area, chlorophyll content, and biomass of *A. mangium* compared with uninoculated control seedlings. The mycorrhizal dependency factor indicated that the growth of *A. mangium* was 57% dependent on *G. occultum*, 47% on *G. mosseae*, and 46% on *G. aggregatum*. The findings indicate the presence of disparity among AMF species with regard to their growth enhancement in a particular mycorrhizal legume. It has also been demonstrated that mycorrhizal dependence and responsiveness of legumes declines with an increase in P added to the soil (Khaliel et al. 1999).

12.5 How Arbuscular Mycorrhizal Fungi Enhance Legumes Performance?

The AM-fungi affects the growth and development of plants both directly and indirectly as listed in Table 12.1 (Gosling et al. 2006; van der Heijden et al. 2008). However, broadly, the principal contribution of AMF to plant growth is due to

Table 12.1 Direct and indirect effects of mycorrhizal fungi on crop productivity in organic farming systems

Direct effects	Indirect effects
Stimulation of plant productivity of various crops	Weed suppression
Nutrient acquisition (P, N, Cu, Fe, Zn)	Stimulation of nitrogen fixation by legumes
Enhanced seedling establishment	Stimulation of soil aggregation and soil structure
Drought resistance	Suppression of some soil pathogens
Heavy metal/salt resistance	Stimulation of soil biological activity (phosphate solubilization)
	Increased soil carbon storage
	Reduction of nutrient leaching

Adapted from Gosling et al. (2006) and van der Heijden et al. (2008)

the uptake of nutrients by extraradical mycorrhizal hyphae (Marschner 1998; Hodge and Campbell 2001; van der Heijden et al. 2006). The most prominent effect of AMF is to improve P nutrition of the host plant in soils with low P levels due to the large surface area of their hyphae and their high affinity P uptake mechanisms (Muchovej 2001). To substantiate this concept of plant growth promotion by AMF, several studies have shown that AM fungi contribute to up to 90% of plant P demand (Jakobsen et al. 1992; van der Heijden et al. 2006). For instance, the P depletion zone around a non-mycorrhizal roots extends to only 1–2 mm, nearly the length of a root hair whereas extraradical hyphae of AMF extends 8 cm or more beyond the root making the P in this greater volume of soil available to the host (Fig. 12.1; Muchovej 2001).There are reports of production of organic acids by AMF that could solubilize the insoluble mineral P (Lapeyrie 1988), an added advantage in terms of improvement of P uptake by host plants. In addition, AMF mycelia have also been shown to increase uptake of many other nutrients, including N, S, B, Cu, K, Zn, Ca, Mg, Na, Mn, Fe, Al, and Si (Clark and Zeto 2000).

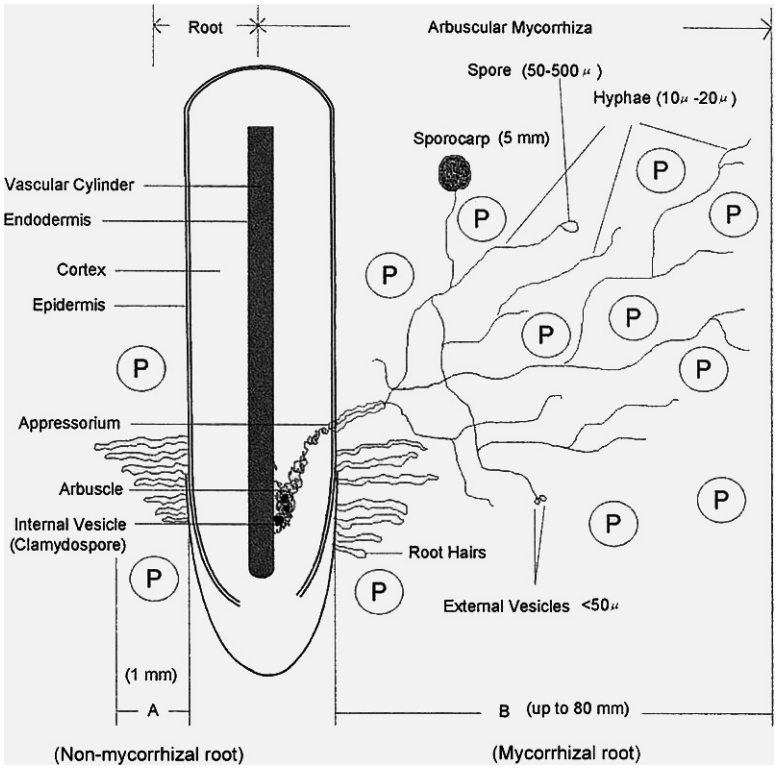

Fig. 12.1 Root colonized by endomycorrhizal fungus. Note the zone of P (or other nutrient) absorption by a non-mycorrhizal root (**A**) and by a mycorrhizal root (**B**) *P* phosphate ion. Adapted from Muchovej (2001)

Apparently, besides providing P to their host plants, AMF can facilitate N_2–fixation by providing legumes with P and other immobile nutrients, such as copper (Cu) and zinc (Zn), essential for BNF (Li et al. 1991; Kothari et al. 1991; Clark and Zeto 2000). There are reports that N fixation can be reduced or even completely inhibited in the absence of AMF at low nutrient availability (Azcón et al. 1991). The improvement in plant growth under both greenhouse and field conditions has also been suggested because of increased photosynthesis and improved C flow to the nodule and to AM sinks, giving rise to more and larger nodules that fix more nitrogen for the plant (Linderman and Paulitz 1990). In some cases, AMF may, however, be responsible for acquiring 100% of host nutrients (Smith et al. 2004). Thus, Marschner (1998) and Hodge and Campbell (2001) have suggested that the overall improvement in plant nutrition following AM inoculation occurs due to the following: (1) increased root surface through extra-radical hyphae, which can extend beyond root depletion zone; (2) degradation of organic material; and (3) alteration of the microbial composition in the rhizosphere. More specifically, mechanisms as to how AMF contribute to plant health have been extensively studied leading to development of several hypotheses (Linderman 1994). The most important ones are as follows: (1) increased nutrient uptake results in higher resistance of the plant to pathogen invasion or a compensation of the symptoms; (2) competition for photosynthates or space; (3) plant morphological changes and barrier formation; (4) changes in biochemical compounds related with plant defense; (5) increased percentage of microbial antagonists in the rhizosphere.

Under conditions of low N and P availability, which exist in many tropical soils, the possible transfer of nutrients from the mycorrhizal plant to another plant via AMF hyphal network may occur. Underground hyphal links can be formed when hyphae of mycorrhizal fungi spread from one infected plant and enter the roots of one or more other plants (Heap and Newman 1980). Studies have ascertained that AM fungi did enhance N transfer from mycorrhizal legumes to another nonleguminous plant (Vankessel et al. 1985). Similarly, Snoeck et al. (2000) demonstrated that nearly 30% of the N fixed by legumes such as *Desmodium* and *Leucaena* was transferred to associated coffee trees.

12.5.1 Alleviation of Environmental Stresses in Mycorrhizal Legumes

Currently, a wide array of environmental stresses (abiotic/biotic) is increasing worldwide due to various types of anthropological activities that have seriously threatened plant distribution and function in a given ecosystem. Although plants have evolved mechanisms to cope such unfavorable factors, they can perform better if grown with the beneficial rhizosphere microbes (Aroca and Ruiz-Lozano, 2009). The role of AM-fungi in the promotion of biological and chemical properties of legumes under stressed environment is briefly discussed in the following section.

12.5.1.1 Tolerance to Salt and Acidity

Worldwide, salinity is one of the most important abiotic stresses that limit crop growth and productivity. For example, Rabie and Almadini (2005) while investigating the effects of dual inoculation of *Azospirillum brasilense* [nitrogen fixing bacterium (NFB)] and AMF (*Glomus clarum*) on *Vicia faba* grown with five levels of NaCl (0.0–6.0 dSm^{-1}) observed that AM inoculated faba plants showed decreases in salinity tolerance, percent of mycorrhizal infection and higher accumulation of proline with increasing levels of salinity. In addition, AMF infection significantly increased mycorrhizal dependency, N and P level, phosphatases enzymes, nodule numbers, protein content, and nitrogenase enzymes of all salinized faba plants compared with control and non-mycorrhizal plants either in the absence or in the presence of NFB. In shoots of non-AM plants, Na^+ concentration was increased while the concentrations of K^+, Mg^{++}, and Ca^{++} were decreased with increasing salinity. AM colonized plants, on the other hand, had greater K^+/Na^+ , Mg^{++}/Na^+, and Ca^{++}/Na^+ ratios relative to non-AM plants at all salinity levels. The Na^+ level in shoots of AM plants showed slight increase with gradual increase in salinity, while a noticeable increase was observed in K^+ and Ca^{++} concentrations especially at higher salinity levels. The results clearly showed that the inoculation of NFB along with AM plants synergistically increased the performance of test legume under salinity stress providing evidence for reducing the salt affected negative impact on legumes as also reported for *Trifolium alexandrinum* plants grown under different salinity levels (2.2, 5, and 10 dS m^{-1}) in a pot experiment under glasshouse conditions (Shokri and Maadi 2009). The ability of crop plants to tolerate low soil pH is other interesting aspect that has become extremely important in the agricultural production systems of the humid tropics as such soils are low in pH (Kamprath and Foy 1985). Studies by Dodd et al. (1990) and Sieverding (1991) show that over 50 field trials with effective AMF in acid soils of varying fertility resulted in an average increase of a 20–25% in yields (3 tons/ha) and a greater stability in production year-after-year. Later on, the influence of soil acidity on the levels of colonization by the microsymbionts and the dependency of pioneer plants on the microsymbionts was investigated in an abandoned quarry of acid sulfate soil (Maki et al. 2008). The levels of AM colonization in pioneer grass, forbs, and legume shrubs grown in the field were assessed, and no significant decline in the levels with an increase in soil acidity was observed. Most of the legume shrubs formed root nodules. Several AM fungi and bradyrhizobia were cultured from the rhizosphere soils of pioneer plants grown in the quarry. Pot experiments revealed that the microsymbionts isolated from the field significantly promoted the growths of pioneer grasses and legume shrubs in acid sulfate soil at pH 3.4.On the other hand, Dodd et al. (1990) supported the idea that increasing the AMF inoculum potential of acid-infertile soils by inoculation or pre-crops can greatly increase the rate of establishment of mycorrhiza-dependent host plants. Thus, from these and other studies, it was suggested that bacterial-AM-legume tripartite symbioses could be a new approach to increase the tolerance of legume plants under stressed environment.

12.5.1.2 Heavy Metal and Drought Tolerance

Working with *Trifolium repens*, Vivas et al. (2003) studied the effect of inoculation with indigenous naturally occurring microorganisms (an AM fungus and rhizosphere bacteria) isolated from a cadmium (Cd) polluted soil. One of the bacterial isolate identified as a *Brevibacillus* sp. showed a marked PGPR activity. Mycorrhizal colonization also enhanced *Trifolium* growth and N, P, Zn, and Ni content and the dually inoculated (AM fungus with *Brevibacillus* sp.) plants achieved further growth and nutrition and less Cd concentration, particularly at the highest Cd level. Interestingly, increasing Cd level in soil decreased Zn and Pb accumulation in shoot. Co-inoculation of *Brevibacillus* sp. and AM fungus increased shoot biomass over single mycorrhizal plants by 18% (at 13.6 mg Cd kg^{-1}), 26% (at 33.0 mg Cd kg^{-1}), and 35% (at 85.1 mg Cd kg^{-1}). In contract, Cd transport from soil to plants was substantially reduced and at the highest Cd level; *Brevibacillus* sp. lowered this value by 37.5% in AM colonized plants. However, the increase in Cd level highly reduced plant mycorrhization and nodulation. On the contrary, strong positive effect of this bacterium was observed for nodule formation in all treatments. In a similar study conducted by Al-Garni (2006), the composite inoculation of AMF and *Rhizobium* significantly increased dry weight, root:shoot ratio, leaf number and area, plant length, leaf pigments, total carbohydrates, N and P content of cowpea (*Vigna sinensis*) plants grown in pots treated with six concentrations of Zn (0–1,000 mg/kg dry soil) and Cd (0–100 mg/kg dry soil) when compared with non-inoculated controls. Moreover, tolerance index of inoculated cowpea plants was greater than uninoculated plants. And microsymbionts dependencies of test plants increased at higher levels of Zn and Cd in polluted soil. Metals accumulated by microsymbionts-infected cowpea plant were mostly distributed in root tissues, suggesting that an exclusion strategy for metal tolerance exists in such organisms. Yet in other study, the influence of *Glomus macrocarpum* Tul. and Tul. on growth, nutrients and Pb uptake by *Bradyrhizobium*-inoculated soybean (var. IAC-14) was assessed in soils treated with different levels of Pb (Andrade et al. 2004). The results revealed that soybean shoot dry biomass were not affected by increasing doses of Pb, but the number of pods decreased significantly. Nodule dry weights of mycorrhizal roots were reduced by soil Pb additions, although the mycorrhizas stimulated plant nodulation significantly. The inoculation of AMF in soybeans provided higher rates of nutrients uptake, mainly P, inducing greater mycorrhizal-soybean growth. Thus, mycorrhizas improved Pb uptake, produced shoots with Pb concentrations 30% lower than those of non-mycorrhizal plants, at the highest Pb concentration added to the soil. AM fungus was, however, more susceptible to the higher Pb rates added to the soil than the soybean plants, decreasing both root AM colonization and spore production. This work indicated that a concentration of 600 mg dm^{-3} of Pb in the soil interfered with the establishment of double symbioses between AMF and *Bradyrizhobium*, and with the fungus perpetuation in soil. Recent surveys indicate that ecosystem restoration of heavy metal contaminated soils practices need to incorporate microbial biotechnology research and development

in order to harness the optimum benefits of bacterial-AM-legume tripartite symbiosis under heavy metal contaminated soils (Al-Garni 2006; Khan 2006).

Water deficit is considered one of the most important abiotic factors limiting plant growth and yields. Several eco-physiological investigations have shown that the AM symbiosis often alters the rates of water movement into, through, and out of the host plants, with consequent effects on tissue hydration and plant physiology (Ruiz-Lazano 2003) and consequently improve water uptake by plants (Aliasgharzad et al. 2006). For instance, in a controlled pot culture experiment performed by Aliasgharzad et al. (2006), soybean plants were inoculated with two species of AM- fungi, *G. mosseae* (Gm) or *G. etunicatum* (Ge), or left non-inoculated (NM) as control in a sterile soil. Four levels of soil moisture (Field capacity, 0.85 FC, 0.7 FC, 0.6 FC) in the presence or absence of *B. japonicum*, were applied to the pots. Relative water content (RWC) of leaf at both plant growth stages (flowering and seed maturation) decreased with the dryness of soil; RWC was higher in all mycorrhizal than non-mycorrhizal plants irrespective of soil moisture level. At the lowest moisture level (0.6 FC), Ge was more efficient than Gm in maintaining high leaf RWC. Leaf water potential (LWP) had the same trend as RWC at flowering stage but it was not significantly influenced by decrease in soil moisture to 0.7 FC during seed maturation stage. Seed and shoot dry weights were affected negatively by drought stress. Mycorrhizal plants, however, had significantly higher seed and shoot dry weights than non-mycorrhizal plants at all moisture levels except for seed weight at 0.6 FC. Root mycorrhizal colonization was positively correlated with RWC, LWP, shoot N and K, and seed weight, implying improvement of plant water and nutritional status as a result of colonization. Shoot K was enhanced considerably by both bacterial and fungal inoculations, particularly in plants with dual inoculations where the highest shoot K levels were found. The relatively higher shoot and seed dry weights in plants inoculated with both *G. etunicatum* and *B. japonicum* could be ascribed to their higher RWC and LWP, suggesting that drought avoidance is main mechanism of this plant–microbe association in alleviation of water stress in soybean. Recently, Aroca and Ruiz-Lozano (2009) also emphasized that phytobeneficial soil microorganisms enhance plant drought tolerance by different mechanisms including decreased oxidative stress, improved water status, or regulation of aquaporins. In addition, the authors further suggested that AM symbiosis improves almost every physiological parameter, such as water status, leaf transpiration, photosynthesis, or root water uptake of the host plant under drought stress. At the same time, AMF in combination with rhizobia or other PGPR results in additive or synergistic effect on plant drought tolerance, although this depends on the compatibility of strains used for inoculation. Therefore, although there is evidence that help us to understand that soil microorganisms induce plant drought tolerance at physiological level, the mechanistic basis of drought tolerance at molecular level is inadequate.

Currently, it is well documented that desertification is a complex and dynamic process, which obviously has a negative environmental impact, particularly in arid, semi-arid, and subhumid areas of the world, where the process is claiming several million hectares per annum (Herrera et al. 1993; Aroca and Ruiz-Lozano 2009).

Consequently, the proportion of plants living under water shortage conditions is increasing. Thus, management of indigenous plant–microbes symbioses assists in restoration of desertified ecosystems (Requena et al. 2001). Legumes are the most appropriate candidates for revegetation of water-deficient, low-nutrient environments/disturbed ecosystems because of their ability to establish tripartite symbiotic associations with N_2 fixing rhizobia and AMF, which improve nutrient acquisition and help plants to become established and cope with stress situations (Herrera et al. 1993; Zaharan 1999). Studies show that useful legume tree species may contribute around 12 tons of dry litter and 190 kg of N ha^{-1} y^{-1} to renovate degraded soil (Franco and De Faria 1997).

12.5.1.3 Tolerance of Soilborne Pathogens

The effects of AMF, *G. mossseae* (Gm) and *G. fasciculatum* (Gf) and *R. leguminosarum* biovar *phaseoli* (Rlp), were examined on the patho-system of *Sclerotinia sclerotiorum* (Lib) de Bary (Ss) and common bean (Aysan and Demir 2009). The colonization and nodulation of two biological control agents exhibited differences as a result of reciprocal interactions of these items as well as the effect of Ss. Nodulation of Rlp decreased in triple inoculation. In addition, colonization of AMF significantly decreased in treatment of Ss + AMF than control AMF. Treatments of single inoculation of AMF and Rlp isolates reduced disease severity by 10.3–24.1%. It was found that single biological control agent's inoculations were more effective than dual inoculations (AMF with Rlp). While comparing the morphological parameters of common beans, all measured morphological parameters were decreased in treatments having pathogen isolate. Besides this, all biological control agents increased total content of P and N in treated plants when compared with the controls. Root colonization by AMF can improve plant resistance/tolerance to biotic stresses. Studies indicate that a range of mechanisms are involved in controlling the pathogen by mycorrhizal roots, such as exclusion of pathogen, lignifications of cell wall, changed P nutrition, exudation of low molecular weight compounds, and others (Morandi 1996; Sharma et al. 2004). Sundaredan et al. (1993) investigated the interaction of *G. fasciculatum* with a wilt-causing soil borne pathogen, *F. oxysporum,* against cowpea plants. It was found that preestablishment by AMF reduced the colonization of the pathogen and the severity of the disease, as determined by reduction in vascular discoloration index. In mycorrhizal plants, the production of phytoalexin compounds was always higher than in the nonmycorrhizal plants and a direct correlation between the concentration of the phytoalexins and the degree of mycorrhizal association was found. It is argued that the production of phytoalexin compounds in mycorrhizal plant could be one of the mechanisms imparting tolerance to the plants against wilt disease. Moreover, multiple lines of evidence reveals that AM-fungi significantly reduced disease symptoms caused by fungal pathogens, such as *Phytophthora*, *Gaeumannomyces*, *Fusarium*, *Pythium*, *Rhizoctonia*, *Verticillium*, and *Aphanomyces* (Demir and Akkopru 2007).

12.6 Inoculum Development and Formulations

Since AM-fungi are obligate biotrophs, the AM inoculum production on a commercial scale via a host plant is still an obstacle and hence limits its utility as inoculants in sustainable agricultural production systems. Despite the limitation in inocula development, certain progress has been made in this direction and some commercial inoculum is currently marketed in the world (Gianinazzi and Vosátka 2004). Currently, there has been a remarkable boom in enterprises producing mycorrhizal fungi inocula and related services for the retail sector, commercial plantations, horticulture and, more recently, the developing agricultural market. There are number of reasons for increasing interest in developing mycorrhizal inocula by the mycorrhizal industry. First, the positive effects of mycorrhizal fungi on plant health, growth, and yield has generated a greater interest among end users of mycorrhizal technology (Gianinazzi and Vosátka 2004). Second, it offers an environmentally friendly and economically attractive option in commercial cultivation (Oberson et al. 1993; Toro et al. 1997). Therefore, AM-fungi are gaining popularity as "biofertilizers"/efficient scavengers of nutrients, "bioprotectors" and "biocontrol" agents (Sylvia 1999), and hence, the industry of mycorrhizal inoculum production is expanding (Todd 2004). However, extensive field trials are required to prove that bioagent indeed is effective, and hence, can be recommended for inoculants development and its consequent application over a wide range of soil, environmental conditions, and crop types (Leggett et al. 2007).

The first consideration in inoculum production involves the selection of fungal isolates endowed with growth promoting activity (Ryan and Graham 2002). Other factors to be considered in the production of inoculum include soil conditions, the host plant used to grow fungus (Sieverding 1991; Ryan and Graham 2002). Several host plants including Sudan grass (*Sorghum bicolor* var. Sudanese), bahia grass (*Paspalum notatum*), guinea grass (*Panicum maximum)*, cenchrus grass (*Cenchrus ciliaris)*, clover *(Trifolium subteraneum)*, straw berry *(Fragaria sp.)*, sorghum *(Sorghum vulgare)*, maize *(Zea mays)*, barley (*Hordume vulgare*), and onion *(Allium cepa)* have been attempted to produce AM inoculum. However, mass propagation of AM fungi varies greatly with root structure and habitat of host plant (Bever et al. 1996). Furthermore, since there are greater variations in soils and climates around the world, the locally available materials for inoculum production should be tested (Sieverding 1991). The traditional and most widely used approach has been to grow the fungus with the host plant in solid growth medium individually or a combination of the solid growth media such as soil, sand, peat, vermiculite, perlite, clay, or various types of composted barks (Sylvia and Jarstfer 1992). For the commercial development of AM inoculants, numerous strategies have been adopted time to time with their own merits and demerits. Some of the recently followed techniques for the production of mycorrhizal inoculum include soil or soilless technologies, such as nutrient film technique (NFT) (Mosse and Thompson 1984), circulation hydroponic culture system, aeroponic culture system (Sylvia and

Hubbell 1986), root organ culture, and tissue culture (Nopamornbodi et al. 1988) are briefly discussed in the following section.

12.6.1 Inoculum Production Strategies

12.6.1.1 Soil-Based Pot Culture Method

Soil-based pot culture is a common method for production of AM fungal inoculum (Menge 1984). Soil inoculum contains all AM fungal structures; this inoculum source is highly infective (Sieverding 1991). The author further suggested that the success for good soil inoculum production depends on the selection of the host plant and the ambient conditions. The soil inoculum (containing AM-infected roots, AM spores, and mycelium) is chopped and homogenized before use. Soil may contain abiotic and biotic components, which makes it undesirable substrate in which to grow and subsequently to distribute the AM fungal inoculum. Inocula containing soil are considered impractical because of their bulk and the risk of contamination by insects, nematodes, and plant pathogens (Sylvia and Jarstfer 1992). However, chopped roots in peat blocks (Warner 1985) and spores within a clay matrix (Dehne and Backhaus 1986) have been proposed for field application. This technique is cost effective with low inputs and thousands of infectious propagules can be extracted in a gram of soil. However, the major disadvantage associated with this technique includes bulk amount, vulnerability of pest to infestation, and nutrient management (Sharma et al. 2000). To overcome these problems, soilless technologies were discovered and are discussed.

12.6.1.2 Nutrient Film Technique (NFT)

In this method, large volume of nutrient liquid in a film is recycled, which flows over the roots of plants. However, any host in the NFT should be grown first in the soil substrate with AM inocula in order to infect the roots. This technique eliminates the possibility of contamination and helps produce large quantities of AM infected roots. However, higher sporulation compared with soil system is not achieved. Yun-Jeong and Eckhard (2005) used NFT culture system for nursery production of arbuscular mycorrhizal horticultural crops. In the NFT system, a thin layer of glass beads was used to provide solid support for plant and fungus growth and nutrient solution was supplied intermittently (15 min, six times per day). A modified nutrient solution (80 μM P) was used and was changed with fresh solution at 3 days intervals. The dry matter accumulation in *G. mosseae* (BEG 107) colonized lettuce (*Lactuca sativa* var. capitata) was significantly higher than nonmycorrhizal lettuce in Perlite during the precolonization period. The root colonization rate was also high at rates up to 80 μM P supply. On the NFT system, growth differences between mycorrhizal and nonmycorrhizal plants were less than in Perlite. However,

root colonization rate was not reduced during the NFT culture period. In this system, high amounts of fungal biomass were produced. The authors suggested that using this technique, metal and other nutrient concentrations in fungal hyphae can be determined. Furthermore, this modified NFT culture system could also be suitable for fungal biomass production on a large scale with a view to additional aeration by intermittent nutrient supply, optimum P supply, and a use of glass beads as support materials. Furthermore, bulk inoculum composition with a mixture of spores, colonized roots, and hyphae grown in soilless media by the modified NFT system might be a useful way to mass-produce mycorrhizal crops and inoculum for commercial horticultural purposes.

12.6.1.3 Aeroponic Method

A culture system that applies a fine mist of defined nutrient solution to the roots of trap plant is termed as aeroponic culture (Zobel et al. 1976). For this, plants are generally inoculated in sand or vermiculite before they are transferred to these systems. Plants have also been inoculated directly in the aeroponic system (Hung et al. 1991). Applying aeroponics, higher number of spores has been produced compared with soil-based pot cultures. Since no substrate is present with the inoculum with aeroponic culture of roots, it is possible to produce inoculum with hundreds of thousands of propagules per dry gram of roots (Sylvia and Jarstfer 1992). The aeroponics has distinct advantage over other AM producing techniques, such as the highly aerated rooting environment of aeroponics stimulates rapid and abundant sporulation of the AM fungi, and this system reduces the risk of contamination but this technique is a costly affair. For example, in an aeroponic culture, root colonization and sporulation of *G. mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *G. intraradices* Schenck and Smith with bahia grass (*Paspalum notatum* Flugge) was found superior relative to a soil-based pot culture (Sylvia and Hubbell 1986). Similarly, Martin-Laurent et al. (1999) designed an experiment to produce *Acacia mangium* saplings associated with AM fungi using aeroponics (a soilless plant culture method). *A. mangium* seedlings were first grown in multipots and inoculated with Endorize (a commercial AM fungal inocula) followed transfer to aeroponic systems or to soil. Aeroponics was found as a better system than soil and doubled the production of tree saplings compared with soil. Moreover, compared with plants grown in soil, aeroponically grown saplings inoculated with AM fungal inoculum exhibited significantly different rates of mycorrhization, leading to an increase in chlorophyll contents in plant tissues. The authors suggested that the aeroponic system is an innovative and appropriate technology, which could be used to produce large quantities of tree saplings associated with soil micro-organisms, such as AM fungi, for reforestation of degraded land in the humid tropics. Aeroponically produced *G. deserticola* and *G. etunicatum* inocula retained their infectivity after cold storage (4°C) in either sterile water or moist vermiculite for at least 4 and 9 months, respectively (Hung and Sylvia 1988).

12.6.1.4 Root Organ Culture System

The root organ culture system is the most attractive and advanced cultivation methodology for AMF development. This technique uses root-inducing transfer-DNA-transformed roots of a host plant to develop the symbiosis on a specific medium in vitro, which provides pure, viable, contamination-free inoculum using less space. Systems utilizing excised roots of various host species as well as different media formulations have been developed to culture glomalean fungi monoxenically (Mugnier and Mosse 1987). Less than 5% of currently known arbuscular mycorrhizal species have, however, been successfully cultivated using such a dual culture approach. *Gigaspora margarita* (Miller-Wideman and Watrud 1984), *Glomus fasciculatum*, *G. intraradices* and *G. macrocarpum* (Declerck et al. 1998), and *G. versiforme* (Diop et al. 1994) have been maintained and sporulated in association with excised tomato roots or roots of carrot transformed by "hairy root" inducing T-DNA from *Agrobacterium rhizogenes*. Evidently, the rate of in vitro spore formation of the AM fungus *G. versiforme* was followed in Petri dishes, using mycorrhizal root-segment inoculum associated with Ri T-DNA transformed carrot roots (Declerck et al. 1996). Three phases of sporulation were observed: a lag phase, a period of intensive spore production and a plateau phase. An average of 9,500 spores per Petri dish was produced after 5 months of dual culture. The root-organ culture system supported extensive root colonization, with many arbuscules and vesicles being formed. The fungus, both within root-segments and as spores produced, was viable and able to complete its life cycle in vitro. However, the mycorrhizal root-segments exhibited higher inoculum potential due to the numerous vesicles and extensive intraradical mycelium. The in vitro propagation on root-organ culture, however, may not change drastically the traditional procedures but will certainly facilitate the quality control of strain purity and improve the supply of massive amounts of spores as starting inoculum (Dalpé and Monreal 2004).

12.6.2 Formulations

Different formulated products are available in the market, which creates the need for the establishment of standards for widely accepted quality control. In most cases, fresh AMF inoculum is applied (Fig. 12.2; Sieverding 1991). In preparation and formulation of mycorrhizal inoculum, the most widely used methods are based on the entrapment of fungal materials in natural polysaccharide gels (Sieverding 1991; Vassilev et al 2005). The potential of such inoculant preparations is illustrated by various studies, which include immobilization of mycorrhized root pieces, vesicles and spores, in some cases, coentrapped with other plant beneficial microorganisms (Vassilev et al. 2001). For example, the applicability of microbial inoculants entrapped in alginate gel is reported (Vassilev et al. 2001). In this method, *Glomus deserticola* (AM) was inoculated into soil microcosms, enriched with rock phosphate, either in free form or entrapped in calcium alginate alone or in

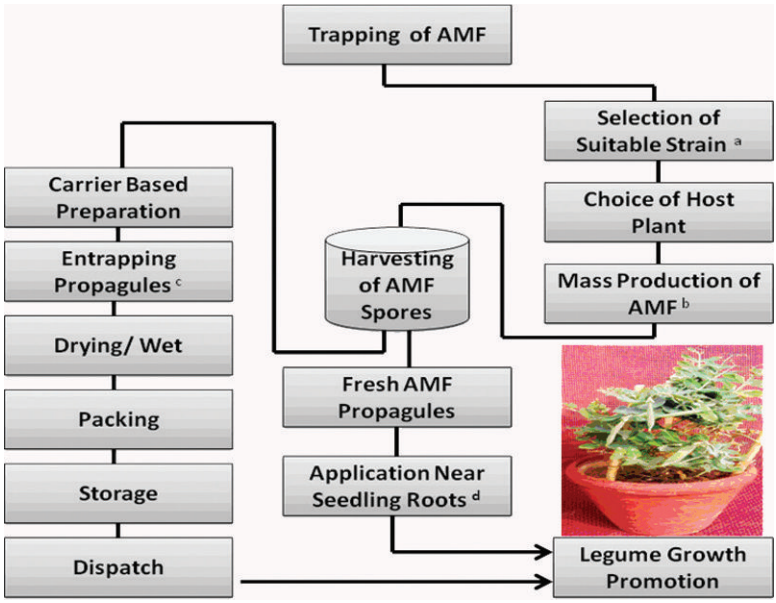

Fig. 12.2 Various stages involved in production and inoculation of AMF: [a]Strain to be selected must be the best greenhouse and field performer, [b]Cultivation using suitable host by employing either conventional soil-based or soilless techniques, [c]Include immobilization of mycorrhized root pieces, vesicles and spores, in some cases co-entrapped with other plant beneficial microorganisms, [d]Supplying propagules near seedlings in soil at appropriate rate

combination with a P-solubilizing yeast culture (*Yarowia lipolytica*). Plant dry weight, soluble P acquisition, and mycorrhizal index were equal in treatments inoculated with free and alginate-entrapped AM. Dual inoculation with entrapped *G. deserticola* and free cells of *Y. lipolytica* significantly increased all measured variables. Highest rates of the latter were obtained when both fungal microorganisms were applied co-entrapped in the carrier. The yeast culture behaved as a "mycorrhiza helper microorganism" enhancing mycorrhization of plant roots. These results indicate that dual inoculation with an AM fungus and a P-solubilizing microorganism co-entrapped in alginate can be an efficient technique for plant establishment and growth in nutrient deficient soils. Likewise, Weber et al. (2005) studied dual inoculation of *Acacia mangium* grown in aeroponic culture using selected strains of *Bradyrhizobium* sp. and *G. intraradices*. A single-step technique with alginate as an embedding and sticking agent for an inoculum composed of AM-infected sheared roots was used to infect plants. This method resulted in the successful establishment of AM in 100% of the inoculated plants after 7 weeks. The results indicated that dual microbial inoculation with *G. intraradices* strain S-043 and *Bradyrhizobium* strain AUST 13C stimulated the growth of *A. mangium* in aeroponic culture. The effects of single and dual microbial inoculations were also evaluated at two levels of P in the nutrient medium. A concentration of 5 mg P kg^{-1}

stimulated the development of AM without affecting plant development or establishment of *Bradyrhizobium* symbiosis. In contrast, saplings supplemented with a higher concentration of P (25 mg kg^{-1}) alone or co-inoculated with *Bradyrhizobium* had lower AM frequencies.

12.7 Conclusion

The great agricultural and environmental importance of legumes due to their ability to form symbiosis make legumes target crops in sustainable agriculture. Beneficial plant–microbe interactions in the rhizosphere are crucial determinants of plant health and soil fertility. Accordingly, using beneficial soil microbes is one of the established, promising, and sustainable low-input soil management ventures. Legumes have a high level of productive diversification and a flexible utilization. In nature, most plants do not only have roots; instead they have mycorrhizas, which also associates quite impressively with legumes. AMF primarily affect the ecophysiology of nodulated legumes, the biota of the surrounding soil, and associated non-legume plants that make them one of the most efficient ecological factors in the ecosystem functions. Research on composite inoculation of legumes with both rhizobia and AMF have shown a profound effect on the overall performance of legumes in different agro-ecological regions. Furthermore, AMF alleviates various types of environmental stresses in legume plants suggesting their roles in helping legumes to adapt and grow in stressful environment. Additionally, AMF play also pivotal roles in rehabilitation of desertified/degraded habitats. Use of arbuscular mycorrhizas inoculum therefore may serve as an alternative to agrochemicals.

While research over many years has broadened our understanding of the multifaceted phytobeneficial roles of AMF, yet, there are several aspects that need to be addressed. Further researches are required to understand the physiology and ecology of the association and host specificity under different agro-climatic conditions. For this, the method to rapidly and specifically identify and select the most efficient AM fungal endophytes is required. Moreover, the molecular mechanisms underlying the process of stress amelioration by AMF in legume plants is limited to partial understanding of only a few genes. Investigating such issues further is likely to lead to a better understanding of the process. Development of suitable methods for production and delivery of inoculants on large scale is urgently required. It is hoped that further investigations, especially in low-input cropping systems, may enable to harness the substantial potential of mycorrhiza for legume productivity.

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Chapter 13
Bacterial Biofilms: Role in *Rhizobium*–Legume Symbiosis

Luciana V. Rinaudi and Walter Giordano

Abstract Biofilms are surface-attached communities of bacteria contained within a self-produced extracellular polymeric matrix. They are composed of a single species or, more commonly, several species of bacteria. This multicellular mode of growth provides a protective measure against adverse environmental conditions and promotes survival of organisms. Biofilms can be established on both abiotic and biotic surfaces, typically under stressful conditions. Bacteria that colonize plant surfaces can have either negative (pathogenic) or positive (symbiotic) effects and are therefore important in agriculture. In this chapter, we review the current knowledge of soil bacterial biofilms, various bacterial functions that influence biofilm formation, and the contributions or effects of exopolysaccharides, quorum sensing, rhizobial proteins, and motility on this process.

13.1 Introduction

Plant roots secrete a wide range of compounds into the surrounding soil, the rhizosphere, which creates nutrient-rich conditions for microbial growth. It is reported that up to 40% of the carbon fixed by plants is converted into root exudates (Lynch and Whipps 1990) which contains ions, free oxygen, water, enzymes, and carbon-based compounds (Bais et al. 2006) such as carbohydrates, amino acids, organic acids, mucilage, and proteins. However, root exudates vary with type of soil and nutrient availability, environmental factors such as temperature, light and soil moisture, and physiological stage of the plant. Root-derived compounds create a niche around the roots and mediate positive and negative interactions among microorganisms. Association of plants with beneficial microbes, collectively called

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as plant growth-promoting rhizobacteria (PGPR), include nitrogen-fixing bacteria, mycorrhizal fungi, and biocontrol agents, which are grouped in positively interacting organisms. Negative interactions on the other hand include associations of plants with pathogenic bacteria or fungi.

Many microorganisms exist in their natural environment not as free-living bacteria but as sessile multicellular communities called biofilms. Biofilms are defined as an assemblage of microbial cells that are irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material allowing growth and survival in sessile environment (Fig. 13.1). Scientists have recently realized that in the natural world, more than 99% of all bacteria exists as biofilms (Costerton et al. 1987). Inside biofilms, bacteria undergo physiological changes in relation to individual, planktonic cells, leading to special proteomes and metabolic activities (Whiteley et al. 2001; Sauer et al. 2002; Vilain et al. 2004). The extracellular matrix, mostly composed of exopolysaccharides (EPS), is believed to play a key role in biofilm endurance (Stoodley et al. 2002). Other components like proteins, DNA, and products from bacterial lysis provide the matrix for biofilm formation, which allows first, attachment of the cells to a solid surface and to each other and later, colonization of such surface/substrate. This sessile lifestyle confers bacteria within the biofilm resistance against certain environmental stresses (such as desiccation, pH changes, UV radiation) as well as tolerance against antibiotics and defense-related compounds from the host, protection from protozoan predation, enhancement of genetic exchange (through horizontal gene transfer), and production of secondary metabolites and exoenzymes (Danhorn and Fuqua 2007). Such transition from planktonic to sessile state is mediated by numerous environmental signals as well as by accumulation of quorum-sensing signals (called autoinducers), which mediate cell–cell communication in bacteria and coordinate communal behavior by regulating specific genes in response to population density. Biofilm formation may be a result of passive

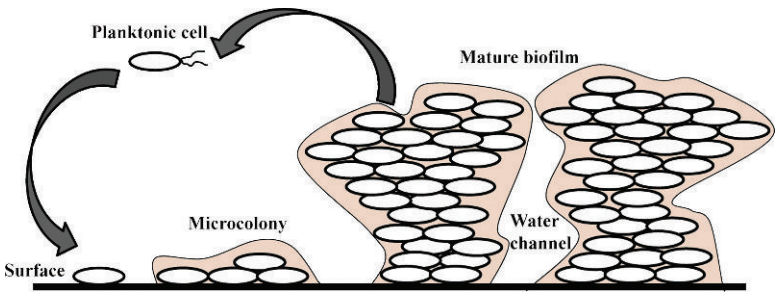

Fig. 13.1 Free-living (planktonic) bacteria are able to attach to a surface and form microcolonies on it. Microorganisms inside such microcolonies start producing the exopolysaccharides that serve as the matrix for biofilms. Finally these communities develop into highly structured communities surrounded by water channels, which allow the exchange of extracellular signals, nutrients and toxic metabolic wastes. Attached bacteria can also return to the swimming mode of life. For this reason biofilm formation and then dispersal of the biofilms are considered as a cycle

deposition and accumulation of bacterial cells on a surface due to, for example, water flow in the rhizosphere or, in other cases, to active processes of bacterial chemotaxis, attachment, and production of exopolymeric substances. No matter the origin of the biofilm, it confers bacteria an adaptive advantage to confront hostile environments, allowing cells within the community to cooperate and realize functions not exhibited by single cells (Morris and Monier 2003).

Root attachment and colonization constitutes the first step during the development of beneficial as well as pathogenic associations between plants and microorganisms. During the *Rhizobium*–legume symbiosis, attachment of rhizobia to legume roots constitutes a requirement for infection and nodulation (Rodríguez-Navarro et al. 2007). Biofilm formation was first reported in rhizobia by Seneviratne and Jayasinghearachchi in 2003, who observed that *Bradyrhizobium* sp. is able to form typical biofilm structures on diverse biotic and abiotic surfaces (Seneviratne and Jayasinghearachchi 2003). Furthermore, roles of EPS (Fujishige et al. 2006b; Russo et al. 2006; Williams et al. 2008; Rinaudi and González 2009), Nod factor (Fujishige et al. 2008) and other mechanisms involved in biofilm formation have been studied in several species of rhizobia as well as the conditioning of biofilm formation by nutrient and osmotic cell status (Rinaudi et al. 2006) (Table 13.1).

Root exudates though provide a carbon-rich environment for microbial growth, yet it initiates cross-talk with soil microbes leading to plant colonization (Bais et al. 2006). Biofilm formation has been reported to be influenced by abiotic factors, as, nutrient availability, osmolarity, temperature, and soil moisture (Stanley and Lazazzera 2004). In addition, biofilm formation is also influenced by nutrient release and exudation at different sites along the roots (Ramey et al. 2004). It has been proposed that more structured biofilms are formed on mature regions of the roots due to high nutrient availability, while lower nutrient availability or the secretion of antimicrobials from the root tip lead to reduced biofilm formation

Table 13.1 Sumary of some of the articles available on biofilm formation by rhizobia

Role described in biofilm formation	Rhizobial species	Source
Exopolysaccharide	*S. meliloti*	Fujishige et al. (2006b), Wells et al. (2007), Rinaudi and González (2009), Rinaudi et al. (2010)
	M. tianshanense	Wang et al. (2008)
	R. leguminosarum	Vanderlinde et al. (2002), Russo et al. (2006), Williams et al. (2008)
	B. japonicum	Pérez-Giménez et al. (2009)
Quorum sensing	*S. meliloti*	Rinaudi and González (2009)
	M. huakii	Wang et al. (2004)
	R. leguminosarum	Edwards et al. (2009)
Rhizobial proteins	*B. japonicum*	Dardanelli et al. (2003)
	R. leguminosarum	Mongiardini et al. (2008)
Motility	*S. meliloti*	Fujishige et al. (2006b)
	R. leguminosarum	Verstraeten et al. (2008)
Nod factors	*S. meliloti*	Fjishige et al. (2008)

(Rudrappa et al. 2008a). In this chapter, the bacterial functions involved in the establishment of beneficial biofilms on the plant root surface as well as the rhizosphere environment are discussed.

13.2 Plant Products Regulating Associations with Microorganisms

Biofilm formation confers some advantages to beneficial and pathogenic bacteria, but how are these interactions regulated is not clear. Interestingly, it has been reported that plants can recognize and attract beneficial organisms, like rhizobia and mycorrizal fungi, by releasing secrete secondary metabolites while prevent the attachment of harmful ones (Ramey et al. 2004). For example, it has recently been shown that *Arabidopsis thaliana* plants infected with *Pseudomonas syringae* can secrete malic acid into rhizosphere, which activates chemotaxis and biofilm formation by *Bacillus subtilis* (Rudrappa et al. 2008b). Consequently, the roots colonized by *B. subtilis* result in protection of *Arabidopsis* plants from infection by foliar pathogen (*P. syringae)* by inducing systemic resistance (ISR) on the host plant (Rudrappa et al. 2008b). Interestingly, biofilm formation by beneficial microorganisms seems to be also regulated by in planta redox potential in the rhizosphere (Rudrappa and Bais 2007). For example, root colonization by *B. subtilis* is suppressed on *A. thaliana NahG* plants through reactive oxygen species (ROS) mediated down-regulation of the *yqxM* and *epsA* operons required for biofilm formation by *Bacillus* (Rudrappa et al. 2007). Plants have, however, evolved several mechanisms to prevent negative interactions. Among these, host plants produce quorum-sensing like signal molecules (plant quorum-sensing mimics) capable of interacting with quorum-sensing systems from different bacterial strains (Teplitski et al. 2000; Mathesius et al. 2003; Keshavan et al. 2005). Such molecules help plants to protect themselves from pathogens and also modify bacterial behavior from pathogens (González and Marketon 2003), such as the formation of biofilms. It has also been reported that plants produce sesquiterpene lactones that are able to inhibit biofilm formation by *Pseudomonas aeruginosa* (Cartagena et al. 2007). Plants also combat bacterial pathogens by secreting antimicrobial compounds through the roots. In this regard, rosmarinic acid secreted by the roots of sweet basil (*Ocymum basilium*) plants upon infection by *P. aeruginosa* showed antibacterial activity against planktonic cells and consequently prevented biofilm formation. However, established biofilms resist microbiocidal effects of rosmarinic acid and ultimately cause plant mortality (Walker et al. 2004). Biofilm formation on *Arabidopsis* roots by the pathogenic *P. aeruginosa* PA14 is affected by the synthesis of salicylic acid, which not only induce plant-defense responses against pathogen attacks but also downregulates the production of several virulence factors on *Pseudomonas* such as the pigment pyocyanin and the exoenzymes protease and elastase (Prithiviraj et al. 2005).

13.3 Mechanisms of Biofilm Formation

13.3.1 Exopolysaccharide Production

During plant–bacterial interactions, EPS are known to be involved in adhesion of bacteria to roots (Michiels et al. 1991), root colonization (Matthysse et al. 2005) and hence, serve as primary factor in the development of biofilms on plant roots (Bianciotto et al. 2001; Ramey et al. 2004; Fujishige et al. 2006a). In the rhizosphere, bacterial EPS contribute further to soil aggregation by cementing particles together (Chenu 1995). Inoculation of plants with EPS-producing rhizobacteria, such as *Rhizobium* sp. YAS34 (Alami et al. 2000) and *Rhizobium* sp. KYGT207 (Kaci et al. 2005), modifies the aggregation of root-adhering soil and eventually improves plant growth. EPS also play an important role in biofilm formation by rhizobia (Fujishige et al. 2006b; Russo et al. 2006; Wells et al. 2007; Wang et al. 2008; Williams et al. 2008; Pérez-Giménez et al. 2009; Rinaudi and González 2009; Rinaudi et al. 2010). For example, *Sinorhizobium meliloti* has the ability to produce two EPS, succinoglycan and EPS II. Biofilm formation by *S. meliloti* Rm1021 (an *expR* mutant) on the contrary seems to be independent of EPS (Rinaudi et al. 2010). However, overproduction of succinoglycan in *exoR* and *exoS* mutants led to an increase in biofilm formation compared to wild type (Fujishige et al. 2006b; Wells et al. 2007). On the other hand, in *S. meliloti* Rm8530 (a strain with an intact copy of the *expR* gene which allows EPS II production) biofilm formation depends on the presence of the low-molecular weight fraction of EPS II, which mediates attachment to abiotic surfaces such as PVC and borosilicate, as well as to roots of the legume host *Medicago sativa* (Rinaudi and González 2009). In this sense, EPS II-producing strains have been found as efficient root-hair colonizers while strains lacking EPS II, or only able to produce HMW fraction of this polymer, form very low levels of biofilm colonizing mostly the principal roots forming patchy colonies (Rinaudi and González 2009). In other studies, *Rhizobium leguminosarum* formed highly structured and organized biofilms on borosilicate when evaluated by Confocal Laser Scanner Microscopy (CLSM) (Russo et al. 2006; Williams et al. 2008). Biofilms formed by cellulose and glucomannan (*celA* and *gmsA*, respectively) are indistinguishable from those of the wild-type strain. However, these mutants were defective in root colonization when incubated with host plant *Vicia hirsuta*, suggesting that interactions between the rhizobia and glass surface are different from those occurring during root cap formation (Williams et al. 2008). A mutant of *R. leguminosarum* bv. *viciae* highly sensitive to desiccation has been isolated (Vanderlinde et al. 2002). This mutant in an ABC transporter shows a reduction in the accumulation of EPS and it is also defective in biofilm formation on polystyrene microplates, which proves the importance of EPS in desiccation tolerance in rhizobia and provided evidence for the role of biofilm formation against environmental stresses. In yet other investigation, an EPS mutant of *B. japonicum*, which lacks UDP-glucose-4′ epimerase activity and produced low levels of a shorter EPS lacking galactose, showed reduced adhesion to soybean (*Glycine*

max) roots compared to wild-type strain, indicating that complete EPS is required for efficient colonization of soybean by *B. japonicum* (Pérez-Giménez et al. 2009). Similarly, EPS-deficient strains of *Mesorhizobium tianshanense* showed low levels of biofilm formation on borosilicate and fail to nodulate *Glycyrrhiza uralensis*, suggesting that EPS are essential for biofilm formation (Wang et al. 2008).

Bacterial attachment by the biocontrol *Pseudomonas fluorescens* CHA0 strain to the external mycelium of *Glomus intraradices,* mycorrhizal, and nonmycorrhizal carrot roots has been evaluated (Bianciotto et al. 2001). In all cases, two mucoid mutants overproducing an alginate-like EPS showed an enhanced attachment to the surfaces compared to that of the wild type (Bianciotto et al. 2001). Biofilm formation and overproduction of the matrix may improve persistence and survival of these mutants in the soil, since it confers resistance to several environmental stresses; however, it has also been proposed that this may not lead to an increased plant protection by biocontrol strains since antifungals or antibiotics may remain trapped within the biofilm and overproduction of EPS may limit diffusion of these compounds to the rhizosphere (Bianciotto et al. 2001). However, overexpression of EPS does not always correlate with an increased biofilm formation capability in all bacteria (Parsek and Fuqua 2004). For instance, *Agrobacterium tumefaciens* is a soil bacterium that forms biofilms on the roots of plants such as tomato, alfalfa, and *A. thaliana* (Matthysse et al. 2005). Cellulose-minus mutants of *A. tumefaciens*, however, fail to attach to tomato roots and showed a reduced colonization of the surface, while overproduction of cellulose resulted in an increased biofilm formation and reduced root colonization when compared to wild type (Matthysse et al. 2005). Although most reports indicate EPS play an important role during biofilm formation this cannot be considered as a rule since EPS production by *Rhizobium* sp. YAS34 is not essential for biofilm formation, either on polypropylene surfaces or on roots of two nonlegume plants, *A. thaliana* and *Brassica napus* (Santaella et al. 2008).

13.3.2 Quorum Sensing

Presence of different quorum-sensing systems has been described in rhizobia, which regulate several phenotypes such as plasmid transfer, nodulation efficiency, nitrogen fixation, EPS production, and swarming motility (Sánchez-Contreras et al. 2007). Recently, quorum sensing has also been shown to play a role in biofilm formation (Wang et al. 2004; Zheng et al. 2006; Edwards et al. 2009; Rinaudi and González 2009). For example, mutant strains in the MrtR-MrtI quorum-sensing system in *M. tianshanense* showed a 60% reduction in root hair attachment efficiency, which may explain the reason why these strains are unable to nodulate the legume host *G. uralensis* (Zheng et al. 2006). While in *R. leguminosarum,* disruption of the CinI/CinR quorum-sensing system led to an increase in biofilm formation (Edwards et al. 2009). This effect seems mediated by the transcriptional regulator ExpR as well as the small protein CinS, coexpressed with the autoinducer

synthase CinI. ExpR and CinS regulate expression of the EPS glycanase PlyB, responsible for the cleavage of the acidic EPS, which has been involved in biofilm formation (Russo et al. 2006; Williams et al. 2008). The presence of an intact ExpR/Sin quorum-sensing system in *S. meliloti* is essential for the formation of large amounts of biofilm on abiotic surfaces and also regulates the structure of mature biofilms (Rinaudi and González 2009). In this way, it has been shown that Rm1021 lacking the *expR* gene forms a flat biofilm with no apparent structure or organization (Fig. 13.2a), while Rm8530, which has an intact ExpR/Sin quorum-sensing system, produces structured and highly organized biofilms (Fig. 13.2b, c).

13.3.3 Rhizobial Proteins

In addition to quorum sensing and EPS, some rhizobial proteins are also involved in biofilm formation. As an example, in *Bradyrhizobium* sp., a rhicadhesin-like protein mediates rhizobial attachment to peanut (*Arachis hypogaea*) roots (Dardanelli et al. 2003). The rhizobial adhesion protein 1 (Rap1), an extracellular calcium-binding protein from *R. leguminosarum* bv. *trifolii* promotes rhizobial autoaggregation through cell poles, and is involved in attachment to the legume host red clover (*Trifolium pratense*) and nonsymbiotic plants such as common bean, alfalfa, and soybean (Mongiardini et al. 2008).

13.3.4 Motility

Various bacterial motility mechanisms, such as swarming, swimming, and twitching, are known to have a profound impact on biofilm formation, including colonization and the subsequent expansion into mature structured surface communities. In a study, nonflagellated and nonchemotactic mutants of *Azospirillum brasilense* showed a strongly reduced colonization of wheat (*Triticum aestivum*) roots as compared to the wild type suggesting that initiation of root colonization requires

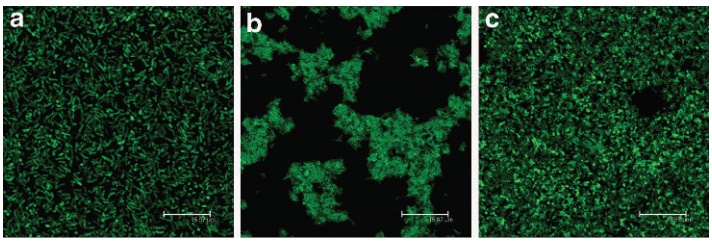

Fig. 13.2 Single-scan images from *Sinorhizobium meliloti* biofilms obtained by confocal laser scanning microscopy. (**a**) "flat" (**b**) "structured", and (**c**) "organized" honeycomb-like biofilms. The size bars indicate 15.8 μm. (Images courtesy of J.E. González)

active bacterial motility (Vande Broek et al. 1998). On the contrary, the motile strains of *P. fluorescens* had a higher rate of survival in soil and attached better to wheat roots than nonmotile strains (Turnbull et al. 2001). Furthermore, the motility allowed movement of bacteria from the roots to the surrounding rhizosphere. This movement was facilitated by signal compounds present in root/seed exudates, known to influence attachment, colonization, and biofilm formation. Similarly, the effect of exudates released by seeds and roots of soybean on chemotaxis and biofilm formation has been studied in the *Bacillus amyloliquefaciens* strain BNM339 with biocontrol activity against several fungi causing crop-related diseases (Yaryura et al. 2008). These and other associated findings thus suggests that chemotaxis, and consequently motility, are regulated by quantitative and qualitative changes in the composition of seed and root exudates. As with other PGPR, Fla^- mutants of *S. meliloti* show a poor biofilm formation capability when compared to wild-type strain Rm1021 (Fujishige et al. 2006b). The *fliP* and *flgH* mutants used in the study showed more than 50% reduction in biofilm formation, being defective in their initial attachment to PVC. Although no assays have been performed on legume roots yet, it may be speculated that such mutants would be impaired as well in biofilm formation on roots, which could explain the delay in nodule development shown by these strains.

13.4 Conclusion

Even though it is yet to establish whether there is a direct relationship between biofilm formation and infectivity, *S. meliloti* succinoglycan-producing strains, though did not colonize alfalfa (*Medicago sativa*) roots as efficiently as EPS II-producing strains, more efficiently invaded the legume (Pellock et al. 2002). This study indicated that biofilm formation may provide rhizobia with an advantageous microenvironment to persist in the soil and eventually colonize root surfaces and establish the symbiosis but it is not essential for legume invasion. On the other hand, plant hosts as well as other soil microorganisms may benefit from the biofilm-forming ability of other plant growth-promoting rhizobacteria since EPS within biofilms improve soil structure and help maintain the soil moisture (Morris and Monier 2003). Additionally, biofilms may also enhance nitrogen and phosphate availability when established on soils, as found with bradyrhizobia and common soil fungi (Sereviratne and Jayasinghearachchi 2005). Moreover, the information strongly suggests that like other bacterial biofilm, rhizobacteria biofilm formation is a complex process and hence, cannot be explained easily at molecular level employing a single mechanism.

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Chapter 14
Role of Metal Tolerant Microbes in Legume Improvement

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Abstract Soils contaminated with heavy metals present a major threat to nodule-forming rhizobia, legumes, and symbiosis formed by the interacting symbionts. The symbiotic relation, as it occurs generally in economically important legumes, has deep impact on human interest. However, in legume–*Rhizobium* symbiosis, maximum yield is possible only when there is suitable condition for both symbiotic partners. Thus, understanding the effects of heavy metals on rhizobia–legume symbiosis will be useful. Although mechanical and chemical processes have been used to clean up metal-contaminated soils, most traditional remediation technologies do not provide acceptable solutions for the removal of metal from soils. The use of metal tolerant/detoxifying microbes offers a viable and inexpensive alternative technology to clean up polluted soils. Metal-tolerant microbes not only help to remediate the contaminated soils, but also provide elements essential to the growing legumes. Given the importance of legumes in animal and human consumption and their role in maintaining soil fertility, attention is paid to understand how rhizobia develops resistance to various heavy metals. Possible role of symbiotic nitrogen fixers in the metal-contaminated soils and how these microbes influence the productivity of various legumes in metal-contaminated soils across different geographical regions are discussed.

14.1 Introduction

Discharge of toxic heavy metals such as cadmium, copper, chromium, lead, arsenic, and nickel from various industrial operations is constantly adding up metals to the soil environment up to dangerous levels for plants, animals, and human beings

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(Sharma and Agrawal 2005; Chen et al. 2010). Heavy metals should be Heavy metals in the fertile layers of soils and are influenced greatly by physicochemical characteristics of soils such as clay minerals and hydrous metal oxides, pH and buffering capacity, redox potential, water content and temperature, cation exchange capacity (CEC), organic matter, biological properties like root exudates (Jung 2008; Xu et al. 2007), duration of exposure, dose and types of metals used (Giller et al. 1998), and other environmental variables (Margesin and Schinner 2007). At low concentrations, metals can serve as important components in life processes, often involved in important enzyme functions. Above certain threshold concentrations, metals can, however, become toxic and induce morphological and physiological changes in the microbial communities (Krishnamurthy 2000; Frostegård et al. 1996). The persistence of heavy metals in soils profoundly affects overall population of microbes (Sobolev and Begonia 2008; Abou-Shanab et al. 2005), their activities (Bamborough and Cummings 2008; Akerblom et al. 2007) and biomass (Liao et al. 2005; Brookes and Mc Grath 1984) and cause a shift in microbial community (Bamborough and Cummings 2008) including nitrogen fixers (Paudyal et al. 2007) and growth and development of various agronomic crops (Liu et al. 2009; Bose and Bhattacharyya 2008) including legumes (Wani et al. 2006, 2007a; Giller et al. 1989). Among numerous heterogeneously distributed plant growth-promoting rhizobacteria (PGPR), the nodule-forming organisms, often collectively referred to as rhizobia assumes a subtle agricultural importance largely because of their exceptional capability to form an effective and functional symbiosis with legumes. Due to greater significance of legumes in dietary systems as it provides a protein rich source for both human and animal, and that it improves soil fertility by adding a substantial amount of nitrogen to soils, emphasis is being placed onto assess the impact of metals both on legumes and their associative symbionts. The accumulating data suggests that metals if present in greater concentration in soils have substantial deleterious effects on both survivability and nitrogen-fixing efficiency of symbiotic rhizobia (Younis 2007; Broos et al. 2004; Alexander et al. 1999). For example, the reduction in the population of *Rhizobium leguminosarum* bv. trifolii able to form symbiosis with white clover (*Trifolium repens* L.) when grown in soils polluted with metals following long-term application of sludge is reported (McGrath 1998). In other studies, the nitrogen-fixing rhizobia though have been found to survive in metal contaminated soils but failed to fix N with clover plants (Hirsch et al. 1993; Giller et al. 1989). Similar reports on the adverse impact of heavy metals on the composition, survival, and metabolic activity of rhizobial strains are reported by others (Pereira et al. 2006a; Chaudri et al. 2000). Like rhizobia, legumes when grown in soils treated intentionally (as experimental strategies) with heavy metals or in soils contaminated previously by toxic metals, suffers heavily from metal toxicity (Wani et al. 2007b, 2008a) and physiological aspects of legumes (e.g., protein and chlorophyll synthesis) have been found to be adversely affected under experimental conditions (Wani et al. 2007a, b). For example, the nitrogen fixation declined significantly in white clover when raised in soil irrigated with sewage sludge (Broos et al. 2005) and chickpea (Wani et al. 2007a), greengram (Wani et al. 2007b), pea (Wani et al. 2007c), and lentil (Wani et al. 2007d), when grown in soils treated with single or mixture of heavy metals. These and

associated data suggests that the strategies should be identified and developed as to how the elevated concentration of metals could be reduced or removed/detoxified in derelict soils in order to facilitate the proper growth and development of legumes.

To address heavy metal stress, even though mechanical and chemical processes can be adopted to circumvent toxicity, such traditional remediation strategies, because of one or the other reasons, do not provide acceptable solutions for the removal of metal from soils. Therefore, natural resources such as the use of plants (phytoremediation) (Hajiboland 2005; Mengoni et al. 2009) or PGPR alone (e.g., *Enterobacter aerogenes* and *Rahnella aquatilis*) (Kumar et al. 2009) or plant–microbe partnership including symbiotic nitrogen fixers and some endophytic bacteria have been considered as an alternative option to traditional methods since they possess the ability to tolerate and transform toxic to less toxic forms of metals (Rajkumar et al. 2009; Nele et al. 2009; Pajuelo et al. 2008; Castro et al. 2008). In this context, there is evidence of rhizobial bacteria promoting the growth of plants at elevated concentrations of a heavy metal via a mechanism other than improved N nutrition (Reichman 2007). The intrinsic potential of rhizobia to express high-level tolerance toward toxic metals along with their ability to transform atmospheric N in to usable form of N, makes them one of the most important organisms in agronomic practices for legume improvement in soils polluted with metals. In addition, the nodule bacteria also facilitate the growth and yield of legumes by other mechanisms, such as synthesis of siderophores and phytohormones (Wani et al. 2007e; Avis et al. 2008), synthesis of ACC deaminase to lower ethylene levels (Duan et al. 2009; Tittabutr et al. 2008), and depression of plant diseases (Khan et al. 2002). Thus, the potential use of rhizobia as growth-promoting bacteria for the remediation of heavy metal contaminated sites is an exciting and more practical area of research for the improvement of legumes in metal contaminated soils.

14.2 Heavy Metal Toxicity to Legume–*Rhizobium* Interactions

The symbiotic association between the roots of legumes and certain soil bacteria, rhizobia, accounts for the development of a specific organ, the symbiotic root-nodule, whose primary function is to transform atmospheric N to usable N (ammonia) and make it available to plants. Thus, given the importance of legumes in animal and human consumption and their use in maintaining soil fertility, attention has been given to the effects that heavy metals exert on nodule-forming rhizobia. Accordingly, toxicity of heavy metals to nodule bacteria and the legume–*Rhizobium* interaction often termed symbiosis has been one of the highly interesting subjects in biological sciences. When rhizobia are used as inoculants for legume improvement, a significantly higher yield is reported (Wani et al. 2007f; Zaidi et al. 2003) provided soil is free from any contaminants and conducive for both legume and

rhizobia. Within soil, some elements, such as heavy metals, though essential for organisms, are harmful if present in excess. For example, the increasing concentrations (50–200 mg kg^{-1} soil) of cobalt, copper, cadmium, and zinc have shown inhibitory effect on the growth, nodulation, and nitrogenase activity of *lablab perpureus* when grown in pot and under field soils treated with these metals. Moreover, the nutrient elements like Na, K, and Ca within shoots were decreased with increasing levels of metals (Younis 2007). Even though, excess metals are known to reduce the formation of root nodules in legumes, currently, little is known about how the legume–*Rhizobium* symbiosis is affected by high metal concentrations. However, the abnormally high concentrations of metal are reported to abate the water and nutrients uptake (Karpiscak et al. 2001; Terry 1981) leading thereby to a stressed situation for growing plants. In addition, after uptake by plants and its translocation to various organs, metals can directly interact with cellular components and disrupt the metabolic activities causing cellular injuries and in some cases even may lead to the death of the plants. For example, cadmium has an adverse effect on legume nodule metabolism even at low concentration. Cadmium is toxic to the microsymbiont (Younis 2007; Pereira et al. 2006b) and inhibits nitrogenase activity, affects the number and biomass of nodules, disrupt nodule ultrastructure, induce nodule senescence and dry matter accumulation in roots, shoot, leaf, and metabolic activities like photosynthesis of legumes (Noriega et al. 2007; Mumtaz et al. 2006; Wani et al. 2006; Bibi and Hussain 2005; Balestrasse et al. 2004). Furthermore, cadmium-induced oxidative stress decreases carbohydrate and soluble protein (leghemoglobin) within nodule and inhibits antioxidant enzyme activity. The increase in lipid peroxidation and thiols has also been found due to cadmium toxicity (Benavides et al. 2005; Balestrasse et al. 2003). In a recent study, the increasing concentrations of heavy metals like cadmium, zinc, and lead significantly decreased index (i.e., the number of nodules per gram of the total fresh biomass) at about 2.64 mg Cd kg^{-1}, 300 mg Zn kg^{-1}, and 130 mg Pb kg^{-1}. From this study, it was proposed that the nodulation index of white clover could serve as a suitable bioindicator of increased heavy metal toxicity in soil (Manier et al. 2009).

The changes in rhizobial populations due to high concentration of metals as well as effects of metals on legume plants are, however, conflicting (Wani et al. 2008a, b; Wani et al. 2007a, b). For example, Paudyal et al. (2007) revealed that aluminum, even in small concentration had negative effect on rhizobial growth kinetics in cultural condition, while Wood and cooper (1988) reported inhibition of multiplication of rhizobial strain at 50 μM Al concentration, as also reported by others (Broos et al. 2004; Chaudri et al. 1993). In contrast, Kinkle et al. (1987) did not find any reasonable changes in dynamics of *Bradyrhizobium japonicum* and growth and nitrogen fixation of its host plant when grown in metal contaminated soils (Smith and Giller 1992; El-Aziz et al. 1991). In other studies, the results from ^{15}N-dilution experiments for the measurement of nitrogen fixation have shown that adverse heavy metal effects were apparent on symbiotic nitrogen-fixation rates for white clover grown in inter-specific competition with ryegrass under mixed sward conditions, compared to white clover grown in pure sward. Further experiments on broad bean and pea indicated a significant, but minor-inhibitory metal-related effect on

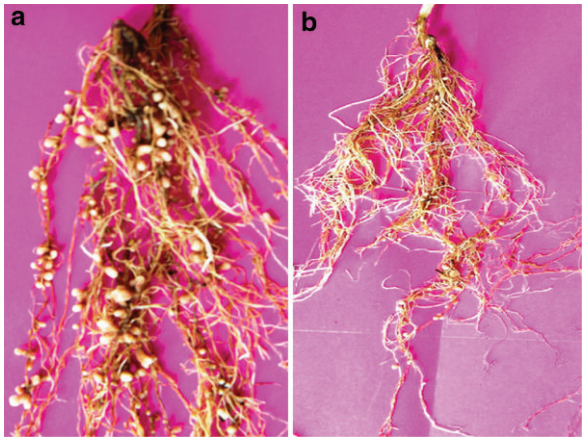

Fig. 14.1 Nodulation on root systems of legume grown in conventional (**a**) and metal-stressed (**b**) soils

the rate of nitrogen fixation compared to untreated soils and soils amended with a relatively uncontaminated sludge (Obbard and Jones 2001). Furthermore, it is suggested that there exist a relationship between *Rhizobium*'s tolerance, heavy metal soil contamination and alterations in protein pool. As a result, the analysis of protein alterations seems to be a good indicator to estimate the level of stress imposed on *Rhizobium* populations submitted to heavy-metal contamination (Pereira et al. 2006b).

Generally, the inhibitory effect of heavy metals on *Rhizobium*–legume symbiosis could probably be due to the direct toxic impact of such metals on the prognosis of nodules (Fig. 14.1) or photosynthetic pigments (Wani et al. 2006; Bibi and Hussain 2005) and Rubisco activity (Sheoran et al. 1990) or indirectly on the survival of rhizobia colonizing the root surfaces of legumes. While affecting rhizobia, such metals are known to inhibit the synthesis of biomolecules essential to growth and proliferation (Breen and Murphy 1995; Asada 1994) by generating reactive oxygen intermediates, like singlet oxygen ($^1O_2$) and hydroxyl radical (HO^*). Oxidative stress due to the cadmium treatment has been reported in pea (*Pisum sativum* L) leaves (McCarthy et al. 2001), while copper is known to interfere with oxidative enzymes in bean (*Phaseolus vulgaris*) leaves.

14.3 How to Overcome Heavy Metal Stress?

Heavy metals due to their nondegrading property are difficult to remove from contaminated soils and hence, persist indefinitely within soil environment. Heavy metals at elevated concentrations exhibit toxicity for both microbes and legumes. In order to clean up metal-poisoned soils, heavy metals should be concentrated and extracted by conventional methods for reuse or for proper disposal. A promising

option to achieve this is through phytoremediation, the use of plants to remove, destroy, or sequester hazardous substances from the environment (Cunningham et al. 1995). The legume plants, for example, alfalfa (*Medicago sativa* L.) has a high phytoremediation potential due to its ability to grow and uptake heavy metals in low pH soils (Peralta-Videa et al. 2002a, b, 2004, 2006). Some of the possibilities related with phytoremediation include phytoextraction, rhizofiltration, phytodegradation, phytovolatilization, and pytostabilization (Khan et al. 2009). The plants used to clean up metal polluted soils, however, should exhibit two basic properties (1) must be able to take up and accumulate high concentrations of metals, and (2) be able to produce a large biomass. Despite these, plants endowed with such properties have been reported to be adversely affected by the higher concentration of available metals in the contaminated soil leading thereby to a substantial decrease in plant biomass. This tendency of plants in fact challenges the efficiency of phytoremediation as a potential metal clean up approach.

The other approach to relieve the toxicity of heavy metals to plants involves the use of plant growth-promoting bacteria (Khan et al. 2009; Belimov et al. 2005). In this context, several PGPR like *Azotobacter chroococcum* HKN-5, *Bacillus megaterium* HKP-1, *B. mucilaginosus* HKK-1, *B. subtilis* SJ-101, *B. pumilus, Brevundimonas* sp. KR013, *Pseudomonas pseudoalcaligenes, P. fluorescens* CR3, *Brevibacterium halotolerans,* etc. (Zhuang et al. 2007; Abou-Shanab et al. 2008), including symbiotic nitrogen fixers like *Rhizobium leguminosarum bv. trifolii* NZP561 (Pereira et al. 2006a, b; Lima et al. 2006) and *Mesorhizobium metallidurans* sp. nov (Vidal et al. 2009) have also evolved mechanisms to circumvent metal toxicity by developing either resistance (the ability of microbes to survive in higher concentrations of toxic metals by detoxification mechanisms, activated in direct response to the presence of heavy metals) or tolerance (the ability to cope with metal toxicity by means of intrinsic properties of the microorganisms) ability (Zhuang et al. 2007). Broadly, the microbes in general protect itself from metal toxicity by one or more of the following mechanisms, like (1) efflux of metal ions outside the cell (2) extrusion – the metals are pushed out of the cell through chromosomal/plasmid mediated events (3) accumulation and complexation of the metal ions inside the cell, and (4) reduction/transformation of the heavy metal ions to a less toxic state (Khan et al. 2009; Wani et al. 2008b; Kinkle et al. 1994). For example, rhizobacteria including rhizobia produce siderophores (Rajkumar and Freitas 2008; Wani et al. 2008a, b; Wani et al. 2007e) that play an important role in sequestering metals and has more affinity to plants. Microbial siderophores are used as metal chelating agents that regulate the availability of iron in plant rhizosphere. This in turn helps plants to alleviate the toxicity of metals as reported for arsenic uptake by fern, *Pteris* taxa, *Pteris aspericaulis*, *Pteris cretica* var. *nervosa*, *P. fauriei*, *Pteris multifida*, *P. multifida* f. *serrulata*, and *Pteris oshimensis* (Wang et al. 2007). Furthermore, the release of exopolysaccharides (EPS) by nodule bacteria (Ahemad and Khan et al. 2009) is considered one of the factors involved in protection of rhizobial species from stressed environment (Lopareva and Goncharova 2007). For example, *Rhizobium etli* M4 recovered from ore rich in manganese produced larger quantities of EPS and oxidized Mn (II) to Mn (IV), the

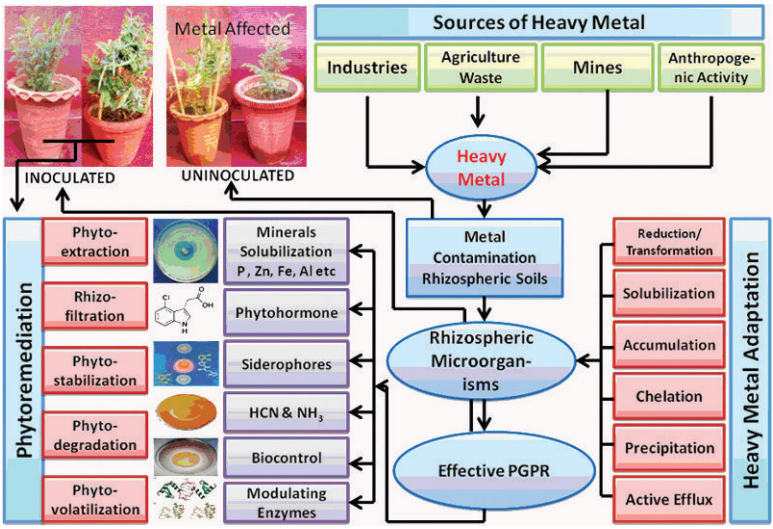

Fig. 14.2 Bioremediation strategies adopted to improve legume productivity in metal contaminated soils

latter as manganese dioxide. One of the physiological functions of the EPS was suggested to trap free Mn (II) ions, a requirement for growth. Furthermore, during growth, cells together with their EPScap, bound approximately 60% of their weight as manganese ions suggesting that the ability of *R. etli* M4 and its EPS to bind a variety of metals could be used as a tool for metal bioremediation as also reported for zinc uptake by hyperaccumulator plant *Thlaspi caerulescens* (Lopareva and Goncharova 2007). Interestingly, it has further been reported that under metal stress conditions, phytoharmones (IAA and ethylene) are released and results in increased uptake of metal ions. The search for arsenic resistance genes has enabled to admit that some *Rhizobium* isolates inhabiting contaminated soils have the genetic information that allows them to survive under such harsh conditions (Sa-Pereira et al. 2007). Considering all these, the rhizobacteria used to remediate heavy metals in association with plants (Fig. 14.2) could be modified/manipulated with three main objectives (1) helping plants to accumulate excessively higher concentrations of metals (2) reducing the uptake of metals, and (3) in situ stabilization of the metals as organocomplexes.

14.4 Performance of Inoculated Legumes in Metal-Stressed Soils

Rhizobia in association with legumes has been practiced by the progressive legume growers over the years as a viable, environmentally friendly and ecologically sound, and inexpensive alternative to widely used chemical fertilizers in order to achieve

optimum productivity of legumes in different ago-ecological systems. By forming symbiosis with legumes, the rhizobia (bacteroid) inhabiting a specialized organ, nodules, generally produced onto the root system, reduces atmospheric nitrogen into ammonia which is then taken up by plants via a process often referred to as biological nitrogen fixation (BNF). Of the two symbiotically interacting partners, rhizobia in particular is reported to tolerate high levels of metals (Wani et al. 2009) and hence, could help to remediate heavy metal polluted soils besides providing a good system to understand metal–microbe interactions (Ike et al. 2007). On the other hand, once symbiosis is established, metals may accumulate in nodules. This would be an alternative and less expensive method to remove metals from the soil. Following this, the *Rhizobium*–legume interaction has been used to remediate soils contaminated with arsenate and other metals (Nie et al. 2002). Moreover, the symbiotic relationship between leguminous plants and rhizobia could be exploited for the improvement of plant abilities by introducing genetically engineered rhizobia to plant roots. Recombinant rhizobia inside nodules of a legume are highly important as the expression of foreign genes may help to sequester metals in contaminated soil. For example, *Mesorhizobium huakuii* subsp. rengei strain B3 (Murooka et al. 2000; Nuswantara et al. 1999; Murooka et al. 1993), able to form symbiotic relationship with *Astragalus sinicus*, a legume used as green manure in rice (*Oryza sativa*) fields in China and Japan, are reported to form nitrogen-fixing root nodules (Chen et al. 1991). This plant has been found to increase N and, at the same time, to remove metals from soil. To increase the metal-binding capacity, an oligomeric metallothioneins (MTs) was constructed, which later on was used for absorption of heavy metals. The dimeric and tetrameric MTs were overproduced in bacteria and bound two and four times cadmium and zinc, respectively, than the hMT monomer. Furthermore, transformation systems were developed both in *M. huakuii* and renge-sou plant. *M. huakuii* subsp. *rengei,* which harbor the oligomeric MT gene, was infected and formed nodules on renge-sou plant. Renge-sou plant was grown in soil treated with 20–500 ppm zinc, copper, or cadmium. Zinc was accumulated in plant ten times more than cadmium, and the transport of metals within plant organs were influenced by types of heavy metals used. For example, a 10% of total cadmium was translocated to shoots, whereas the transport of zinc from roots to shoots was 50%. This finding suggested that transgenic renge-sou plant harboring the tetrameric MT gene could remove 20 times heavy metals than the wild-type plant grown in metal-stressed soil. In other studies, the gene encoding metal-binding protein, tetrameric metallothionein (MTL4) (Hong et al. 2000) or *Arabidopsis* phytochelatin synthase (PCS) (Cobbett 2000; Zenk 1996; Rauser 1995) was introduced into *M. huakuii* subsp. Rengei strain B3 (Sriprang et al. 2003, 2002) which expressed under the control of a bacteroid-specific promoter, nifH or nolB (Perret et al. 1999; Ruvkun et al. 1982). Resultant recombinant strain enhanced the accumulation of cadmium in free-living cells. The MTL4 and PCS proteins were later on detected by immune staining of bacteroids in mature nodules produced on *A. sinicus* roots (Sriprang et al. 2003, 2002).

Inoculated leguminous species are often used in the remediation of contaminated sites not only because of their ability to fix nitrogen and improve soil fertility but

also due to some other growth-promoting traits expressed by bacterial partners during symbiosis, as shown in Table 14.1. For example, after the toxic spill that occurred at Aznalcóllar pyrite mine (Southern Spain), a wide area of croplands near the Doñana Wild Park was contaminated with 4.5 million m^3 of slurries composed of acidic waters loaded with toxic metals and metalloids. And even 6 years after the spill, the concentration of toxic elements in such soils was very high despite the measures adopted to clean up the zone. However, some plant species were found to colonize this contaminated area, where nodules positive legumes dominated the crop plants. In order to remediate and making this area suitable for cultivation, *Rhizobium*–legume symbiosis was used. In this study, of the total about 100 *Rhizobium* strains, 41 strains demonstrated greater resistant to high concentrations of As (300 mg l^{-1}), Cu (100 mg l^{-1}) and Pb (500 mg l^{-1}). Following the PCR and Southern blot hybridization tools, the metal resistance genes were confirmed in

Table 14.1 Plant growth regulators synthesized by nodule bacteria and affecting growth of legumes both in conventional and stressed environment

Symbiotic nitrogen fixers	Stressor molecules	Plant growth-promoting substances	References
Rhizobia		ACC deaminase	Duan et al. (2009)
Rhizobium leguminosarum var. phaseoli		IAA	Etesami et al. (2008)
R. leguminosarum var. viciae		Gibberellic acid and IAA	Erum and Bano (2008)
Mesorhizobium sp. RC3	Chromium (vi)	IAA, Siderophore	Wani et al. (2009)
Rhizobium sp.	–	Catechole siderophores	Sridevi et al. (2008)
Rhizobium sp. strain TAL1145		ACC deaminase	Tittabutr et al. (2008)
Rhizobium spp.		IAA, GA and zeatin	Boiero et al. (2007)
Mesorhizobium loti MP6		Siderophore HCN, IAA , P-solubilizing ability	Chandra et al (2007)
Rhizobium etli USDA9032		Phenazine, Antibiotic	Krishnan et al. (2007)
Rhizobium spp.		IAA, ammonia, catalase	Joseph et al. (2007)
Rhizobium sp. RL9	Zinc	IAA, Siderophore, Ammonia, HCN	Wani et al. (2007d)
Bradyrhizobium sp. RM8	Nickel, Zinc	IAA, Siderophore, Ammonia, HCN	Wani et al. (2007e)
Rhizobium sp. RP5	Nickel, Zinc	IAA, Siderophore	Wani et al. (2007c)
B. japonicum	–	IAA	Shaharoona et al. (2006)
R. leguminosarum LARI 917		Schizokinen Siderophores	Storey et al. (2006)
*Rhizobium i*solates		HCN, P- solubilization, volatile compounds	Arfaoui et al. (2006)
R. leguminosarum		P solubilization	Alikhani et al. (2006)
Mesorhizobium ciceri BICC 651		Siderophores	Raychaudhuri et al. (2005)
R. leguminosarum bv. *viceae*.		Biocontrol activity	Bardin et al. (2004)

some of the tested strains and several of these rhizobial strains were found symbiotically effective in the contaminated soils (Carrasco et al. 2005). In other studies, rhizobial species isolated from nodules of greengram (*Bradyrhizobium* spp.), lentil (*Rhizobium* spp.), chickpea (*Mesorhizobium* spp.), and pea (*Rhizobium* spp.) have shown greater tolerance to one or more of the heavy metals and when tested under pot house environment, substantially increased the growth, symbiotic properties, and nutrient uptake of inoculated legumes (Wani et al. 2009, 2008a, b, 2007d), grown in metal treated soils. Furthermore, a substantial reduction in metal uptake by various plant organs was also observed in the inoculated legumes, which in turn decreased the metal toxicity and consequently improved the overall performance of legumes in metal contaminated soils. However, the toxicity of metals to each legume and remediating ability of rhizobia in general varied according to legume, types of metals, their concentration, and intrinsic ability of nodule bacteria. The increase in growth and yields of such legumes was attributed to metal reducing potential through adsorption/desorption mechanism of rhizobial strains (Mamaril et al. 1997) besides their ability to fix N, synthesize growth regulators, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Khan et al. 2009; Belimov et al. 2005; Uchiumi et al. 2004; Ma et al. 2003). The (ACC) deaminase induce physiological changes in plants by metabolizing ACC to ketobutyrate and ammonia (Penrose and Glick 2001), and hence lowers the toxic effects of abnormally higher concentration of ethylene on plant, which otherwise inhibits plant growth (Duan et al. 2009; Tittabutr et al. 2008; Belimov et al. 2002; Yang and Hoffman 1986). Thus, the potential of nitrogen-fixing bacteria in metal resistance/reduction and their ability to facilitate legume growth by several mechanisms other than nitrogen fixation in metal-stressed soil make them one of the most suitable choices for cleanup of the metal contaminated sites and hence may further help in reducing problems associated with the legumes when grown in derelict soils.

14.5 Conclusion

The introduction of metal tolerant rhizobial species into metal contaminated soils has facilitated the vegetative growth, nitrogen-fixing efficiency, yields, and grain quality of various legume crops. In addition, the metal tolerant rhizobia has been found to profoundly reduce the accumulation of toxic metals in plant organs, and, consequently in grains. Rhizobia due to their multifaceted activities like ability to tolerate higher concentrations of varied metals, ability to synthesize plant growth-promoting substances in addition to their intrinsic property of fixing atmospheric nitrogen, and ability to remove heavy metals from contaminated sites, could therefore, serve as an ideal inoculant for raising the productivity of legumes in metal-poisoned soils. The use of nitrogen-fixing rhizobia in decontaminating heavy metal enriched soils is hence, an evoking field of research that in turn could lead to promote the productivity of legumes in different production systems.

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Chapter 15
Legumes–Microbes Interactions Under Stressed Environments

Hamdi H. Zahran

Abstract Legumes and their associated microbes are common and exist in different environments. Microbes have evolved many mechanisms, which enable them to cope with changing environment. Resilience to these changes is essential to their survival and depends on rapid and efficient control of genetic expression and metabolic responses. Legumes establish several mutual, antagonistic, and beneficial interactions with microbes, which are occasionally subject to unfavorable (stressed) environmental conditions. Stressed terrestrial environments include, deserts with arid climate (warm and dry), salt-affected soils, alkaline and acidic soils, soils contaminated with toxic metals, and nutrient deficiency. During the course of development, microbes inherit traits that enable them to survive under undesirable conditions. Legumes, however, are stress-sensitive plants, and only few of them can withstand stressed environments. Legume rhizospheres colonized by a consortium of microbes are influenced by nutrient-rich root exudates. Legumes and microbes exhibit mutual relationships such as, association, symbiosis, and parasitism and live together in one habitat for long periods. The associated microorganisms include plant-growth-promoting rhizobacteria (PGPR), which are either nitrogen-fixing or not, and many fungi. Symbiotic organisms include mycorrhiza and the root-nodule bacteria (rhizobia). Recent molecular and genetic tools have assisted in discovering new effective stress-tolerant microbes. This chapter broadens the scope of microbes interfering with growth of legumes – a relationship that has been misunderstood to be restricted to rhizobia. Therefore, future investigations have to consider a consortium of microbes in order to improve productivity of legume crops.

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15.1 Introduction

Nitrogen (N) is one of the major liming nutrients for most crops and non-crop plant species. The acquisition and assimilation of biologically-fixed nitrogen is important but only second to photosynthesis for plant growth. Biological nitrogen fixation (BNF) involves the conversion of atmospheric N_2 to ammonium, a form of N that is easily utilized by plants. Many diverse biological associations contribute to BNF in both soil and aquatic systems. However, BNF is in the sole domain of certain bacteria (diazotrophs), which contain nitrogenase, the enzyme complex that catalyzes the conversion of gaseous N_2 to the combined form. The ability of a plant to supply all or part of its N requirements from BNF in its roots can be a great competitive advantage over non-N_2-fixing neighbors (Vessey et al. 2005). An essential element of agricultural sustainability is the effective management of N in the environment. This usually involves at least some use of biologically fixed N_2 because N from this source is used directly by the plant, and so is less susceptible to volatilization, denitrification, and leaching (Graham and Vance 2000). In most agricultural systems, the primary source of BNF (ca. 80%) occurs via the symbiotic interactions between legumes and rhizobia of the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium.* The actinorhizal (*Frankia*) and *Anabaena–Azolla* types of interactions mainly contribute the other 20%. Legumes provide approximately 35% of worldwide protein intake and that ca. 250 million ha of legumes grown worldwide. There is great potential for all legumes to increase N derived from N_2 fixation as well as to enhance the total N_2 fixed through improved management and genetic modification of the plant. Legume N_2 fixation is a variable, but valuable process in agriculture, contributing almost 20% of the N needed for world grain and oilseed production. About 100 Tg N is required annually for the production of the world's grain and oilseed crops (Herridge and Rose 2000; Sadowsky 2005).

Legumes and their bacterial-nodules evolved about 60 and 58 million years ago, respectively (Sprent 2006, 2008). Nodulation is one of the interesting characteristic features of legumes, but non-nodulation remains common in Caesalpinioideae, but lesser in Mimosoideae and Papilionoideae. Legumes are within the order Fabales and represented by a single family, the Fabaceae (formerly the Leguminosae); however, most of the more than 650 genera in the family contain species that can form nodules (Vessey et al. 2005). Nodules are highly specialized organs formed by rhizobia on roots or stems of legume plants under N limited conditions. Within nodules, rhizobia are transformed into an endosymbiotic form - the bacteroids - in which N_2 is reduced to ammonia. The legume–rhizobia symbiosis is hence, of great ecological and agronomic importance.

Optimization of the symbiosis between legumes and their respective microsymbionts (the rhizobia or nonrhizobial bacteria) requires a competitive, infective, and highly efficient N_2-fixing rhizobial strains in sufficient numbers to maximize nodulation. Over 70 species of rhizobia varying in symbiotic and physiological characteristics are now identified (Vessey and Chemining'wa 2006) and relatively

high degree of genetic diversity has been reported for rhizobia. The infection and nodulation process in rhizobia–legumes symbioses involves an intimate interaction of macro- and microsymbionts, mediated by bidirectional molecular communications between both symbionts. The rhizobia induce two types of nodules on legumes: determinate and indeterminate. The indeterminate nodules are formed most commonly on temperate legumes (e.g., pea, clover, alfalfa, etc.), inoculated with the fast-growing rhizobia, whereas determinate nodules are normally induced by bradyrhizobia on tropical legumes (e.g., soybean, common bean, etc.). Rhizobia infect host plants, and induce root- or stem-nodules, using three fundamentally different mechanisms: via root hairs, entry through wounds, cracks, or lesions, and via cavities located around primordia of adventitious roots.

Over the last two decades, advances in molecular biology and genetics have helped identify a large number of genes having symbiotic functions. In the fast-growing species, symbiosis-related genes are clustered on one or several relatively large plasmids, whereas in the bradyrhizobia, these genes are chromosomally-located (Debelle et al. 2001; Gualtieri and Bisseling 2000). Symbiotic N_2 fixation requires the coordinated interaction of two major classes of genes, the *nif* and *fix* genes. The *nif* genes encode the molybdenum-based enzyme system having structural and functional relatedness to the N_2-fixation genes of *Klebsiella pneumoniae*. In most rhizobia, *nif* genes are plasmid-borne, but located on the chromosome in the bradyrhizobia. Nitrogen fixation in symbiotic and free-living microbes is catalyzed by nitrogenase, an enzyme system encoded by the *nif*DK and *nif*H genes. Nitrogenase itself consists of a molybdenum-iron protein (MoFe), called component 1, and an iron-containing protein (Fe), called component 2. Environmentally, *nif*-gene expression is regulated by both O_2 and fixed N level. Moreover, several other genes in the rhizobia including those for exo-polysaccharide, hydrogen uptake, glutamine synthase, dicarboxylate transport, nodulation efficiency, B-1,2-glucans, and lipopolysaccharides, either directly or indirectly influence N_2-fixation (Sadowsky 2005). Legumes are genetically polymorphous for the balance between symbiotrophic and combined types of N nutrition. Wild-growing populations of legumes occasionally exceed crops in the activity of symbiotic N_2 fixation (Al-Sherif et al. 2004; Zahran 2006a). Legume species vary greatly in N_2 fixation ability and the amounts of fixed N under optimal conditions are several folds higher than the amount of N_2 usually fixed in the field. The major approaches for symbiotic N_2 fixation improvement are the selection and construction of effective rhizobial strains, and the breeding of the symbiotically active plants (Zahran 2006a, b, 2009). The amount of N_2 fixed by legumes–rhizobia symbioses may increase by 300% due to crop breeding and management practices (Vance 1998).

To advance analysis of the microbe–legume interactions, several model organisms, which provide either genomic or expressed sequence tags (EST), have been chosen – a prerequisite for large-scale protein identification by peptide fingerprinting. Two model legumes include *Medicago truncatula* and *Lotus japonicus*, which have EST databases with about 180,000 and 32,000 entries, respectively, and whose genome is being sequenced (Rolfe et al. 2003). Proteomic analysis has mainly focused on *M. truncatula*, for which a proteome reference map has been established

(Mathesius et al. 2001). On the other hand, the model symbiotic bacterium *Sinorhizobium meliloti*, able to infect both *M. truncatula* and its relative alfalfa (*Medicago sativa*), was chosen. *S. meliloti* genome consists of a 3.7 Mb chromosome and two megaplasmids of 1.4 and 1.7 Mb. The genome sequence contains 6,294 protein-coding frames, which provide a better understanding of the possible functions of *S. meliloti* (Galibert et al. 2001). However, the gene sequence alone often reveals little about the function of the gene products. Thus, functional proteomics is beginning to play a role in the identification and analysis of gene networks at the level of protein expression.

Among grain crops, pulses, or food legumes rank third after cereals and oilseeds in world production, and represent an important dietary constituent for humans and animals. Grain legumes are mainly cultivated in developing countries accounting for 61.3 million ha in 2002, compared to 8.5 million ha in developed countries (Graham and Vance 2003). Grain legumes play a crucial role in sustainability of agricultural systems and in food protein supply in developing countries (Zahran 2006b). In this chapter, various responses of legumes and their associated microbes to stressed environments is reviewed and discussed.

15.2 Arid and Saline Environments

Arid environments include desert areas characterized by water deficiency due to the atmosphere dryness and low rainfall, resulting in seriously degraded vegetation, and progressive reduction in biological diversity in the ecosystem. Vast tracts of arid and semiarid lands in the world are barren because the vegetation suffers due to water deficits. Under drying conditions, the soil water potential decreases and so does the soil hydraulic conductivity. It is more difficult for plants to extract water and, consequently, the plant water potential tends to decrease. This decrease may directly affect the physical aspects of some physiological processes. Such lands usually lack water supplies for supplemental irrigation, except ground water which is often very deep and saline and aquifers are low yielding. Despite these hostile living conditions being far from optimal, a considerable number of animal and plant species succeeded in adapting to these unhydrobiotic conditions, associated in some areas to high salinity. The rehabilitation of these degraded lands is limited to two possibilities (Tomar et al. 2003): first, the exploitation of plants native to arid environments and second, devising efficient systems for using limited saline water resources either by preventing its unproductive evaporation loss due to dry environment or drainage below rooting zone. Arid and semiarid regions offer optimal light and temperature conditions for most crops, but insufficient precipitation causes extensive reliance on irrigation. Plants developing in the Mediterranean climate (hot and dry summer), for example, are periodically subject to a combination of stresses including, not only the lack of water and high temperature coupled to high evaporative demand and high light intensity in summer but also limitation in the content of N, P, and other nutrients (Sánchez-Diaz 2001).

Irrigated lands are particularly prone to salinization, and salinity has profound effects on crop production. Reducing salinity and increasing salt tolerance of high yielding crops are becoming important global issues. Soil salinity is a wide spread problem representing the most serious forms of land degradation. It is the major cause of declining agricultural productivity and restricting plant growth and biomass production (Apse et al. 1999). In addition, excess salts in soil can bring drastic changes in some of the soil's physical and chemical properties resulting in the development of an environment unsuitable for cultivation. Soils having salts in the solution phase and/or sodium ions (Na^+) on the cation exchange sites exceeding the specified limits are called salt-affected soils. Major cations in salt-affected soils are Na^+, Ca^{2+}, Mg^{2+}, and to a lesser extent K^+. The major anions are Cl^-, $SO_4{}^{2-}$, $HCO_3{}^-$, $CO_3{}^{2-}$, and $NO_3{}^-$. These soils are generally divided into three broad categories: saline, sodic, and saline–sodic. A soil having electrical conductivity of saturated paste extract (EC_e) $\geq$ 4 dS/m and sodium adsorption ratio (SAR) <13 is called saline soil. Soils having $EC_e < 4$ dS/m and SAR $\geq$ 13 are designated as sodic soils. If a soil has $EC_e \geq 4$ dS/m and SAR > 13, it is categorized as a saline–sodic soil. Several means are used to ameliorate saline soils; cropping, in conjunction with leaching, is among those methods that are found to be the most successful and sustainable in ameliorating saline soils (Hamdy 1990; Qadir et al. 2000). About 23% of the 1.5×10^9 ha cultivated land considered saline and about half of all the existing irrigation systems of the world (3×10^8 ha) are influenced by secondary salinization, alkalization, and waterlogging. Further, about 10×10^6 ha of irrigated lands are abandoned each year because of the unfavorable effects of secondary salinization and alkalization (Dajic 2006). Approximately 400 million ha of agricultural lands throughout the world are affected by salinity (FAO 2005). The intensive irrigation of croplands under an arid climate is the main reason for secondary soil salinization in Egypt. Crops in Egypt are 100% irrigated, as precipitation is very scarce and evaporation is very high. According to government reports, almost 35% of the agricultural lands (ca. 1 M ha) in Egypt suffer from salinity, wherein the electrical conductivity of the extract from saturated soil is higher than 4 dS/m (Kotb et al. 2000). The major cause of soil salinization of the Nile Delta and Valley may include a high water table resulting from either over-irrigation or insufficient drainage system, irrigation with salty drainage and ground water, accumulation of surface runoffs in low-lying areas, and overuse of salt-generating agrochemicals (Kotb et al. 2000). Soil salinity problems usually relate to irrigation with low quality (saline) water occurring when salts accumulate in the crop-root zone and, consequently, the available water in soil for the crop is reduced. Such unfavorable soils of low fertility are generally unsuitable for agricultural production, causing unacceptable yield reduction, and in some cases, not being reasonably utilized. Because of the increased need for food production and increasing areas of salt-affected soils, research on plant responses to salinity has rapidly expanded in recent decades. The identification and use of plants adapted to saline environments is, therefore, of increasing importance if such areas are to remain productive. Recent investigations of plant tolerance to salt stress are focusing on

improvement of breeding and modification of the genetic structure of existing crops, aiming at enhanced adaptation to salinity conditions.

Saline soil contains very little N and is thus not suitable for cultivation. An appropriate solution to this situation would be cultivation of salt-tolerant plants able to fix N through symbiotic systems (Zahran 1991, 1999, 2001). However, generally considered only marginally salt-tolerant, a number of legume trees have been used in the remediation of degraded land area, including salinized soils. Examples of these legume trees are *Albizzia lebbeck*, *Acacia auriculiformis*, *Acacia farnesiana*, *Acacia nilotica*, *Acacia tortilis*, *Cassia gluca*, *Cassia javanica*, *Cassia alata*, *Dalbergia sissoo*, *Glyricidia maculate*, *Prosopis juliflora,* and *Sesbania* spp. (Sharma et al. 2001, Zahran 2001; Giri et al. 2002; Tomar et al. 2003). However, legume trees usually exhibit considerable dependence on mycorrhiza for adequate supply of P, which enable them to thrive under salt stress conditions (Giri et al. 2003). Similarly, some herb legumes, such as *Medicago intertexta* and *Melilotus indicus,* are growing naturally in salt-affected soils (Al-Sherif et al. 2004; Zahran et al. 2007) or on seashores, e.g., the halophytic herb *Canavalia rosea* (Chen et al. 2000), are salt tolerant. Thus, rehabilitation of arid soils with salt-tolerant legume tree species will not only render these abandoned soils to be productive but will also ensure conservation and improvement of these lands.

15.3 Legume–Rhizobia Associations

15.3.1 The Rhizobial Bacteria

The Rhizobial bacteria exhibit several different lifestyles. It may colonize the soil environment as well as the root–soil interface (rhizosphere), and live within the root nodule. They can live in soils either as free-living saprophytic heterotrophs or as legume-host-specific N_2-fixing symbionts. These general features give rhizobia several distinct advantages with respect to survival and persistence over most other soil bacteria. A legume host may not be needed for persistence (saprophytic competence) of rhizobia and many of the rhizobia (bacteroids) released from nodules survive and persist in the soil indefinitely as free-living, heterotrophic saprophytes until they colonize the susceptible legume host (Lindström et al. 1990). Rhizobia are traditionally known to be highly stress resistant organisms compared to their compatible host legumes (Zahran et al. 2003; Vriezen et al. 2006, 2007) and some salt-tolerant rhizobia occasionally form functional symbiosis with their hosts. Salt-tolerant rhizobia may include *Sinorhizobium* sp. from the halophytic herb *Canavalia rosea*, grown at 3.5% NaCl (Chen et al. 2000), *Mesorhizobium* strain CCNWGX035 having high tolerance to NaCl, pH, and temperature (Wei et al. 2008), the halotolerant rhizobia from seedlings of *Acacia gummifera,* and *Acacia raddiana* grown at about 6% NaCl (Essendoubi et al. 2007). Similarly, *Bradyrhizobium* sp. from lupine grew at 5% NaCl and survived at acidic (pH 4–5) and alkaline (pH 9–10) conditions (Raza et al. 2001).

Abiotic stresses such as salt, osmosis, and heat may modify the synthesis pattern of some essential cellular components (e.g., proteins and lipopolysaccharides) of the salt-tolerant rhizobia (Zahran et al. 1994). For example, the salt-tolerant *Rhizobium etli* strain (EBRI 26) formed 49 differentially expressed proteins at 4% NaCl (Shamseldin et al. 2006), of which 14 were overexpressed and 35 downregulated. Proteins induced in response to stress may have an important role in homeostasis and maintenance of vital cellular functions (Wankhade et al. 1996). The mechanisms underlying salt tolerance have, however, not been completely elucidated in rhizobia and functional aspects of salt-stress proteins (SSPs). Like proteins, the synthesis pattern of phospholipids in *Bradyrhizobium* strain (SEMIA 6144) cells were modified under saline and temperature stresses and are suggested to be involved in the bacterial response to environmental stress (Medeot et al. 2007).

Rhizobia exposed to increased salinity can maintain osmotic equilibrium across the membrane by exclusion of salts and via intracellular accumulation of inorganic and/or organic solutes (Csonka 1991). For example, *Rhizobium meliloti* overcomes osmotic stress-induced growth inhibition by accumulating compatible solutes, such as K, glutamate, proline, glycine betaine, proline betaine, trehalose, and the dipeptide, *N*-acetylglutaminylglutamine amide (Boscari et al. 2002; Vriezen et al. 2007). Some compatible solutes are used as either N or C sources by rhizobia suggesting that their catabolism is regulated to prevent degradation during osmotic stress. However, the type of osmolytes and their concentrations depend on the level of osmotic stress, growth phase of the culture, C source, and the presence of osmolytes in the growth medium (Smith et al. 1994). Many bacteria are equipped with systems that facilitate the efficient transport of osmoprotectants under stressed conditions, and several of these osmoregulated systems have been identified (Wood et al. 2001). BetS, a system involved in the uptake of proline betaine (PB) in *S. meliloti*, is a Na^+-coupled secondary transporter with high affinity for glycine betaine and proline betaine (Boscari et al. 2004). This system is activated posttranslationally by osmotic stress and plays a crucial role in the rapid response to osmotic upshock. The salt tolerance of a salt-sensitive *Bradyrhizobium japonicum* strain was improved after transformation with *bets* gene of *S. meliloti*. An increased tolerance of transformant cells to a moderate NaCl concentration (80 mM) was detected in the presence of glycine betaine or proline betaine, whereas the growth of the wild-type strain was totally eliminated at 80 mM NaCl (Boscari et al. 2004).

Adaptation of rhizobia to salt is a complex multilevel regulatory process involving many genes (Nogales et al. 2000; Wei et al. 2004). As an example, Rüberg et al. (2003) determined that the prolonged exposure of *S. meliloti* 1021 to 380 mM NaCl activated genes related to polysaccharide biosynthesis and transport of small molecules (amino acids, amines, peptides, anions, and alcohols). In this bacterium, 137 identified genes showed significant changes in gene expression resulting from the osmotic upshift; 52 genes were induced and 85 were repressed. Similarly, sudden increase in external osmolarity of *S. meliloti* cultures, elicited by addition of either NaCl or sucrose stresses, induced large number of genes having unknown functions and in repression of many genes coding for proteins with known functions (Domínguez-Ferreras et al. 2006). Of the genes upregulated, 64% were located on

plasmid (pSmbB) and 85% of the genes downregulated were chromosomal. This finding suggests the role of *S. meliloti* plasmid in osmoadaptation. Further, they reported that ribosomal genes and tricarboxylic acid cycle genes are repressed. Interestingly, 25% of all genes specifically downregulated by NaCl encode ribosomal proteins. Five salt-tolerance genes of *Sinorhizobium fredii* RT19 were identified by construction and screening of a Tn5-1063 library (Jiang et al. 2004). Na^+ intracellular content measurements established that *phaA2*, *phaD2*, *phaF2,* and *phaG2* are mainly involved in the Na^+ efflux in *S. fredii* RT19. Growth recovery of the *metH* mutants grown with different NaCl concentrations, obtained by addition of methionine, choline, and betaine, showed that the *metH* gene is probably involved in osmoregulation in *S. fredii* RT19 (Jiang et al. 2004).

Nodulation factors or Nod factors (lipochitooligosaccharides) of rhizobia are communication signals with leguminous plants and are major host-specificity determinants that trigger the nodulation program in a compatible legume host. Nod factor activities and cloning of genes required for their initiation, lead to an understanding of the first steps in signaling pathways and symbiotic interactions (Geurts et al. 2005; Mulder et al. 2005; Chen et al. 2006). Nod factors, which possess hormone-like properties, stimulate the plant to produce more *nod*-gene inducers to deform root hairs on their respective host plant, and initiate cell division in the root cortex. However, Nod factors from different *Rhizobium* species differ in the number of *N*-acetylglucose amine residues, the length and saturation of the acyl chain, and the nature of modifications on the basic backbone (e.g., sulphate, acetate, fucose, etc.). These differences define the host specificity observed in the symbiosis. The production of Nod factors and excretion of *nod* metabolites by *Rhizobium leguminosarum* bv. *trifolii* have been found to be disrupted by pH, temperature, and both P and N concentration (McKay and Djordjevic 1993). For instance, *Rhizobium tropici* strain CIAT899 grown under acid conditions formed 52 Nod factors, 37 of which differed from the 29 formed under neutral conditions (Morón et al. 2005). Under salt stress conditions, 46 different Nod factors were identified in a *R. tropici* CIAT899 culture, 14 different new Nod factor structures identified were not produced under neutral or acid conditions. High concentration of sodium enhanced *nod* gene expression (using a *nodP*::*lacZ* fusion) and Nod factor biosynthesis (Estevéz et al. 2009). Stimulation or suppression of Nod factors under stressed conditions might affect the rhizobia–legumes symbioses.

15.3.2 Legume–Rhizobia Symbioses

15.3.2.1 Effects of Water, Osmotic, and Desiccation Stresses

In nature, plants are frequently exposed to adverse environmental conditions that have a deleterious effect on their survival, development, and productivity. Drought and salinity are considered the most important abiotic factors limiting plant growth and yield in many areas of the world. Osmotic stress refers to a situation where

insufficient water availability limits growth and development of plants (Zhu et al. 1997). Soil water content directly influence growth of rhizospheric microbes by decreasing water activity below critical tolerance limit and indirectly by altering plant growth, nutrient concentration, root architecture, and exudates.

Microbial cells are able to withstand lower water potentials than most higher-plant cells. Generally, the root-nodule bacteria (rhizobia) are more resistant to soil-water deficit (drought) than the plant itself and hence, the impact of drought stress conditions on N_2 fixation might be due to direct influence on the microsymbionts (Serraj et al. 1999; Hungria and Vargas 2000). Consequently, from the beginning of infection by rhizobia until the functioning of differentiated nodules, the most important factors limiting the fixation under water stress will probably depend on the host plant. The work done on different lucerne (*M. sativa*) cultivars suggests that those adapted to dry conditions are likely to show less water stress effects on N_2-fixation than those less adapted cultivars (Aguirreolea and Sánchez-Díaz 1989). Species of rhizobia, however, differ in their susceptibility to the detrimental effects of desiccation in natural soils. For example, slow-growing rhizobia is generally thought to survive desiccation better than fast-growing rhizobia (Zahran 2001). As far as the effect of water stress on symbiosis is concerned, it affects nodule establishment, C and N metabolism, nodule O_2 permeability, nitrogenase activity, and total plant N_2 fixation ability (Zahran and Sprent 1986; Aguirreolea and Sánchez-Díaz 1989; Sadowsky 2005). However, N_2-fixation is widespread in arid land legumes (e.g., *Acacia* and *Prosopis* species) and drought tolerant rhizobial strains have been reported for both tree and crop legume species (Nijiti and Galiana 1996). Differences exist between rhizobial species, with respect to drought or osmotic stress tolerance and the capacity to infect plants and fix atmospheric N as seen in *Acacia mangium* (Galiana et al. 1998), *Gliricidia sepium* (Melchior-Marroquin et al. 1999), *Sesbania* (Rehman and Nautiyal 2002), *Albizzia adianthifolia* (Swaine et al. 2007), and *Retama raetam* (Mahdhi and Mars 2006; Mahdhi et al. 2008).

Like bacterial partners, plants may also alleviate the impact of stress (e.g., osmotic), if grown with soil microorganisms like PGPR and AM-fungi (Valdenegro et al. 2001; Ruiz-Lozano 2003). The AM-fungi have improved ability for nutrient uptake and tolerance to biotic and abioic stresses. Tree legumes form an association with AM-fungi and rhizobia. This association could further be beneficial if they are used with PGPR. In this regard, *Medicago arborea*, a leguminous tree used for re-vegetation purposes under semi-arid conditions, was inoculated either singly or in combination with microorganisms [three *Glomus* species, two strains: wild type and genetically-modified *S. meliloti*, and PGPR (Valdenegro et al. 2001)]. Mycorrhizal fungi were effective in all cases, while PGPR inoculation was only effective when associated with specific mycorrhizal endophytes (*G. mosseae* plus wild type rhizobia and *Glomus deserticola* plus genetically-modified rhizobial strain). The effect of double inoculation with two species of AM-fungi (*G. deserticola and G. intraradices*) and two strains of *S. meliloti* (wild type and its genetic variant) was examined in three *M. sativa* (*Mimosa nolana*, *Mimosa rigidula,* and *Mimosa rotata*) plants. Nodulation and mycorrhizal dependency changed in each plant

genotype in accordance with the *Sinorhizobium* strain and AM-fungi involved. Plants inoculated with both the AM-fungi and the genetically-modified *S. meliloti* were better adapted to drought stress (Vázquez et al. 2001).

AM-fungal symbiosis can also alleviate drought-induced reductions in nodule activity and senescence. The most remarkable observation was the substantial reduction in oxidative damage to lipids and proteins in nodules of mycorrhizal plants subject to drought as compared to the nodules of non-mycorrhizal plants. Mycorrhizal protection against the oxidative stress caused by drought is perhaps one of the most important mechanisms by which the AM symbiosis increases the tolerance of plants against drought (Ruiz-Lozano et al. 2001). The AM symbiosis considerably increased the glutathione reductase activity (an important component of the ascorbate glutathione cycle) both in roots and nodules of soybean plants subject to drought stress (Porcel et al. 2003). The AM-soybean plants respond to drought stress by down regulating the expression of two plasma-membrane intrinsic proteins (PIP) genes (Ruiz-Lozano et al. 2006). This is likely to be a mechanism to decrease membrane water permeability and to allow cellular water conservation. The role of AM-fungal symbiosis in the regulation of *Phaseolus vulgaris* root hydraulic properties and root plasma membrane aquaporins was evaluated under different stress (drought, salinity, and cold) conditions (Aroca et al. 2007). Hydraulic conductance and plasma-membrane intrinsic proteins (PIPs, proteins regulate the whole water transport through plant tissues) remained unchanged under various stress conditions in AM plants. The expression of each *PIP* gene responded differently to each stress and was dependent on the AM fungal presence. This finding indicates a specific function and regulation of each gene of AM symbiosis under the specific conditions of each stress tested.

The use of genetic engineering technology could lead to more effective gene-based approaches for improving crop tolerance to drought. Certain genes are expressed at elevated levels when a plant encounters stress–specific proteins such as water channel proteins, key enzymes for osmolyte biosynthesis, detoxification enzymes, and transport proteins (Vinocur and Altman 2005) are induced by abiotic stress. However, tolerance to complex stress like drought is very unlikely to be under the control of a single gene. Therefore, the successful strategy may be the use of genetic engineering to switch on a transcription factor regulating the expression of several genes related to abiotic stress (Bartels and Sunkar 2005; Chinnusamy et al. 2005). Transgenic plants over-express the *P5CS* (Δ^1-pyroline-5-carboxylate synthetase) gene from *Vigna aconitifolia*, accumulate high proline levels, and are more tolerant to osmotic stress (Kishor et al. 2005). Two *P5CS* genes have been isolated from the model legume *M. truncatula* (Armengaud et al. 2004): *MtP5CS1* (encode a developmental "housekeeping" enzyme) and *MtP5CS2* (shoot-specific osmoregulated isoform). *M. truncatula* transformed with the *P5CS* gene from *V. aconitifolia* (Verdoy et al. 2006). Over-expression of *P5CS* genes accumulates high levels of proline in tissues of *M. truncatula*, which display enhanced osmotolerance (Verdoy et al. 2006). Transgenic legume models allow analysis of some biochemical and molecular mechanisms that are activated in the nodule in response to high osmotic stress and ascertain the essential role of proline in the maintenance of

nitrogen-fixing activity under these conditions. Recent molecular investigations thus indicate the active role of proline in alleviating the effects of osmotic stress on *Rhizoium*–legume symbiosis. A transcription factor DREB 1A from *Arabidopsis thaliana*, driven by the stress inducible promoter from the *rd29A* gene, was introduced in a drought-sensitive peanut cultivar JL24 through *Agrobacterium tumefaciens*-mediated gene transfer. All transgenic events were able to maintain a transpiration rate equivalent to the well-watered control in soils dry enough to reduce transpiration rate in the wild type (Bhatnagar-Mathur et al. 2007).

15.3.2.2 Effects of Salt Stress

Salinity is considered a significant factor affecting crop production and agricultural sustainability in arid and semiarid regions of the world. Soil infertility is often due to the presence of large quantities of salt and the introduction of plants capable of surviving under these conditions, is worth investigating(Soussi et al. 1998). Salt tolerance in plants is a complex phenomenon that involves morphological and developmental changes as well as physiological and biochemical processes. Salinity disrupts cell function, through the toxic effects of specific ions and by osmotic effects, or both (Munns 2005). Specific ion effect results from a reduction in metabolic activity, due to the presence of excessive concentrations within cells, and causes plant death when a critical salinity level exceeds. Osmotic effects, however, are manifest by water deficit due to reduction in cell turgor. The complexity of the plant response to salt stress is partially explained by the fact that salinity imposes salt toxicity in addition to osmotic stress (Hasegawa et al. 2000). Sodium is toxic to many organisms, except to halotolerant organisms such as halobacteria and halophytes, which possess specific mechanisms that keep intracellular sodium concentrations low. Sodium accumulation in the cytoplasm is prevented by restricting its uptake across the plasma membrane and by promoting its extrusion or sequestration in halophytes (Hasegawa et al. 2000). Therefore, a better understanding of physiological responses under salt conditions can be of value in programs conducted to breed salt-tolerant crop varieties. In the following section, plant responses to soil salinity are discussed with emphasis on molecular mechanisms of signal transduction and on the physiological consequences of altered gene expression. Understanding the mechanisms by which plants perceive and transduce stress signals to initiate adaptive responses is essential for engineering stress-tolerant crop plants (Xiong and Zhu 2001). Thus, in addition to the existing salt-tolerant crop genotypes, research is needed to develop genotypes with increased tolerance to salinity (Qadir et al. 2000). Genetic variability within a species offers a valuable tool for studying mechanisms of salt tolerance. One of these mechanisms depends on the capacity for osmotic adjustment. A general feature of many plants growing in a saline environment is that they decrease osmotic potential by accumulation of inorganic and/or compatible solutes in their cells.

High soil salinity can limit legume productivity by adversely affecting the growth of the host plant, the development of root-nodule bacteria, and finally the

N_2-fixation capacity (Zahran 1999). Furthermore, high salinity causes suppression of photosynthesis, reduces the yield of dry mass of stems, roots, and nodules, decreases the survival of root-nodule bacteria in soil and rhizosphere, increases generation time, and disrupts the cell ultrastructure (Novikova and Gordienko 1999). The identification of tolerant genotypes that may sustain a reasonable yield in salt-affected soils has thus been a strategy adopted by scientists to overcome salinity. On the contrary, numerous reports are available that explain the formation of the symbiosis between root-nodule bacteria and various legume species under salinized soils (Soussi et al. 1998, 1999; Zahran et al. 2003). The root-nodule bacteria grown under saline conditions may have specific traits, which enable them to establish a symbiotic interaction under salt stress (Zahran 2005). For example, 15 isolates of *S. meliloti* recovered from nodules of wild species of alfalfa, melilot, and trigonella, preferably formed symbiosis with a salt-tolerant legume grown in both salinized and nonsalinized soils (Ibragimova et al. 2006). It appears that the efficiency of symbiotic interaction under salinized conditions depends on the symbiotic efficiency of the isolates under standard conditions, but this did not correlate with the source of nodule bacteria (soil or nodule) or their salt tolerance.

Legume trees such as *Acacia*, *Prosopis*, *Sesbania,* and legume herbs such as *Melilotus* and *Medicago*, are salt-tolerant (Shamseldin and Werner 2005; Zahran et al. 2007). These legumes establish a symbiotic association with a wide range of rhizobia (*Rhizobium*, *Mesorhizobium*, and *Sinorhizobium*), welladapted to the drastic conditions of arid climates (Marcar et al. 1991; Räsänen and Lindström 2003; Nguyen et al. 2004). Grain legumes recognized as either sensitive or only moderately tolerant to salinity: *Cicer arietinum*, *Lens culinaris*, *P. vulgaris,* and *Pisum sativum* are sensitive to salt stress, while *Glycine max* and *Vicia faba* plants are particularly moderate salt-tolerant grain legumes (Zahran and Sprent 1986; Ashraf and Waheed 1990; Bouhmouch et al. 2005, Phang et al. 2008). The high sensitivity of the legume–*Rhizobium* symbiosis to salinity has been recognized, and the necessity to develop salt-tolerant symbioses has been emphasized (Sprent and Zahran 1988; Zahran 2005; Ibragimova et al. 2006; Zahran 2009). The limitation of N_2 fixation imposed by environmental factors could be resolved through the selection and breeding of improved legume cultivars. On the other hand, the unsuccessful symbiosis under salt stress may be due to a failure in the establishment of rhizobia populations in the rhizosphere, the failure of the infection process, and the inhibition of nodulation (Sprent and Zahran 1988; Bouhmouch et al. 2005). A best symbiotic N_2 fixation under salinity conditions is achieved if both symbiotic partners, as well as the different steps of their interaction (recognition, root colonization, infection, nodulation, and nitrogen fixation), are all tolerant to the imposed stress factor.

Annual pasture legumes and the naturally-growing annual herb legumes include salt-tolerant species, which usually adapt to increasing soil salinity. Al-Sherif et al. (2004) and Zahran et al. (2007) reported the existence of *M. indicus* and *M. intertexta* in salt-affected lands of Egypt. Some annual pasture legumes (e.g., *Melilotus siculus* and *Medicago polymorpha*) persist in saline soils ($EC_e > 8$ ds/m) of Australia (Boschma et al. 2008; Nichols et al. 2008). The ability to germinate and

establish seedlings on saline lands is particularly important for annual pasture legumes; however, this point has received less attention compared to the work related to mature plants (Rogers et al. 2008). Seed germination of *T. subterraneum* and *Trifolium michelianum* was significantly reduced by about 50% at 110 mM NaCl (Rogers and Noble 1991), while *M. siculus* had no significant reduction in germination at 200 mM NaCl (Marañón et al. 1989). The mechanism of salinity tolerance and avoidance at germination of five self-regenerating annual pasture legumes of Mediterranean origin in Australia was studied (Nichols et al. 2009). The maximum NaCl concentrations, for which no reduction in germination percentage occurred, were 300 mM for *M. siculus*, 240 mM for *M. polymorpha*, and 120 mM for *T. subterraneum*, *Trifolium tomentosum*, and *Trifolium michelianum*. The results emphasized that *M. siculus and M. polymorpha* are among adapted annual pasture legumes for highly saline soils. The tropical pasture legume (*Stylosanthes humilis*) is a salt-sensitive legume, though significant differences in salt-tolerance were found between populations. The estimated concentrations that reduced shoot dry mass by 50% and 25% varied between populations from 84 to 108 and from 49 to 83 mM NaCl, respectively (Lovato et al. 1999). Populations from arid climate with saline soils show higher salt tolerance than those from nonsaline soils. In another study, salinity affected the germination, survival of seedlings, dry matter accumulation and yield of lentil, *L. culinaris* (Karterji et al. 2001). In soil with an EC_e of 2 dS/m and 3 dS/m (slightly saline soil), yield reduction in *L. culinaris* was about 20% and 90–100 %, respectively. Soybean is an important crop, and its productivity (growth, nodulation, and yield) was significantly hampered by salt stress. The final yield of soybean which is classified as a moderate salt-tolerant crop, reduced when salinity exceeded 5 dS/m (Ashraf 1994). Soybean production was inhibited by 52.5% and 61% when grown under moderate (14–15 dS/m) and high (18–20 dS/m) soil salinity, respectively (Chang et al. 1994). To cope with salt stress, soybean developed several tolerance mechanisms including maintenance of ion homeostasis, adjustment in response to osmotic stress, restoration of osmotic balance, and other metabolic and structural adaptations (Phang et al. 2008).

Under stress, plants maintain low concentration of Na^+ and high concentration of K^+ in the cytosol. However, Na^+ toxicity is not only due to toxic effects of Na^+ in the cytosol, but also because K^+ homeostasis is disrupted possibly due to the ability of Na^+ competing for K^+ binding sites. Plants possess a number of mechanisms to prevent accumulation of Na^+ in the cytoplasm that include minimizing Na^+ influx, intracellular compartmentation of Na^+, and maximizing Na^+ efflux as well as precirculation of Na^+ out of the shoot by the phloem (Ward et al. 2003; Bartels and Sunkar 2005). Salt tolerance correlates to an efficient Na^+ and Cl^- exclusion mechanism and to a better maintenance of leaf K^+ concentration at high levels of external NaCl (Sibole et al. 2003; Garthwaite et al. 2005). The tree legume, *A. nilotica*, however, exhibited different salt tolerance mechanism, which enabled the adjustment of osmotic potential by accumulation of Na^+, K^+, Cl^-, and proline under salt stress (Nabil and Coudret 1995). Salt-tolerant plants achieve the Na^+–K^+ balance in the cytosol by regulating the expression and activity of Na^+ and K^+ transporters and H^+ pumps that generate the driving force for transport (Zhu 2003).

Na^+ transporters, include the NHX and SOS families (salt overly sensitive) of Na^+/H^+ exchangers, HKT proteins, as well as components of the signaling pathway that regulate these transporters, such as SOS2 and SOS3 proteins (Horie and Schroeder 2004; Pardo et al. 2006). Proper regulation of ion flux is necessary for cells to maintain low concentrations of toxic ions and to accumulate essential ions. The vacuolar sodium sequestration is mediated by an Na^+/H^+ antiport at the tonoplast. Sequestration or compartmentalization of Na^+ into the vacuole through vacuolar Na^+/H^+ antiporters uses the proton motive force generated by the vacuolar H^+-translocating enzymes, H^+-adenosine triphosphate (ATPase), and H^+-inorganic pyrophosphatase (PPiase), to couple the downhill movement of H^+ with the uphill movement of Na^+ against the electrochemical potential (Blumwald and Gelli 1997). The presence of Na^+/H^+ antiporter activities has been physiologically characterized in tonoplast vesicles and is molecularly represented by six *Arabidopsis* genes *AtNHX1-6* (Blumwald et al. 2000; Yokoi et al. 2002). The first Na^+/H^+ exchanger identified was *AtNHX1*, a member of a family of six genes (*AtNHX1–AtNHX6*) that show sequence homology to mammalian and yeast NHE or NHX exchangers, respectively (Yokoi et al. 2002). Many reports have indicated the existence of Na^+/H^+ antiporters in plant vacuoles (Blumwald et al. 2000; Zörb et al. 2005). Several studies dealing with the occurrence, expression, and activity of Na^+/H^+ antiporters and NHX genes under salt stress were reviewed (Zahran et al. 2007). Overexpression of *AtNHX1* enhances salt tolerance in crop plants (e.g., tomato, rice, cotton, sugar beet, barley, sunflower, wheat, and maize) as well as in some halophytic plants (*Atriplex*, *Suaeda,* and *Thellungiella*) and this antiporter catalyzes both Na^+/H^+ and K^+/H^+ exchange. Among legumes, a vacuolar antiporter (*MsNHX1*) was cloned from alfalfa whose gene was induced by NaCl and ABA treatments (Yang et al. 2005). The involvement of Na^+/H^+ transporters in *M. intertexta* and *M. indicus*, growing in salt-affected cultivated soils of Egypt (Zahran 1998; Al-Sherif et al. 2004) have been investigated. NaCl induced gene expression of three genes in *M. intertexta* and one gene in *M. indicus*. *NHX* gene triggered in *M. intertexta* plants to cope with tissue Na^+ accumulation, while in *M. indicus*, the absence of Na^+ accumulation and the lack of induction of *NHX* genes in response to NaCl indicated that this species relied on different mechanisms to cope with salt stress.

Under stress conditions, proline, glycine betaine, sucrose, mannitol etc., are switched on to protect major processes such as cell respiration, photosynthetic activity, nutrient transport, and N and C metabolism (Zhu 2002). Trehalose (a nonreducing disaccharide found in a wide variety of organisms, including bacteria and plants) plays an important role as an abiotic stress protectant, stabilizing dehydrated enzymes and membranes as well as protecting biological structures from desiccation damage (Benaroudj et al. 2001; Sampedro and Uribe 2004). Accumulation of trehalose in crop plants improved their tolerance to drought and salinity (Romero et al. 1997). A significant increase in trehalose content was detected in nodules (bacteroids) of soybean (Müller et al. 1994), *M. truncatula,* and *L. japonicus* (López et al. 2008) in response to salt stress. These findings support the possible role of this disaccharide as an osmoprotectant against abiotic

stress. Plant growth parameters and nitrogenase activity decreased in nodules of the model legumes (*M. truncatula* and *L. japonicus*) after treatment with 50 mM NaCl (López et al. 2008). Carbon metabolism in the *L. japonicus* nodule was less sensitive to salinity than in *M. truncatula*, as enzymatic activities responsible for C supply to the bacteroids to fuel nitrogen fixation such as sucrose synthase (SS), alkaline invertase (AI), malate dehydrogenase (MDH), and phosphoenolpyruvate carboxylase (PEPC), were less affected by salt than the corresponding activities in *M. truncatula*.

Cytokinins are a group of adenine derivatives that play a major role in many aspects of plant growth and development. Exogenous cytokinins induce cortical cell divisions in legume roots and the expression of several nodulin genes, thus enhancing legume–*Rhizobium* symbiosis (Jimenez-Zurdo et al. 2000; Mathesius et al. 2000; Gonzalez-Rizzo et al. 2006). Cytokinin levels decrease under adverse environmental conditions. However, application of exogenous cytokinin counteracts the negative physiological effects of salt stress (Hare et al. 1997). A new cytokinin receptor homolog (*MsHK1*) was induced in *M. sativa* seedlings by exogenous application of the cytokinin trans-zeatin (Coba De La Peña et al. 2008). *MsHK1* expressed in roots, leaves, and nodules of *M. sativa* under salt stress, and transcript accumulation in the vascular bundles pointed to a putative role in osmosensing for *MsHK1* receptor homolog (Coba De La Peña et al. 2008). Similarly, exogenous abscisic acid (ABA) pretreatment to plants subject to salinity improved growth parameters and ameliorated the effects of salt on nodule weight and nitrogenase activity of a salt-sensitive cultivar of the common bean (*P. vulgaris*) (Mills et al. 2001; Khadri et al. 2007). ABA treatment seems to limit sodium translocation to shoot resulting in the maintenance of high K^+/Na^+ ratio in salt-stressed plants. Therefore, ABA may function as a stress signal and play an important role in the tolerance of plants to salinity.

Molecular studies of rhizobia–legumes symbioses under stressed environments have sparked increasing interest in recent years. In this context, several kinds of genes or markers associated with stress tolerance have been identified in both bacteria and host legumes (Nguyen et al. 2004). For example, the *typA* gene (an orthologue of *typA*/*bipA* genes found in a wide range of bacteria and which is required for general housekeeping functions) of *S. meliloti* was described (Kiss et al. 2004). The *typA* gene is required for the establishment of nitrogen-fixing symbiosis with certain *M. truncatula* and *M. sativa* cultivars (Kiss et al. 2004). The *typA* gene is required for survival of *S. meliloti* under certain stress conditions such as growth at low temperature or low pH and in the presence of sodium dodecyl sulphate (SDS). In a recent study, Patankar and González (2009) explored the regulatory role of SMc04032 locus, named as nesR (one of the orphan LuxR-type response regulators): it causes the bacteria to cope with specific nutritional, environmental, and stress conditions. Through expression and phenotypic analysis, *nesR* was determined to affect the active methyl cycle and to influence nutritional and stress response activities in *S. meliloti*. These results suggest that *nesR* potentially contributes to the adaptability of *S. meliloti* when it encounters challenges such as high osmolarity, nutrient starvation, and/or competition for nodulation, thus

increasing its chances for survival in the stressful rhizosphere. For plants, many genes encoding PR-5 proteins (proteins known to function as protein-based defensive system against abiotic and biotic stress) have been identified from a variety of plants, indicating that PR-5 is broadly distributed throughout higher plants. The involvement of PR-5 proteins in protection against abiotic stresses, such as osmotic imbalance has been suggested (Kononowics et al. 1992). Novel soybean genes, *GmOLPA* and *GmOLPB* (*G. max* osmotin-like protein), encoding an acidic homolog of PR-5 protein (Onishi et al. 2006) and neutral homolog PR-5 protein (Tachi et al. 2009), respectively, were highly induced in the leaves of soybean plants under conditions of high salt stress. An alfalfa cDNA library was induced by salt stress constructed by suppression subtraction hybridization (SSH) technology (Jin et al. 2009). 119 positive clones were identified by reverse Northern dot-blotting resulting in 82 uni-ESTs. Most of the annotated sequences were homologous to genes involved in abiotic or biotic stress in plants. In addition, several ESTs, similar to genes from other plant species, closely involved in salt stress were isolated from alfalfa, such as aquaporin protein and glutathione peroxidase.

The production of reactive oxygen species (ROS) is yet another major damaging factor, which disrupts normal metabolism through oxidative damage of lipids and proteins in plants exposed to different environmental stresses. Plants with high concentrations of antioxidants (e.g., ascorbate peroxidase APOX, catalase CAT, peroxidase POD, and superoxide dismutase SOD), have greater resistance to these oxidative damages (Jiang and Zhang 2002). Nodules are particularly rich in both quantity and diversity of antioxidant defenses that may protect the nodule structures from high rates of nodule respiration, as well as, conserve nitrogenase activity (Becana et al. 2000; Blokhina et al. 2003). Nitrogenase is O_2 sensitive; therefore, nodules have evolved mechanisms to downregulate their permeability to O_2 and maintain the infected cell O_2 concentration at approximately 5–50 nM compared to 250 μM for cells in equilibrium with air (Minchin 1997). Salinity induces the production of stress proteins or antioxidant enzymes in nodules to minimize damage caused by ROS such as, H_2O_2, O_2, and OH (Porcel et al. 2003). Salt stress (50 mM NaCl) or osmotic stress (50 mM mannitol) reduced plant growth, nitrogen fixation, and the activities of the antioxidant defense enzymes of common bean (*P. vulgaris*) nodules (Tejera et al. 2004; Jebara et al. 2005). The maintenance of sucrose synthase, together with isocitrate dehydrogenase, associated with a suitable antioxidant defense may be relevant for osmotic tolerance in *P. vulgaris* N_2 fixation (Sassi et al. 2008). The performance and responses to osmotic stress (50 mM mannitol) have been evaluated recently in chickpea-*Mesorhizobium* symbiosis (Mhadhbi et al. 2008). Nodular POX and APOX activities were significantly enhanced in chickpea plants under osmotic stress. The increase of POX and APOX inversely correlated with the inhibition of aerial biomass production and nitrogen-fixing capacity, suggesting a protective role for these enzymes in nodules. In a similar report, salinity (75 mM NaCl) significantly increased the nodule conductance in four genotypes of *S. meliloti* inoculated *M. truncatula* plants (Aydi et al. 2004). Thus, sensitivity to salinity appears to be associated with an increase in nodule conductance that supports the increased respiration of N_2-fixing nodules under salinity. In contrast,

salinity did not change the nodule conductance and nodule permeability of the salt-tolerant variety of chickpea (L'taief et al. 2007). Salt tolerance of this variety appears to be associated with stability in nodule conductance and the capacity to form nodules under salt constraint. Nodule conductance to O_2 diffusion has been found to be a major factor in the inhibition of N_2 fixation by salinity that severely reduces the production of legumes.

15.3.2.3 Effects of pH and Temperature

Acid soils limit agriculture production, and as much as 25% of crops suffer from soil acidity (Munns 1986). Tolerance to acid conditions in rhizobia often correlates to the strain's ability to maintain internal pH approaching neutrality (Graham et al. 1994). Generally, bradyrhizobia are more acid-tolerant than rhizobia (Brockwell et al. 1991; Sadowsky and Graham 1998), although some strains of *R. tropici* are very acid-tolerant (Graham et al. 1994) due to the production of glutathione to grow in extreme acid stress conditions (Riccillo et al. 2000). Using Tn5 mutagenesis, acid-sensitive mutant of *S. meliloti* was isolated and some genes involved in acid tolerance have been characterized (Tiwari et al. 1996). Rrhizobia are sensitive to acidity (Hungria and Vargas 2000), but acidity also influences both the growth of the legume plant and the infection process (Munns 1986). This effect is, in part, most likely due to a disruption of signal exchange between macro- and microsymbionts (Hungria and Stacey 1997) and repression of nodulation genes and excretion of Nod factors in the rhizobia (Richardson et al. 1988). Stress parameters such as soil acidity affect rhizobial persistence, nodulation efficiency and N_2 fixation of some legumes (Graham and Vance 2000). Rhizobial strains nodulating *P. vulgaris* under arid conditions were analyzed for pH tolerance (Priefer et al. 2001). One strain (RP163) exhibiting high nodulation efficiency and broad pH tolerance was mutagenised by Tn5 and the resulting mutants unable to grow on extreme pH media were isolated. In these mutants, a suitable well-characterized promoter is now available to drive expression of rhizobial stress-tolerance genes. In a similar approach, promoters and genes inducible under extreme pH values were identified in *R. leguminosarum* bv. *viceae* VF39 (Priefer et al. 2001) - among them *gabT* encodes the GABA (γ-aminobutyrate) transaminase which is induced under acidic conditions.

Soil nutrient status has a tremendous influence on rhizobium–legume symbiosis. A nutrient stress is indirectly caused by changes in soil matric potential or acidity, which in turn limit nutrient bioavailability, rather than to the lack of the presence of nutrients per se (Sadowsky 2005). Stress conditions apparently increase requirements for essential elements, such as Ca^{2+}, P, and N, in both plants and microbes. The presence of Ca^{2+} may offset the deleterious influence of low pH on root growth while ion uptake increases *nod*-gene induction and expression, and concurrently affects the attachment of rhizobia to root hairs and nodule development (Richardson et al. 1988; Alva et al. 1990; Smit et al. 1992). Phosphorous (P) availability is another limiting factor for N_2-fixation and symbiotic interactions (Saxena and

Rewari 1991) and about 33% of the arable land in the world is P deficient, especially in low pH soil (Graham and Vance 2000). There are marked differences in rhizobial and plant requirements for P and the slow-growers are more tolerant to low P than the fast-growing rhizobia (Beck and Munns 1985).

High soil temperature has a marked influence on survival and persistence of rhizobial strains in temperate climate (Boumahdi et al. 2001). However, strains from naturally-growing legumes in tropical regions survive better at higher temperatures (Zahran et al. 1994). The influence of temperature on rhizobia appears to be strain dependent. For example, *Bradyrhizobium* sp. (lupine) was less susceptible than *R. leguminosarum* bv. *trifolii* to high soil temperature (Sadowsky 2005). However, rhizobial strains at elevated temperatures lose infectivity (Segovia et al. 1991). Moreover, excessive temperature shock cures plasmids in fast-growing strains, and some strains which were isolated from warm environments, had a Fix^- phenotype (Moawad and Beck 1991; Hungria and Franco 1993). Soil temperature greatly influences competition for nodulation (Triplett and Sadowsky 1992). However, some high-temperature (up to 40°C)-tolerant rhizobia formed effective nitrogen-fixing nodules with *P. vulgaris* (Hungria et al. 1993; Michiels et al. 1994), *Prosopis* (Kulkarni and Nautiyal 1999), and *Acacia* (Zerhari et al. 2000). Each *Rhizobium*–legume combination has an optimum temperature relationship around 30–40°C; exposure of both symbiotic partners to temperature extremes much above or below these critical temperatures impairs infection, nodulation, nodule development, and general nodule functioning as well as plant growth and productivity (Michiels et al. 1994). Elevated temperatures directly influence the production or release of *nod*-gene inducers as reported for soybean and bean (Hungria and Stacey 1997) where it altered nodule functioning particularly leghemoglobin synthesis, nitrogenase activity, and H_2 evolution, and in addition, hastened nodule senescence (Hungria and Vargas 2000). Therefore, to obtain most competitive and effective bacterial strains, bacteria need to be isolated and screened from the pool of indigenous microbes that could adapt to a wide range of climatic conditions and hence increase growth and enhance nutrient uptake by plants in disturbed soils.

15.3.3 Effects of Metal Toxicity

Worldwide, contamination of soil and ground water by heavy metals is a severe problem. Soil contamination is a particularly serious environmental concern, as the majority of superfund sites are highly contaminated with heavy metals. To remediate such contaminated sites, conventional remediation methods such as, soil excavation followed by coagulation-filtration or ion exchange are applied. Such approaches are however, expensive and disruptive to the sites. On the contrary, in situ bioremediation is gaining momentum as it is a low-cost and effective method for restoration and remediation of polluted site (Khan et al. 2009a). In this context, the use of plants for rehabilitation of heavy-metal-contaminated soils is an emerging area of interest because it is an ecologically sound and safe method (Wu et al.

2006). During rhizoremediation, exudates released from plants can help stimulate the survival and action of bacteria, which subsequently results in a more efficient degradation of pollutants (Kuiper et al. 2004). The root system of plants helps spread bacteria through soil and facilitate penetration to otherwise impermeable soil layers. A suitable solution is to combine the advantages of microbe–plant associations in soil into an effective cleanup technology.

Soils contaminated with heavy metals present a major threat to sustainable agriculture, and legumes growing in these environments suffer heavily from metal toxicity. The effects of heavy metals, like, copper, cadmium, and chromium, used both separately or as mixtures, on growth of pea (*P. sativum*) inoculated with *Rhizobium* sp. was studied (Wani et al. 2008a). Copper was the most toxic of the three metals for pea plants and decreased seed yield by about 15%. Nevertheless, in another study (Wani et al. 2008b) some species of pea-nodulating *Rhizobium* proved to be tolerant to nickel and zinc. This study suggested that the intrinsic ability of N_2-fixation, growth promotion, and the ability to reduce toxicity of nickel and zinc of the tested strain could be of practical importance in augmenting the growth and yield of pea in polluted sites (Wani et al. 2008b). Furthermore, an expression of a metal-binding peptide (EC20) in *Pseudomonas putida* 06909 not only improved cadmium binding but also alleviated the cellular toxicity of cadmium (Wu et al. 2006). Arsenic (As) contamination of natural resources is a global environmental problem. Arsenic-contaminated ground water was reported in over 20 countries (Reichman 2007). Legumes have, however, been identified as naturally occurring pioneer species on arsenic-contaminated sites, and free-living rhizobia are commonly found in soils with high arsenic content (Macur et al. 2001; Carrasco et al. 2005). Legumes and their symbiotic rhizobia are often desirable species, during and after the remediation of arsenic-contaminated lands. For example, excess As reduced the formation of root-nodules and dry weight of roots and shoots of soybean plants (Reichman 2007). However, inoculation of soybean plants by *B. japonicum* had significantly larger dry weights than noninoculated soybean plants. It is hypothesized that *B. japonicum* stimulated the growth of soybean via the production of growth-promoting hormones at elevated concentrations of a heavy metal via mechanisms other than improved nitrogen nutrition. Therefore, the potential use of rhizobia as growth promoting bacteria for the remediation of heavy-metal contaminated sites is an exciting new area of research.

15.4 Legume–Bacteria Associations Under Stressed Environments

Rhizosphere microorganisms influence plant growth, development, productivity, and environmental adaptation. The inoculation with bacterial mixtures provides a more balanced nutrition and improves nutrient uptake by plants (Belimov et al. 1995; El-Komy 2005). The use of beneficial microbes in agriculture production

systems was started about 60 years ago and there is now increasing evidence that it can enhance plant resistance to adverse environmental stresses (Sheng 2005). Among heterogeneously distributed microbes in soils, plant growth promoting rhizobacteria (PGPR) facilitate plant growth and development directly or indirectly (Khan et al. 2009b). Direct stimulation may include providing plants with nutrients through nitrate reductase activity and nonsymbiotic N_2-fixation, phytohormones (indole acetic acid, zeatine, gibberellic acid, and abscisic acid), iron sequestered by bacterial siderophores, and soluble P. Indirect stimulation of plant growth includes preventing phytopathogens, allelopathy, antibiotic production, and competition with deleterious agents (Egamberdiyeva and Islam 2008).

In the rhizosphere of legumes, there are abundant nonsymbiotic rhizobia, which are not able to infect plants but which play a significant role in the rhizosphere of plants. Strains of rhizobia within a single species can have three different genetically determined strategies (Denison and Kiers 2004): mutalistic rhizobia provide N to their legume hosts, parasitic rhizobia infect legumes, but fix little or no N, and nonsymbiotic strains unable to infect legumes at all. Successful growth of legumes at various environments is not dependent only on the symbiotic activities of rhizobia, but may be stimulated by other PGPR. Of the most significant PGPR is the genus *Azospirillum*, a free-living, surface colonizing (sometimes living as endophyte) diazotroph. *Azospirillum* bacteria are capable of increasing the yield of important crops growing in various soils and climatic regions and a significant increase (5–30%) in the yield has been reported (Castro-Sowiniski et al. 2007). *Azospirillum* inoculation improves root development and enhanced water and mineral uptake due to the secretion of indole-3-acetic acid (Spaepen et al. 2007). Many reports have focused on the ability of *Azospirillum* species to promote plant growth and increase agricultural productivity through certain mechanisms that act additively or synergistically with BNF to enhance the overall performance of plants. For example, *Azospirillum* significantly improved yield of legumes when coinoculated with other effective, N_2-fixing bacteria. It has been shown (Rodelas et al. 1996, 1999) that dual inoculation of *Rhizobium* with *Azospirillum* and other PGPR (e.g., *Azotobacter*) significantly increased nodulation and N_2-fixation of legumes (e.g., *V. faba*). Inoculation of chickpeas and faba bean with *Azospirillum brasilense* has shown to significantly reduce the negative effects on growth and nodulation caused by irrigation with saline water (Hamaoui et al. 2001). During interactions, rhizobia synthesize lipochitooligosaccharides (LCOs), also called Nod factors, consisting of approximately 2–60 different individual structures (D'Haeze and Holsters 2002). Nod factors allow rhizobia to enter the root and cortical cells, and induce nodulin gene expression and cell division, leading to nodule formation (Cooper 2007). In a follow up study, the effects of *A. brasilense* inoculation on plant growth, nodulation, and production of flavonoids and LCOs was reported for a *Rhizobuim–P. vulgaris* interaction under salt stress (Dardanelli et al. 2008). *A. brasilense* promoted root branching in seedlings of *P. vulgaris* and increased secretion of *nod* gene-inducing flavonoid species. The negative effects detected under salt stress on gene expression and on Nod factor production were relieved in coinoculated plants. Moreover, insoluble P compounds in the rhizosphere are

converted into available P for plant uptake by bacteria. A range of bacterial genera, including *Bacillus, Mesorhizobium, Pseudomonas, Rhizobium,* and *Sinorhizobium,* are active acid producers and involved in P solubilization (Rodriguez and Fraga 1999; Zaidi et al. 2009). The effects of the P-solubilizing *P. putida* on the symbiosis between rhizobia and legumes (e.g., soybean and alfalfa), usually grown in arid climates, were investigated (Rosas et al. 2006). Modification of shoot and root system dry weights occurred in soybean but not in alfalfa in the presence of *Pseudomonas* strains. A greater number of nodules and dry weight were recorded for soybean when coinoculated with *P. putida* and *B. japonicum*. In addition to N_2 fixation and phytohormone biosynthesis, *A. brasilense* produces specific polyamines. Among polyamines, cadaverine (1,5-diaminobentane) has been identified in *A. brasilense* and some α-proteobacteria (Bohin et al. 2005; Perrig et al. 2007). Cadaverine correlates with root growth promotion and osmotic stress mitigation in some plant species, like *V. faba* (Liu et al. 2000), *Lactuca sativa* (Barassi et al. 2006), and *Oryza sativa* (Cassán et al. 2009).

Certain PGPR produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which regulate ethylene production by metabolizing ACC (an immediate precursor of ethylene biosynthesis in higher plants) into α-ketobutyrate and ammonia (Glick 2005). Bacterial strains containing ACC deaminase alleviates stress-induced ethylene-mediated negative impact on plants (Safronova et al. 2006). PGPR containing ACC deaminase activity sustains plant growth and development under stress conditions by reducing stress-induced by ethylene production (Saleem et al. 2007). Some rhizobacteria (e.g., *Bacillus* species) associated with plants in saline soils, grew and fixed N_2 at 5% NaCl (Zahran et al. 1995; Egamberdiyeva and Isalm 2008). Seed inoculation with the salt-tolerant bacteria *B. japonicum*, *Bacillus polymyxa*, *Bacillus amyloliquefaciens*, *Mycobacterium phlei*, and *Pseudomonas alcaligenes* significantly increased shoot growth, root length, uptake of N, P, and K, and yield of soybean, pea, and wheat as compared to the control (Egamberdiyeva and Hoflich 2003).

15.5 Legume–Fungal Associations under Stressed Environments

Besides N, phosphorus availability is very important for crop productivity. One of the benefits of the Arbuscular mycorrhizal (AM) fungi is the improvement of P uptake by the plant. AM-fungi effect on plant water status has also been associated with improved host nutrition, particularly P. Better understanding of the interactions between AM-fungi and other microorganisms is necessary for the development of sustainable management of soil fertility and crop production. The implication of these interactions on sustainable agriculture has been reviewed (Johansson et al. 2004). Nitrogen-fixing bacteria clearly have the potential to influence AM-fungi. *Rhizobium* species may act synergistically with AM-fungi on their plant hosts. Further intracellular interactions could be important because

they allow rapid exchange of energy and nutrients between plant roots, mycorrhizal fungi, and associated bacteria (Johansson et al. 2004).

Arbuscular mycorrhizal fungi have been shown to promote plant growth and salinity tolerance mainly by enhancing nutrient acquisition, producing plant growth hormones, improving rhizospheric and soil conditions, altering host physiological and biochemical properties, and defending roots against soil-borne diseases (Ghorbanli et al. 2004; Rabie 2005). AM-fungi protect plants against salt stress via better access to nutritional status and plant physiology modification (Rabie and Almadini 2005) and are considered as bio-ameliorators of saline soils (Yano-Melo et al. 2003; Tain et al. 2004). In saline environments (e.g., saline–alkali soils), vesicular AM plant root colonization is host-dependent and significantly affected by various amendments (e.g., PGPR amendments) given to reclaim such soils (Raghuwanshi and Upadhyay 2004). Double inoculation with rhizobia and an endomycorrhizal complex increased tolerance of *Acacia cyanophyla* plants to salinity (Hatimi 1999). The leguminous plants possessing high levels of vesicular AM colonization (50–70%) in saline–alkali soil included *A. nilotica*, *A. lebbeck, and D. sissoo* (Raghuwanshi and Upadhyay 2004). Mycorrhhizal seedlings of two species of *Sesbania* (*Sesbania aegyptica and Sesbania grandiflora*) had significantly higher root and shoot dry biomass, chlorophyll content, nodule number, and increased concentrations of P, N, and Mg^{2+}, but lower Na^+ concentration, than nonmycorrhizal seedlings (Giri and Mukerji 2004). Mycorhizal fungus (*Glomus fasciculatum*) alleviated the deleterious effects on growth of *A. nilotica* plants grown in saline soils that might be related to improved P nutrition (Giri et al. 2007). The reduction of Na^+ uptake, together with concomitant increase in P, N, and Mg^{2+} absorption and high chlorophyll content in mycorrhizal plants, may be important salt-alleviating mechanisms for plants growing in saline soil. Under saline conditions (150 mM NaCl), the halotolernat legume (*Lotus glaber*) colonized by *Mesorhizobium loti* and *Glomus intraradices*, was more dichotomous and total biomass increased (Echeverria et al. 2008). The improved K^+/Na^+ ratios in root and shoot tissues of mycorrhizal *A. nilotica* plants may help in protecting disruption of K-mediated enzymatic processes under salt stress conditions. Exposure of pigeonpea (*Cajanus cajan*) plants to salinity stress (up to 8 dS/m) markedly decreased nodule mass, acetylene reduction activity (ARA), and leghemoglobin content (Garg and Manchanda 2008). However, AM-fungi inoculation significantly improved nodulation, nitrogenase activity, and leghemoglobin content of salt-stressed pigeonpea plants. Under salt stress, soybean plants inoculated with salt pretreated AM-fungi showed increased SOD and POD activity in shoots relative to those inoculated with the nonpretreated AM fungi (Ghorbanli et al. 2004). Further, activities of enzymes involved in the detoxification of O_2^- radicals and H_2O_2 (superoxide dismutase SOD, catalase CAT, and peroxidase POX), and enzymes of the ascorbate glutathione pathway responsible for the removal of H_2O_2 (glutathione reductase GR and ascorbate peroxidase APOX) increased markedly in AM-salt stressed plants (Garg and Manchanda 2008).

Drought resistance of mycorrhizal plants is independent of plant P concentration (Peña et al. 1988; Sánchez-Diaz et al. 1990). In *Medicago–Rhizobium–Glomus*

symbiosis, subjected to drought stress, nodule activity in infected plants was significantly higher than in noninfected plants (Peña et al. 1988). AM-fungi may increase drought resistance of plants by several mechanisms including enhancing water uptake due to hyphal extraction of soil water and lowering leaf osmotic potential for greater turgor maintenance by regulating photosynthesis (Sánchez-Diaz et al. 1990; Ruiz-Lozano and Azcón 1995). However, this effect is independent of the P nutrition in plant tissues.

Mycorrhizal colonization is recognized further as the key to plant growth and fitness in stressed environments and in sustainable soil–plant systems. AM-fungi and bacteria, isolated from metal-contaminated sites, are often more resistant to metals than those collected from uncontaminated environments. In certain cases, the plant growth promoting effect of bacteria in the presence of nickel is attributed to the bacterial ability in reducing the detrimental Ni-induced stress on plants (Burd et al. 2000). Therefore, the combination of Ni-adapted AM-fungal isolates with those Ni-tolerant bacteria could increase phytoremediation potential. However, prolonged exposure of microorganisms to heavy metals may lead to reduced growth rate or to the loss of several beneficial properties, such as the N_2-fixing ability in the case of rhizobia (Zahran 1999; Biró et al. 2001). Thus, selected metal-tolerant saprophytic and symbiotic microorganisms may play an important role for plant establishment in metal-contaminated soils. For instance, the dual symbiosis between AM-fungi and N_2-fixing rhizobia showed a synergistic effect and significantly increased plant tolerance to heavy metals (Biró et al. 2000). Growth of clover (*Trifolium repens*)–*R. leguminosarum* bv. *trifolii*, and uptake of N, P, and Ni , was studied in Ni-contaminated soil (Vivas et al. 2006). Dual inoculation of clover with the Ni-tolerant bacteria (*Brevibacillus brevis*) and AM-fungus (*Glomus mosseae*) increased shoot and root plant biomass and nodule number that was highly depressed, and substantially reduced the specific absorption rate for Ni compared to plants grown in soils inoculated only with *G. mosseae*. These results suggest that selected bacterial inoculation improved the mycorrhizal benefit in nutrient uptake and in decreasing Ni toxicity, and inoculation of adapted beneficial microorganisms (e.g., *B. brevis* and *G. mosseae*), used as a tool to enhance plant performance in soil contaminated with nickel.

15.6 Conclusion

The legume–rhizobia symbiosis is of tremendous ecological and agronomic importance. Optimization of symbiosis between the legumes and their respective microsymbions requires the competitive, infective and highly efficient N_2-fixing rhizobial strains in sufficient numbers to maximize legume productivity. Advances in molecular biology and genetic tools have helped elucidate numerous genes having symbiotic functions. The major approaches employed for improving N_2-fixation include the selection and construction of effective rhizobial strains and breeding symbiotically-active plants. Proteomics is another approach used in

the identification of proteins involved in *Rhizobium*–legume symbiosis. Soil salinity, a wide spread problem, is the major cause of declining agricultural productivity in different ecological niches. The identification and use of plants adapted to saline environments is of increasing importance for raising the productivity of crops in these areas. Recent investigations on plant tolerance to salt stress have focussed on improvement of breeding and modification of the genetic structure of existing crops aiming at enhanced adaptation to salinity conditions. In this regard, cultivation of legumes, especially the nitrogen-fixing trees, is recommended for the rehabilitation of arid saline soil. This solution is not only likely to make abandoned soils productive but will also ensure conservation and improvement of the environment. The legume trees such as, *Acacia*, *Prosopis*, and *Sesbania* and legume herbs such as, *Melilotus* and *Medicago*, have been found to be salt-tolerant. These legumes establish a symbiotic association with a wide range of rhizobia (*Rhizobium*, *Mesorhizobium*, and *Sinorhizobium*) and can adapt to the unfavorable arid climates. On the other hand, salt-tolerant rhizobia can also change the pattern of cellular constituents such as proteins, phospholipids, and polysaccharides, which are essential for nodulation and maintenance of physiological events. Under salt or acid stress, the rhizobia are reported to form different new Nod factors the stimulation or suppression of which under stressed conditions might affect the rhizobia–legumes symbioses.

Molecular studies on rhizobia–legumes symbioses for understanding how such interaction works under stressed environments are receiving increasing interest. Because of these studies, several kinds of genes or markers associated with stress tolerance have been identified both in bacteria and legumes. For example, the *typA* gene required for the establishment of nitrogen-fixing symbiosis with certain *M. truncatula* and *M. sativa* cultivars and for survival of *S. meliloti* under certain stress conditions is reported. A locus, named as nesR, potentially contributes to the adaptability of *S. meliloti* when it encounters challenges such as high osmolarity, nutrient starvation, and/or competition for nodulation, thus increasing its chances for survival in the stressful rhizosphere. In addition, rhizosphere microorganisms influence plant growth, development, productivity, and environmental adaptation. The composite inoculation of beneficial microbes therefore, provides more balanced nutrition and improves growth and yield of crops. The role of AM-fungi in improving growth and N_2-fixation of legumes is another alternative in sustainable agricultural production systems. The AM-fungi promote plant growth and salinity tolerance mainly by enhancing nutrient acquisition, producing plant growth hormones, altering host physiological and biochemical properties, and defending roots against soil-borne diseases. In combination with other PGPR, the AM-fungi have shown dramatic increase in yield of legumes by increasing salinity tolerance, P level, phosphatases, nodule numbers, N level, protein content, and nitrogenase activity, in comparison to the sole application of AM-fungi or PGPR. Leguminous species are often used in the remediation of contaminated sites because of their capacity to fix N and enhance soil fertility. The rhizobial bacteria facilitate growth of the host legume via the production of phytohormones in derelict soils besides improving N nutrition. Therefore, the potential use of rhizobia–legume associations

as growth promoting organism for the remediation of stressed sites is an exciting new area of research, which, however, urgently requires further testing under field environments.

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Chapter 16
Role of *Azospirillum* in the Improvement of Legumes

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Abstract A rapid and sustained increase in crop production is one of the essential steps to meet the food demands of the growing human population all over the world. Constant and unbalanced use of chemical fertilizers used to boost the crop productivity on the other hand is causing decrease in nutrient uptake leading to decreased crop yields. Moreover, the use of chemical fertilizers has several other drawbacks, like they are expensive and cause ground water, soil, and atmospheric pollution. Long-term sustainability in agriculture is possible only through the use of low cost farm grown inputs, which work in harmony with the nature. Biofertilizers in this regard act as perpetually responsible input helping in better maintenance of crop nutrient as well as soil health. *Azospirillum*, an associative symbiotic nitrogen-fixing bacterium, has a higher nitrogen-fixing potential. Though, a number of papers have highlighted the potential application of this microbe, further more research is still needed on *Azospirillum* to understand the mechanism by which the introduced microorganism benefit the crop. The present review addresses the central issues of *Azosprillum* application either alone or in combination with other plant growth-promoting rhizobacteria for the benefit of the crops.

16.1 Introduction

Nitrogen (N) is most often the limiting nutrients for crop production, since only a fraction of atmospheric N is available to plants through biological nitrogen fixation (BNF). It is now well established that microorganisms play an important role in the nitrogen cycle of natural ecosystem. As the cultivation of new modern varieties of crop plants is increasing, it simultaneously is raising the use of chemical fertilizers.

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At the same time, it is also evident that chemical fertilizers generate much more greenhouse gases, such as N_2O, and use at a higher rate leads to the problem of soil health deterioration, ground water, and atmospheric pollution. Moreover, due to declining availability of fossil fuels, the price for chemical fertilizers is increasing due to high capital investment, maintenance, and transport costs. At the same time, there are problem of losses in fertilizer after application through leaching, volatilization, and also through denitrification. Overall, effect of these problems requires more concentration on greater access to inexpensive biofertilizer technologies, as they are ecologically sound and their application could help to minimize the global warming as well as to reduce the fertilizer input in farming practices. Since the rediscovery of *Azospirillum* by Dobereiner and her collaborators in the 1970s, the species *Azospirillum* has gained the reputation of being the most studied plant-associative bacterium. Apart from direct agricultural application, *Azospirillum* is an excellent model for genetic studies of plant-associative bacteria in general. The largest portion of *Azospirillum* literature consists of genetic studies on almost all aspects of the bacterium and its association with plants. As the most researched associative bacterium, Azospirilla has become a corner stone of rhizosphere research unrelated to its questionable field application (Bashan and Holguin 1997). Apart from being a general plant colonizer (Bashan et al. 2004), *Azospirillum* is remarkably versatile. *Azospirillum* not only fixes atmospheric N (Dobereiner and Day 1976), but can also mineralize nutrients from the soil, sequester Fe, survive under harsh environmental conditions, and support beneficial mycorrhizal–plant associations (Bashan et al. 2004). In addition, *Azospirillum* can help plants minimize the negative effects of abiotic stresses. The recent advances made on the mechanisms as to how *Azospirillum* promotes the growth of plants is highlighted. Furthermore, the role of *Azospirillum* used either alone or in combination with other plant growth-promoting rhizobacteria (PGPR) affecting the performance of legumes in different ecological niches is discussed.

16.2 Taxonomy, General Characteristics, and Host Range

Among the diverse range of microbial species studied extensively as biofertilizer is *Azospirillum,* an obligate endophyte, isolated more recently, and have attracted the attention of scientists working in this field. For their ability to stimulate growth they are known as plant growth-promoting bacteria (PGPB). As early as in 1925, Beijerinck first isolated this bacterium from sandy soil and named it as *Spirillum lipoferum* (Beijerinck 1925). Later, this bacterium was renamed as *Azospirillum lipoferum* (Tarrand et al. 1978). *Azospirillum* spp. is included into the alpha subclass of Proteobacteria belonging to the IV rRNA superfamily (Xia et al. 1994). This group of free-living rhizobacteria encompasses ten species, each one classified according to its particular biochemical and molecular characteristics: *A. lipoferum* and *A. brasilense* (Tarrand et al. 1978); *A. amazonense* (Magalhães et al. 1983); A. *halopraeferens* (Reinhold et al. 1987); *A. irakense* (Khammas et al.

1989); *A. largimobile* (Dekhil et al. 1997; Sly and Stackebrandt 1999); *A. doebereinerae* (Eckert et al. 2001); *A. oryzae* (Xie and Yokota 2005); *A. melinis* (Peng et al. 2006); and recently *A. canadensis* (Mehnaz et al. 2007), as reviewed by Barassi et al. (2007). Very recently, Lavrinenko et al. (2010) isolated a novel nitrogen-fixing bacterial strain BV–ST, able to grow optimally at pH 7.5 and 37°C, from a sulfur bacterial mat collected from a sulfide spring of the Stavropol Krai, North Caucasus, Russia. Phylogenetically, this strain belonged to the genus *Azospirillum* within the family *Rhodospirllaceae* of the class Alphaproteobacteria. Within the genus *Azospirillum*, strain BV–ST was most closely related to *Azospirillum doebereinerae* DSM 13131T, *Azospirillum picis* DSM 19922T, and *Azospirillum lipoferum* ATCC 29707T with a 16S rRNA gene sequence similarity of 97.7, 97.7, and 97.4%, respectively. Furthermore, the DNA–DNA hybridization of strain BV–ST showed reassociation values of 38 with *A. doebereinerae* DSM 13131T, 55 with *A. picis* DSM 19922T, and 42% with *A. lipoferum* ATCC 29707T, while similarities between nifH gene sequences of strain BV–ST and *Azospirillum s*pecies ranged from 94.5 to 96.8%. Chemotaxonomic characteristics (Q-10 as quinone system, C18:1 7c as major fatty acid) and G + C content of the DNA (67 mol%) were similar to members of the genus *Azospirillum*. In contrast to the known *Azospirillum* species, the new strain BV–ST is capable of mixotrophic growth under microaerobic conditions with the simultaneous utilization of organic substrates and thiosulfate as electron donor for energy conservation. Oxidation of sulfide was accompanied by deposits of sulfur globules within the cells. Based on these observations, strain BV–ST is considered as a representative of a novel species of the genus *Azospirillum*, for which the name *Azospirillum thiophilum* sp. nov. is proposed with the type strain BV–ST (=DSM 21654T = VKM B – 2513T).

Azospirillum are Gram-negative, vibrio or spirillum shaped, and 1 μm in diameter, possessing peritrichous flagella with short wavelengths used for swarming and a polar flagellum used for swimming. Poly-β-hydroxbutyrate granules fill most of the bacterial cell and colonies develop a pink pigment. *Azospirillum* proliferates under both anaerobic and aerobic conditions, but it is preferentially microaerophilic in the presence or absence of combined N_2 in the medium (Okon 1994) and occurs in about 90% of tropical soils and in about 60% of soils in the temperate zone (Reynders and Vlassak 1982). *Azospirillum* has been isolated from the major cereals like wheat (*Triticum aestivum*), maize (*Zea mays*) (corn), sorghum (*Sorghum bicolor*), and dry rice (*Oryza sativa* L) in Brazil (Baldani and Dobereiner 1980; Baldani 1984), India (Nayak and Rao 1977; Kavimandan et al. 1978; Nayak et al. 1979; Kulasooriya et al. 1981), Egypt (Hegazi and Monib 1983) and several temperate climatic regions (DeConinck et al. 1988; Vlassak and Reynders 1983), Philippines (Watanbe et al. 1979), and France (Bally et al. 1983). They are common in rhizosphere and roots of topical forage grasses in Central and South America and in South Africa (Tyler et al. 1979) and have been isolated from roots of cactaceous plants in Mexico (Mascrua-Esparaza et al. 1988) and from sweet potatoes (*Ipomoea batata*) and tropical fruit trees in Brazil and India (Döbereiner 1978). They have also been isolated from cold climate grass roots in Finland (Haahtela et al. 1981)

and from maize and wheat root samples collected in unfertilized fields in Europe and USA. *Azospirillum* was also detected in the roots of coconut (*Cocos nucifera*) grown under diverse agronomic practices (George 1990) and within the stem nodules, root nodules, and stems of *Aeschynomene indica* and *A. aspera* (Sing 1992). Claim for *Azospirillum* specificity for certain cereal plant is now found inapplicable. They have no preference for crop plants or weeds or annual or perennial plants but can be successfully applied to plants that have no previous history of *Azospirillum* colonization in their roots. *Azospirillum lipoferum and Azospirillum brasilense* enhance the growth of sun flower and oak seedling respectively (Fages and Arsac 1981; Zaady et al. 1993). From these observations it is evident that, this microbe is a general root colonizer but not a plant specific bacterium (Bashan et al. 2004).

16.3 *Azospirillum* Interaction with Soil

Though, azospirilla has a number of host plants, most of which are annual (Bashan and Holguin 1997), this bacteria can survive even in the absence of their hosts. For this reason, *Azospirillum* shows a number of physiological activities like cyst formation (Bashan et al. 1991a, b), floc formation (Neyra et al. 1995), production of melanin (Givaudan et al. 1993) and poly-β-hydroxybutyrate (PHB) (Okon and Itzigsohn 1992), polysaccharide synthesis (Del Gallo and Idaegi 1990), and protection inside ectomycorrhizal fungal spores (Li and Catellano 1987) to survive during unfavorable conditions. The general survival characteristics of *Azospirillum* were related mainly to the geographical origin of the soil and not to the prevailing environmental conditions. For example, in soil from arid, semiarid, or mountainous region in Israel, viability of *A. brasilense* rapidly declined and the population disappeared completely below detectable level within 35 days after incubation (DAI). In contrast, population from arid soil of Baja California (Mexico) remained stable or even increased during the first 45 DAI. In soil from nonarid central Mexico, viability slowly decreased with time (Bashan et al. 1995a). According to other study, on a number of host plants it has been found that, in Brazil, this rhizobacteria can survive well in soil. But in Israel and in some other American and Canadian soil, the bacteria survive poorly (Bashan and Levanony 1987). *A. brasilense* Cd was well adsorbed on light and heavy-textured soil but poorly adsorbed on quartz particles (Bashan et al. 1991a, b). More detailed laboratory experiments showed that in Israel soil, *Azospirillum* species survived for less than 15–20 days in light-textured sandy soil and 9 days in heavy-textured sandy soil in the absence of their host plants (Bashan and Levanony 1987, 1988). In wet quartz sand, *Azospirillum* inoculation at higher numbers (10^8 cfu/g sand) could not be detectable for more than 20 days (Bashan et al. 1991a, b).

The survival of *A. brasilense* Cd and Sp245 was evaluated in plant free soil types obtained from a wide range of environmental condition and also in the rhizosphere soil of the plant growing in the same soil type. The survival rate was analyzed for

15 common soil variables. The bacteria survived well in all the rhizosphere tested, regardless of soil types and bacterial strain. In as little as 15 days, the bacterial population in plant free soil began to decline rapidly reaching undetectable levels of about 60 DAI, depending on *Azospirillum* species (Bashan et al. 1995a, b). Physicochemical composition of the soil (clay, sand, organic matters etc.) plays an important role in adsorption of bacteria where net negative surface charge may prevent bacterial adsorption. However, clay particle posses positively charged edges which helps in bacterial adsorption. An increase (>5%) in the clay or organic matter in the soil increases the adsorption to a level similar to that of many other species of soil bacteria. Early observations indicated that azospirilla require neutral pH for abundant occurrence (Dobereiner and Day 1976; Dobereiner et al. 1976), while 70% of the soils whose pH was between 5.8 and 6.2 contained azospirilla, their presence was only observed in 40% of soils with pH between 4.8 and 5.1 (Döbereiner 1978). Roots however, seem to provide suitable environment for azospirilla in acid soil also. Iron deficiency in the rhizosphere where oxidative condition prevails, is an important factor for microbial competition. The roots of grasses excrete siderophore under iron-limiting conditions (Romheld and Marcher 1986; Neilands 1984) and most of the microorganism including *Azospirillum* exudes siderophores (Perrig et al. 2007; Pedraza et al. 2007). This chelator forms a complex with iron and assimilates them with high efficiency. Addition of microbial chelators such as ferrichromes and ferrichrysin caused a stimulation zone on agar medium without supplying iron, using *A. brasilense* Sp245. But *A. brasilence* Sp7 or other species of *Azospirillum* did not show this effect. The growth optimum temperature for *Azospirillum* is between 32 and 36°C, which explains the much more generalized occurrence of this bacterium in subtropical and tropical regions. There are however, differences between strains isolated in their capacity for growth at low temperature (Hartmann 1988). Another additional soil variable that may affect adsorption of *Azospirillum* spp. is the cation exchange capacity (CEC) of the soil (Govindarazan and Purushothanam 1989) and the soil redox potential (Charyulu and Rajaramamohon 1980). In addition, protease enzyme, EDTA, various bacterial inhibitors, agitation, exposure to high temperature (42°C), etc has adverse effects on bacterial adsorption.

16.3.1 Interaction of Azospirillum *with Plants*

The process of association between *Azospirillum* and the host plant (Fallik et al. 1994) involves the following events (1) the bacterium is chemotactically attracted by the root exudates, both specifically (by a proteic compound and by selective C compounds) and unspecifically. (2) It then loosely adheres to the root surface by flagella and some glycocalix compounds (phase 1 adhesion). During this step, agglutination can be induced by plant lectins. However, it is not known whether this phenomenon is a positive or a negative response of the plant. (3) After attachment, an exchange of message occurs between plant and bacterium (are

flavones/flavonoids involved, like in *Rhizobium*–legumes symbiosis?). It could be the binding of other lectins and polysaccharides. (4) As a result of signal exchange, cellulose fibrils are produced by Azospirillum, which anchor the bacteria more tightly to the root surface (phase 2 adhesion) leading to (5) the viable and fully established association. Consequently, production of plant growth-promoting substances by the bacterium and a stimulation of the endogenous plant hormones production takes place.

16.4 How Azospirilla Facilitate Plant Growth?

Despite extensive research conducted worldwide for the past 30 years (Bashan and Holguin 1997; Bashan et al. 2004), the mechanism as to how exactly *Azospirillum* facilitate the growth of plants has not been clearly understood. Instead, various mechanisms, other than BNF, such as phytohormone production (Somers et al. 2005; Ona et al. 2005; Perrig et al. 2007) and nitrate reduction (Steenhoudt and van der Leyden 2000) have also been suggested to explain how *Azospirillum* enhances growth and development of plants. Some of the mechanisms involved in growth promotion of plants mediated by *Azospirillum* are briefly discussed.

16.4.1 Nitrogen Fixation

Nitrogen fixation was thought to be the main mechanism to explain improved plant growth following inoculation with *Azospirillum*. This was mainly because of an increase in the number of nitrogenase compound and nitrogenase activity in inoculated plants. A nitrogen-fixing bacterium entraps atmospheric nitrogen and converts the unreactive N_2 molecules to NH_3, a form that is readily utilized by the plants. This process is termed as BNF and is catalyzed by the O_2-sensitive enzyme nitrogenase, present within the bacteria, by the following reaction:

$$N_2 + 8H + 8e^- + 16\ ATP \xrightarrow{\text{Nitrogenase}} 2NH_3 + H_2 + 16\ ADP + 16\ Pi.$$

The ability of an endophyte to fix atmospheric nitrogen within a host has been proved using different approaches: acetylene reduction assay, 15N isotope dilution experiments, $^{15}N_2$ reduction assay, or ^{15}N natural abundance assay. The experimental details and short comings of these assays have been discussed (Dalton and Kramer 2006). *Azospirillum* has been proposed as the major organism responsible for the nitrogenase activity in different plants. Detailed studies, however, showed that the contribution of nitrogen fixation by the *Azospirillum* to the plant is minimal and ranged from 5 to 18% of total plant increase (Bermner et al. 1995) and the observed plant growth-promoting effects can be attributed to other mechanisms like

phytohormone production and displacement of pathogens. The nitrogenase activity of *Azospirillum* has been found to increase when grown in mixed cultures with other bacteria, even if they come from completely different habitats (Holguin and Bashan 1996; Khammas and Kaiser 1992). Apparently, some mixed cultures provide conditions more suitable for nitrogen fixation than those present in pure cultures. An example for an extremely unlikely association is the mixed culture of *A. brasilense* Cd and the non-nitrogen-fixing, marine mangrove rhizosphere bacterium *Staphylococcus* sp. that increased the nitrogen fixation of the former. The effect was stronger when diluted *Straphylococcus* supernatant was added to *A. brasilense* culture and was partially due to release of aspartic acid from the *Staphylococcus* sp. cells (Holguin and Bashan 1996).

Nitrogen fixation was the original proposed mechanism by which *Azospirillum* affects plant growth. In recent years, only a few studies have focused on the nitrogen cycle within the cell, apart from the genes involved. Mutants of common *A. brasilense* strains Sp7 and Sp245, defective in flocculation, differentiation into cyst-like forms, and colonizing roots, had a higher nitrogenase expression than wild strains when associated with wheat. Apparently, the ability of Sp7 and Sp245 mutant strains to remain constantly in vegetative forms (spirillum and rods) improved their ability to express exceptional nitrogenase activity rates. Restoring cyst formation and a normal colonizing pattern to the spontaneous mutant Sp7S reduced nitrogenase activity rates to the level of the wild Sp7. This suggests that retention of bacterial cells in the vegetative state provides faster metabolism, which directly affects nitrogen fixation of the bacterium (Pereg-Gerk et al. 2000). Nitrogen fixation by aerobic bacteria is a very energy-demanding process, requiring efficient oxidative phosphorylation, while O_2 is toxic for the nitrogenase complex. Azospirilla and other well-known nitrogen-fixing soil bacteria have evolved a variety of strategies to deal with and overcome this apparent "O_2 paradox." The question is whether the specific environmental adaptations of azospirilla are sufficient to allow optimal proliferation and nitrogen fixation in their natural habitat. Could improving O_2 tolerance of the nitrogen-fixing process contribute to the development of more efficient strains for inoculation of plants? (Marchal and Vanderleyden 2000). This is left for future research. Murray et al. (2007) and Tirichine et al. (2007) reveal that activation of a plant hormone signaling pathway in the legume *Lotus japonicus*, most likely by rhizobial bacteria, is sufficient to activate nodule formation. This discovery could pave the way to transferring this symbiotic process into other plant species.

16.4.2 Growth Hormones

In pure culture, *Azospirillum* have been shown to produce mainly auxins like IAA (Lambrecht et al. 2000; Spaepen et al. 2007), gibberellins, cytokinin-like substances (Tien et al. 1979), and ethylene (Stezelczyk et al. 1994). Sometimes, external application of synthetic hormones or hormones purified from bacterial

culture imitated the positive effects of *Azospirillum* on root development and morphology. Many studies have suggested the involvement of auxin produced by *Azospirillum* in root morphology (Baca et al. 1994; Pattern and Glick 1996). Inoculation of *Azospirillum* on *Arabidopsis thaliana* for example increased the length of individual root hairs by at least two folds (Dubrovsky et al. 1994). It was obvious that phytohormones, especially IAA secreted by *Azospirillum* play an essential role in plant growth stimulation in general and in stimulating symbiosis between legumes and rhizobacterium. However, to attribute a phenomenon of nonspecific growth promotion of numerous plant species resulting from *Azospirillum* inoculation to one substance is oversimplistic, albeit useful as a research tool for understanding the mechanisms of action of the bacteria (Itzigsohn et al. 1993). In short, several evidences support the involvement of IAA produced by *Azospirillum* in the promotion of plant growth. However, there are no reports showing to what extent IAA produced in the rhizosphere originates from *Azospirillum* (Steenhoundt and Vanderleyden 2000). Recently, cadaverine, a polyamine compound, synthesized by *A. brasilense* Az39 strain has been shown to promote root growth and helped mitigate osmotic stress in rice seedlings (Cassán et al. 2009).

The other feature of *A. brasilense* is its ability to modify plant root architecture. In plants, nitric oxide (NO), a bioactive molecule, mediates cross-talk with traditional phytohormones like IAA leading to both lateral (LR) and adventitious (AR) root formation. In this context, Creus et al. (2005) reported the NO production (6.4 nmol g^{-1} of bacteria) as detected by electron paramagnetic resonance and confirmed by the NO-specific fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA), by *A. brasilense* Sp245 when grown under aerobic conditions. In addition, *Azospirillum* inoculated tomato roots incubated with a NO-specific fluorescent probe displayed higher fluorescence intensity compared to noninoculated roots. Fluorescence was mainly located at the vascular tissues and subepidermal cells of roots (Creus et al. 2005). Moreover, the *Azospirillum*-mediated induction of LRF appears to be NO-dependent since treatment of inoculated seedlings with the NO scavenger – (4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide completely blocked this effect (Creus et al. 2005). In a similar study, Molina-Favero et al. (2008) reported aerobic NO production by *A. brasilense* Sp245 wild type (wt) and its mutants Faj009 (IAA-attenuated) and Faj164 (periplasmic nitrate reductase negative), and correlation of NO with tomato root growth-promoting effects. The wt and Faj009 strains produced 120 nmol NO per gram of bacteria in aerated nitrate-containing medium. In contrast, Faj164 produced 5.6 nmol NO per gram of bacteria, indicating that aerobic denitrification could be considered an important source of NO. Furthermore, biopriming of tomato seedlings with both wt and Faj009 induced LR and AR development. On the contrary, Faj164 mutant did not promote LR or AR when seedlings were grown with nitrate. In the absence of NO, both LR and AR formation were inhibited, suggesting that NO mediated *Azospirillum*-induced root branching. These and associated data (Correa-Aragunde et al. 2004) suggest a novel physiological role for NO in the organogenetic process leading to the establishment of root architecture in plants.

16.4.3 Enhancement of Mineral Uptake

Inoculation with *Azospirillum* results in enhanced mineral uptake (Bashan and Levanony 1990). Both greenhouse and field experiments showed that inoculation with *Azospirillum* results in enhanced uptake of K^+, H_2PO_4, and other microelements (Fages 1994). The main reasons behind these effects are due to the proliferation of root hairs and altered cell arrangement in the outer 4–5 layers of the root cortex, as observed in cross-section of inoculated root (Fallik et al. 1994). Although, some studies showed accumulation of nitrogen and other minerals in the inoculated plants, others showed that enhanced growth of soybeans (*Glycine max*) was not necessarily because of a general enhancement of mineral uptake (Bashan and Levanony 1990). The avenue has modestly been pursued in recent years.

16.4.4 Management of Plant Diseases

Though it has not yet been clearly established that *Azospirillum* can function as a biocontrol agent of soil borne plant pathogen, some studies however, have revealed that *A. brasilense* can be helpful in managing phytopathogens causing crown gall-producing *Agrobacterium* (Bakanchikova et al. 1993), bacterial leaf blight of mulberry (Sudhakar et al. 2000), bacterial leaf and/or vascular tomato diseases (Bashan and de-Bashan 2002a, b), and *Pseudomonas syringae* pv. tomato, causing bacterial speck of tomato (Bashan and de-Bashan 2002b). Tapia-Hernandez et al. (1990) in their study reported that 27 *Azospirillum* isolates produced bactericides that inhibited the growth of several indicator bacteria. Furthermore, *A. lipoferum* M produced catechol-type siderophore under iron-starved conditions that exhibited antimicrobial activity against various bacterial and fungal isolates (Stah et al. 1992). In other study, when wounded tissue of grapevines carrot disks were preinoculated with the live cells of *A. brasilence* Sp7, the development of crown gall disease was inhibited and the protection for the disease lasted over 24 h (Bakanchikova et al. 1993). In yet other investigation, inoculation of *A. brasilense* Sp245 on micropopagated plants of *Prunus cerasifera* L. clone Mr. S 2/5 protected the plants from pathogens like *Rhizoctonia* spp. attack. The results showed 100% survival rate of the plant as compared to the negative control. The biocontrol effect of *A. brasilense* Sp245 of the fungal rhizopheric community has been confirmed by denaturing gradient gel electrophoresis (DGGE) profile of the rhizospheric microbial community (Russo et al. 2008). The antibacterial activities exhibited by *Azospirillum* have been found to be related with its ability to synthesize bacteriocins (Tapia-Hernandez et al. 1990), siderophores (Tapia-Hernandez et al. 1990; Perrig et al. 2007), and phenylacetic acid (PAA), an auxin-like molecule with antimicrobial activity (Somers et al. 2005). These and other associated studies thus suggest that species of *Azospirillum* as inoculants could be useful in the management of various pant diseases as well.

16.4.5 Vitamin Production and Degradation of Toxic Residues

As reported by many workers, *Azospirillum* produces a number of vitamins like thiamin, niacin, pentothanic acid, and riboflavin in large quantities (Rodelas et al. 1993; Dahm et al. 1993; Russel and Muszyski 1995). It has been found that vitamin production is largely affected by the presence of carbon sources and the age of the culture. It was also found that *A. lipoferum* can reduce 4-chloronitrobenzine, an aromatic compound used in manufacturing of pesticides, dyes, explosives, and industrial solvent, which is an environmental pollutant (Russel and Muszyski 1995). The further elucidation of the different mechanisms involved in plant growth promotion will help to make associative diazotrophs a valuable partner in future agriculture.

16.5 Azospirillum as Synthetic Inoculants

Apart from traditional inoculants, application of new synthetic inoculants of *Azopirillum* made up of alginate, are now starting to evoke. This process involves the entrapment of living cells of *Azopirillum* in alginate beads and air dehydration (Paul et al. 1993). These inoculants are easy to handle and store and can be used in the field as potent nitrogen-fixing inoculants. Application and commercialization of these synthetic inoculants under agricultural and industrial condition is now starting to proceed. The role of azospirilla in legume improvement is discussed in the following section.

16.5.1 Inoculation Effects of Azospirillum on Legumes

Legumes are vital in agriculture as they form associations with bacteria that fix atmospheric nitrogen and hence, are the main reason that legumes are richer in proteins than all other plants. Legumes consume about 10% of net photosynthetic output for nitrogen fixation of plants. Roots of leguminous plants often associate with bacteria to generate highly specialized structures – nitrogen-fixing nodules. Bacterial cells within the nodule fix the atmospheric nitrogen and produce ammonium that is assimilated by the plant. In return, the plant supplies carbon compounds derived from photosynthesis, for maintenance of the bacteria. Positive effect of combined inoculation with *Azospirillum* and *Rhizobium* has been reported for different legumes. The beneficial response of dually inoculated plants in terms of dry matter production and N content of legumes plants and favorable influence of *Azospirillum* on nodule number, development, dry weight, and nitrogen fixation has been reported (Holguin and Bashan 1996; Burdman et al. 1997; Bashan et al. 2004). The most significant effect of *Azospirillum* on *Rhizobium* legume nodulation

process, under gnotobiotic condition, was demonstrated by Yahalom et al (1984). The efficiency of *Rhizobium* to infect and nodulate pouch-grown plant roots was stimulated by *Azospirillum* inoculation. It was shown that *Medicago polymorpha* inoculated with a rhizobia suspension containing 10 cfu/ml gave no nodulation, whereas the application of *Azospirillum* (10^6 cfu/ml), 24 h prior to *Rhizobium* inoculation resulted in noticeable nodule formation on plant root. A possible reason for enhanced susceptibility to plants to rhizobium infection following *Azospirillum* inoculation may have been the stimulation of the formation of greater number of epidermal cells that differentiate into infectable root hairs. In another study, nodule formation from clover roots on plant grown in petri dishes under gnotobiotic conditions was either inhibited or stimulated by dual inoculation with several *Azospirillum* and *Rhizobium trifolii* strains, depending on concentrations and timings of inoculation. Simultaneous application of *Azospirillum* and *Rhizobium trifolii* at any ratio caused no stimulation of nodule formation, and in most cases even had an inhibitory effect. However, application of *Azospirillum* 24 h (or more) prior or after rhizobium inoculation, all cell ratio between 1:250 and 1:1,000 (*Rhizobium*: *Azospirillum*), simulated nodule formation. Ratios above 1:2,000 were inhibitory. When *Azospirillum* was applied before and after *Rhizobium*, *Azospirillum* cells may enhance or create more infection sites, thereby increasing root potential for nodule formation. Itzigsohn et al. (1993) demonstrated with Fahraeus slides that *Azospirillum* increased the root hairs and root diameters, but no increase in total number of infection threads were observed. Stimulation of nodulation may occur as a result of a direct response of the plant to *Azospirillum* inoculation. For example, an increase in number of root hairs, root hair branching, and lateral roots could be partly mimicked by an amendment of auxins in appropriate concentrations. The observed inhibition of nodulation at *Rhizobium*: *Azospirillum* ratios above 1:200 was explained by abnormal root hair coiling, branching, and swelling. Similarly, it was found that inoculation of graminae with very high levels of *Azospirillum* could lead to slight inhibition of plant root growth. *Azospirillum* cause root hair distortion at multiple sites which could inhibit or abort *Rhizobium* infection. Yahalom et al. (1991), in a study demonstrated that reduction of *Medicago polymorpha* L. root growth at high inoculum level of *Azospirillum* (10^9 cfu/ml) was the result of decreased cell division in the apical meristem of the root that reduced root potential for nodule formation. Alternatively, reduced nodulation can also be explained by general dose competition between *Rhizobium* and *Azospirillum* in clover rhizosphere. *Azospirillum* can colonize root within few hours of inoculation and cells may colonize roots hairs before *Rhizobium* bacteria arrive and prevent the initiation of nodules by *Rhizobium*. This assumption is supported by the fact when nodulation was initiated, no marked root hair curling was observed (Volpin and Kapulnik 1994).

Combined inoculation of pouch grown *M. polymorpha* with *Rhizobium* and an appropriate number of *Azospirillum* (10^6 cfu/ml) caused a significant shift in average distance of the uppermost nodule on main root, demonstrating that *Azospirillum* caused earlier nodulation; however, no change in total number was observed. In the same experimental system, cell free extracts and culture supernatants prepared

from *Azospirillum* caused similar effect as viable *Azospirillum* cell when applied in conjugation with *Rhizobium*. Furthermore, the effect of *Azospirillum* could be mimicked by the addition of cytokinin, benzyl adenine (10^{-8}–10^{-9} M), but not by Indole acetic acid (IAA). These observations suggest that substances, probably cytokinins, excreted from *Azospirillum* cause early nodulation and change in root morphology Plazinksi and Rolfe (1985). However, they found no effect on clover nodulation when a nonviable cell preparation of *Azospirillum* strains was applied in conjugation with *R. trifolii*, as compared with treatments with living cells. When pouch grown *M. sativa* seedlings were inoculated with either *Rhizobium* alone or in combination with *Azospirillum luteolin* (5–10^{-6}M) or benzyl adenine (10^{-8}M), neither of the tested compound were capable of mimicking the early nodulin observed with *Azospirillum*. Differently, luteolin had effects similar to those of *Azospirillum* in increasing the main root, nodule number while benzyl adenine reduced nodulation. The different results obtained with benzyl adenine in this study as compared with the above mentioned, could relate to different experimental systems used in each case. The effect of *Azospirillum* on nodulation and on specific activity of nodule nitrogen fixation leading to growth promotion may be attributed to each or all of the following: early nodulin, increase in total nodule number and/or a general improvement in mineral and water uptake by roots (Volpin and Kapulnik 1994).

In legume *Rhizobium* system, *Azospirillum* application promoted plant growth that was generally followed by increased nodule formation. However, under gnotobiotic conditions, concomitant application of *Azospirillum* and *Rhizobium* did not always result in promotion of nodulation, under some circumstances, even inhibited the ability of *Rhizobium* to nodulate its host. These results suggest that cell-to-cell interactions between the two bacteria are not essential to obtain a positive plant response and in some cases even have a negative effect. Beneficial growth response under gnotobiotic conditions was achieved in most following applications of *Azospirillum* in the right number and ratio, prior or posterior to inoculation with *Rhizobium*. Therefore, it is reasonable to conclude that *Azospirillum* exerts its effect through the host plant, and not through direct interactions with *Rhizobium*. This again is supported by the fact that *Azospirillum* alone is capable of inducing a plant growth response and morphological changes in the root that could lead to improved nodulation by *Rhizobium*. Under gnotobiotic system, the beneficial response achieved in the field of greenhouse studies was in most cases obtained following the simultaneous application of *Azospirillum* and *Rhizobium*. Furthermore, the promoted legume growth following *Azospirillum* inoculation was preceded by enhanced nodulation of the plant. One possible explanation is that *Azospirillum* has strong microaerophillic attraction to the rhizospheric niche of the legume root and faster motility than *Rhizobium*. These two findings can lead to conclusion that legume root may be occupied primarily by *Azospirillum*, permitting preconditioning of root prior to *Rhizobium* colonization (Volpin and Kapulnik 1994). Mixed inoculation of faba bean (*Vicia faba* L.) with four different *Rhizobium/Azospirillum* and *Rhizobium/Azotobacter* combinations led to changes in total content, concentration, and/or distribution of the mineral macro- and micronutrients, K, P, Ca, Mg,

Fe, B, Mn, Zn, and Cu, when compared with plants inoculated with Rhizobium only. The effects varied to a great extent among the *Azotobacter* and *Azospirillum* strains selected for combined inoculation (Rodelas et al. 1999).

The plant growth-promoting *Azospirillum* has also been used in combination with certain plant growth-promoting/biocontrol fungi. For example, Mehmet et al. (2005) in their study evaluated the effect of single and combined inoculation of *A. brasilense* Sp7 and a biocontrol fungus (*Trichoderma harzianum* Rifai 1295–22), which could also solubilize insoluble phosphorus, on dry bean (*Phaseolus vulgaris*) and wheat (*Triticum aestivum* L.) grown in soil. A pot experiment with bean and a field experiment with both bean and wheat were designed. In contrast to single inoculation of *Trichoderma*, the single inoculation of *Azospirillum* and the double inoculation did not significantly increase nodule numbers and nodule mass at 45 days after planting in pot grown beans. However, the *Azospirillum* inoculation with supplementary P significantly increased nodule mass. There were no significant differences among the inoculation treatments for plant dry weight, total plant N, and total plant P at 45 days after planting in both pot and field experiments with bean. The combined inoculation and rock phosphate (RP) application at 1 Mg ha^{-1} on the other hand quite impressively enhanced bean seed yield, total seed N and P in the bean field trial. This treatment more than doubled the measured properties compared to the control. The microbial inoculations, with the exception of the combined inoculation, significantly increased total seed N, but never affected seed yield in the wheat field trial. It was suggested from this study that the combined inoculation had obvious advantages over single inoculation provided that RP was supplied at an amount not exceeding 1 Mg ha^{-1}. Higher RP application rates, however, decreased many plant and yield parameters. A similar study was conducted in Tokat (Turkey) in 2001–2002 to determine whether inoculation with *A. brasilense*, *Trichoderma harzianum*, sole, or in combination, and/or the application of P fertilizers can enhance micronutrient concentrations of field-grown bean (*Phaseolus vulgaris*) and wheat (*Triticum aestivum*). In beans, *Azospirillum* inoculation combined with P fertilization dramatically increased seed concentrations of Mn, Zn, and Cu, from 8.8, 22.6, and 7.0 mg kg^{-1} in the control to 10.3, 28.3, and 11.0 mg kg^{-1}, respectively. *Trichoderma* inoculation alone significantly reduced the concentrations of Fe, Mn, Zn, and Cu and the cumulative plant uptake of Fe and Zn in 45-day-old bean plants. However, it significantly increased bean-seed Cu content and accumulation. The double inoculation resulted in significantly higher micronutrient concentrations than *Trichoderma* inoculation alone in 45-day-old plants. In contrast to beans, the effects of microbial inoculations were less in wheat. However, dual inoculation increased Zn content by 45% and Zn accumulation by 40% above the uninoculated control. This study thus suggested that inoculation with plant growth-promoting microorganisms appears to be a promising strategy to combat micronutrient deficiencies (Mehmet and Fatih 2006). Recently, Cassán et al. (2009) observed that *A. brasilense* strain Az39 and *Bradyrhizobium japonicum* strain E109, when used either singly or in combination, showed the capacity to promote seed germination, nodule formation, and early development of corn and soybean seedlings. The growth promoting effect was suggested to be due

to the synthesis of IAA, gibberellic acid (GA3), and zeatin (Z) by both strains, as observed under in vitro conditions at a concentration sufficient to produce morphological and physiological changes in young seed tissues.

The nitrogenase activity of *Azospirillum* has been found to increase when grown as mixed cultures with other bacteria, even if they come from different habitats. For example, when *A. brazilense* Cd and the non-N_2 fixing mangrove rhizosphere bacteria *Staphylococcus* spp. are mixed, it increases nitrogen fixation of the former. Development of *Azospirillum* strains having better rhizo-competence and coinoculation of these strains with other bacteria is an evoking field of agro-technology. Still most studies regarding the coinoculation of *Azospirillum* spp. have been performed under laboratory and in the greenhouse and there is a need to extend it further to the field, as well as to optimize coinoculation procedures so that its actual benefit is realized in agricultural practices. In this regard, the combined inoculation of *Rhizobium* and *Azospirillum* significantly increased both nodulation and nitrogen fixation of common bean plants grown in pot trials compared with single inoculation of *Rhizobium*. At an *Azospirillum* concentration of 10^8 cfu ml^{-1}, the combined inoculation reduced dry matter accumulation in plant organs (root and shoot) in comparison to *Rhizobium* alone and uninoculated controls. However, when the combined inoculation was performed using a lower *Azospirillum* concentration (5×10^6 cfu ml^{-1}), positive effects on plant growth were observed, although the enhancement of nodulation and nitrogen fixation were not as great as observed with the higher *Azospirillum* concentration (Burdman et al. 1997). However, while using mixed inocula, the compatibility of interacting strains determines the overall performance of the plants. In another study, *Azopirillum–Rhizobium* was applied for two genotypes of common beans, BAT477 and DOR364, grown in station and farm field conditions in different regions in Cuba (Remans et al. 2008a, b). The mixtures of the two cultures increased the amount of N fixed across all sites by DOR364. But for BAT477, a negative effect of coinoculation was observed on most of the sites of the studies. Compared to single *Rhizobium* inoculation, coinoculation of *Azopirillum–Rhizobium* increased the number of root hairs, the amount of flavonoids exudates by the roots, and the number of nodules formed (Remans et al. 2008a, b, 2007). Under field conditions, coinoculation have shown the potential to increase grain yield of various other legumes and the improvement of nodulation and nitrogen fixation is a major goal in breeding projects of legume.

16.6 Conclusion

There are many challenges to be faced in maximizing and popularizing BNF using *Azospirillum* for the common goods. Improvement and further modification of existing nitrogen-fixing bacteria will be a useful strategy to meet the ever increasing nitrogen demands of the world. An expanding knowledge base and the use of powerful tools of biotechnology may help in gaining further insight into the application of *Azospirillum* as inoculants. Progress must, therefore, be made to

address certain issues before the benefits of *Azospirillum* application is realized under real soil situation. Such scientific challenges include (1) as fertilizer N generally inhibits BNF in both symbiotic and nonsymbiotic systems, how the effects of N application could be reduced when applied with microbial inoculants (2) how to pinpoint specific factors causing low nitrogen-fixation activity in legumes and activity improvement in desired cultivars (3) development of cultivar-strain resistant to stress conditions, acid and alkaline soils, nutritional deficiency, salinity, high temperature, presence of toxic elements, etc. are required (4) development of biochemical approaches for the enhancement of nodule initiation using more efficient strains of *Azospirillum* and extension of this technique to the farmer's field and (5) how to improve inoculation of *Azospirillum* into plant cell suspensions and regeneration of embryos and eventually into plants. Moreover, extensive field experiments are also needed, which will allow the discovery of new strains of azospirilla and also the discovery of Azospirilla-genotype combinations that shows little or no N-deficiency symptoms in the absence of N fertilizer. Testing of mixtures of these strains for effects on yield as well as testing the inocula dose required for better yield responses, also need to be studied properly and elaborately. Plant breeding for increased nitrogen fixation and comparison of such genotypes with nonfixing genotypes will help us to identify the most efficient plant genotype-*Azospirillum* combination. It is suggested that the direction in which *Azospirillum* research should proceed, to gain the full potential of this association, is toward more basic understanding of the underlying fundamental components of the system and less toward full-scale field experiments. We assume that this approach will be the best in ultimately harnessing *Azospirillum* activity for the benefit of mankind.

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Chapter 17
Role of Arbuscular Mycorrhizal Fungi in Nitrogen Fixation in Legumes

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Abstract Most herbaceous legumes of family Papillionaceae are symbiotic with nitrogen-fixing rhizobia and nutrient-absorbing arbuscular mycorrhizal (AM) fungi. Rhizobia and AM-fungi often interact synergistically resulting in better root nodulation, nutrient uptake, and plant yield compared to plants symbiotic with either organism used alone. Increased phosphorus and other nutrients, enhanced photosynthesis, beneficial interaction with rhizospheric microorganisms, and alleviation of environmental stresses due to AM colonization could account for enhanced nitrogen fixation and grain yield of legumes. Few studies have also been carried out to investigate the effect of AM-fungi on free-living nitrogen fixers in the rhizosphere of legumes; however, no definite conclusion could be drawn. The effectiveness of co-inoculation of AM-fungi and rhizobia and AM-fungi with free-living diazotrophs depends upon the compatibility among interacting partners in the rhizosphere that varies greatly with physicochemical characteristics of soil, test microorganisms, plant genotypes, and substances exude from host plant species. In this chapter, role of AM in symbiotic and non-symbiotic nitrogen fixation in legumes and the mechanisms involved are described.

17.1 Introduction

17.1.1 Importance of Legumes

Legumes are plants that bear seeds in pods. They markedly differ from non-legume crops because much of the nitrogen they require is produced through fixation of atmospheric nitrogen by bacteria inhabiting nodules borne on their roots.

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World-wide, more than 16,000 species of legumes are known, including herbs, shrubs, and trees, but only about 200 are cultivated. Historically, legumes have been part of dietary system throughout the world. They are second only to the cereals in providing food crops for world agriculture. The total world value for leguminous crops is thought to be approximately two billion US dollars per annum (Duranti 2006). Peanut (*Arachis hypogaea* L.) and lima bean (*Phaseolus lunatus* L.) have been used for centuries in South America. Among others, soybeans [*Glycine max* (L.) Merr.] and mung beans [*Vigna radiata* (L.) Wilczek] have been a key part of Asian dishes throughout the history. The Middle East is the origin of broad beans (*Vicia faba* L.), chickpeas (*Cicer arietinum* L.), and lentils (*Lens culinaris* Medik). Grain legumes occupy an important place in human nutrition, especially in the dietary pattern of low-income groups of people in developing countries. They are a valuable source of food proteins (Duranti 2006). Proteins in legume seeds range from about 20% (dry weight) in pea (*Pisum sativum* L.) and beans up to 38–40% in soybean and lupin (Guéguen and Cerletti 1994). Quite often, they represent a necessary supplement to other protein sources (Duranti and Gius 1997). Legumes are typically low in fat, contain no cholesterol, and are high in folate, potassium, iron, and magnesium (Andersen et al. 1984; Grusak 2002). Being a good source of protein, they could serve as a substitute for meat, which has more fat and cholesterol. Several reports claim that inclusion of legumes in the daily diet has many beneficial physiological effects in controlling and preventing various metabolic diseases such as, diabetes mellitus, coronary heart disease, and colon cancer (Tharanathan and Mahadevamma 2003).

The use of legumes in rotation with cereal and oilseed crops is a well-established practice to increase soil fertility and crop yields (Lupwayi and Kennedy 2007; Ncube et al. 2009). Many legumes, such as *Lupinus* spp., *Medicago* spp., and *Trifolium* spp., are used as fodders, green manures, and forages (Graham and Vance 2003; Pecetti et al. 2009). Legumes are also utilized for a variety of other purposes including timber, medicine, tannins, and gums. Various species of *Lonchocarpus* and *Derris* are the source of rotenone, which is used as an insecticide, fish poison, or molluscicide. Some legume trees yield valuable resins, used in varnishes, paints, and lacquers. In addition to the direct benefits from nitrogen fixation, legumes have long been known to have a longer-term effect on soils, via such means as reduction of pathogen load (Mourad et al. 2009). Moreover, in regard to greenhouse gases, legumes can have a very positive effect, not only by reducing emission of nitrous oxide from excess fertilizer nitrogen, but also by sequestering carbon in soils (Sprent 2009).

17.1.2 Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi form a mutually beneficial symbiosis with most herbaceous plants. These fungi are ubiquitous in terrestrial ecosystem and have been widely accepted for their role in maintaining structure and functions of

ecosystem (Smith and Read 2008). The symbiotic association of AM fungi and plant roots is known since vascular plants first appeared on land (Stubblefield et al. 1987). These fungi may influence plant fitness and inter- or intra-competition, leading to relative shift in the plant community structure (Urcelay and Diaz 2003). Increased AM fungal diversity has been shown to increase plant community biodiversity and productivity (van der Heijden et al. 1998). Asexual mycorrhizal spores viz. chlamydospores, renamed as glomerospores (Goto and Maia, 2006), are transitorily dormant, persistent propagules that remain infectious in the absence of host plants and can survive under unfavorable conditions (Klironomos and Hart 2002). Spores of AM fungi, upon germination, produce thick-walled hyphae that penetrate the host root causing internal infection. After entering into the root, the hyphae spread inter- and/or intra-cellularly in the root cortex without damaging the integrity of the cells (Strack et al. 2003). On passing to the inner cortical layers, specialized haustoria-like structures called arbuscules are formed within the cells where exchange of metabolites occur between the fungus and host cytoplasm (Parniske 2000). Usually, later on, vesicles are also formed as terminal or intercalary swellings in the cortical cells and function as nutrient storage organs or as propagules in root fragments. They are inter- and/or intra- cellular, depending on the host species. The AM fungal hyphae also extend from the roots out into the soil where they interface with soil particles. These extraradical hyphae function as absorptive structures for mineral elements and water. Since they traverse several centimeters away from the roots, they can effectively bridge over the zone of nutrient depletion around roots and absorb immobile elements from the bulk soil (Bethlenfalvay and Linderman 1992).

Arbuscular mycorrhizal fungi constitute an essential component of sustainable and low-input agricultural systems where nutrient availability is a limiting factor for plant growth (van der Heijden et al. 1998). They play a vital role in the nutrition and growth of plants in many agricultural ecosystems (Javaid et al. 1993; Cavagnaro et al. 2006; Hu et al. 2009). The effects of increased uptake of P and Zn, as well as Cu and N, on the growth of plants were the direct results of fungal colonization and are often related to increased growth when the nutrients in question is limiting (Smith and Read 2008; Javaid 2009). They also provide other benefits to plants – like (1) enhance enzymatic production (Adriano-Anaya et al. 2006), (2) increase photosynthetic rate (Wu and Xia 2006), (3) enhance nitrogen fixation by symbiotic or associative nitrogen-fixing bacteria (Javaid et al. 1994a,b; Meghvansi et al. 2008), (4) help plants to survive under drought stress (Farahani et al. 2008), (5) increase resistance to pests (Chandanie et al. 2009) and help tolerate various abiotic stress factors (Javaid, 2007, 2008; Porras- Soriano et al. 2009), (6) production of certain secondary metabolites (Schliemann et al. 2008), (7) improve soil structure through soil aggregation (Wu et al. 2008; Kohler et al. 2009), (8) increase vase life of cut flowers by reducing ethylene level (Besmer and Koide 1999) and (9) increase reproduction and offspring survival (Koide and Dickie 2002).

17.2 Arbuscular Mycorrhiza and Nitrogen-Fixing Bacterial Associations

While forming a symbiotic association with host plants, AM-fungi live both within and outside the root tissues in soil. Probably for this reason, they interact directly with other soil organisms, or could influence those soil inhabitants indirectly by modulating host plant physiology. The nutritional profile of the host tissues changes in response to altered uptake of minerals from soil. This in turn changes the architecture and biochemical composition of root cells that alter membrane permeability and thus affect the quality and quantity of root exudation. Altered exudation induces changes in the composition of microorganisms in the rhizospheric soil, now appropriately called the mycorhizosphere (Linderman 1988).

17.2.1 Interaction of AM-Fungi with Symbiotic Nitrogen-Fixing Rhizobia

17.2.1.1 Mycorrhizal Colonization in Legumes

Most herbaceous legumes forms symbiotic relationships with both rhizobia and AM-fungi, and results in tripartite relationship of host–rhizobia–AM-fungi (Zaidi et al. 2003). This situation of both rhizobia and AM fungi interacting with the same legume might appear advantageous for competition during mutualistic relationship, since both nodules and mycorrhizae are maintained by the energy provided by the plants to its roots. Nevertheless, even though relatively few studies have been carried out to assess the role of AM fungi on the performance of the legume-rhizobial symbiosis (Chalk et al., 2006), these studies, however, have clearly demonstrated that when legumes symbiose with both rhizobia and AM-fungi, plant growth, yield, and nitrogen nutrition are generally much greater than plants inoculated either with rhizobia or AM fungi alone (Javaid et al. 1993; Antunes and Goss 2005; de Varennes and Goss 2007; Meghvansi et al. 2008; Kaschuk et al. 2009a). In a study, Asai (1944) first suggested that mycorrhizae were a necessary precondition for effective nodulation in many legumes. He showed that several legumes grew poorly and failed to nodulate in autoclaved soil unless they were mycorrhizal. Direct evidence of mycorrhizae increasing nodulation in legumes was obtained by Crush (1974). He found that AM strongly stimulated the growth and nodulation of *Centrosema pubescens, Stylosanthes guyanensis*, and *Trifolium repens*. Since then, several workers from across the world have reported increased plant growth and yield, nodulation and nitrogen fixation, and P and N content in *Vigna unguiculata* (Islam et al. 1980), *Medicago sativa* (Barea et al. 1980) and *Leucaena leucocephala* (Manns and Mosse 1980) when the plants were inoculated with AM-fungi and given rock phosphate. Similar observations were also made for *G. max* (Antunes et al. 2006b), *Lens*

culinaris (Xavier and Germida 2002), *Arachis hypogaea* (Lekberg and Koide 2005), *Vicia faba* (Jia and Gray 2008), and *Cajanus cajan* (Manjunath and Bagyaraj, 1984) when AM-fungi was applied with superphosphate. Recently, Wu et al. (2009) reported that dual inoculation of *Glomus mosseae* and *Rhizobium* elicited a synergistic effect resulting in enhanced N, P, and K content by *M. sativa* grown on three types of coal mine substrates. This indicates that inoculation with AM-fungi may be a promising approach for revegetation of coal mine substrates. The flavonoids daidzein, genistein, and coumestrol have been identified as possible signals for regulating the establishment of the tripartite symbiosis (Antunes et al. 2006a). Moreover, at least eight of the genes encoding signal transduction components downstream of the NF receptors are also required for mycorrhizal establishment and are referred to as common symbiosis (*SYM*) genes (Herder and Parniske 2009).

17.2.1.2 Performance of AM-Fungi Under Field Conditions

Most of the studies regarding the role of AM-fungi in the performance of the legume-rhizobial symbiosis have been conducted in artificial or sterilized growth media. Several authors have questioned the relevance of these pot studies conducted in sterilized medium, and were doubtful that results could be duplicated in the field because of natural mycorrhizal colonization. However, the results obtained in the field trials give cause for optimism with respect to the efficacy of mycorrhizal inoculation (Chalk et al. 2006). Agronomically significant increases in the amounts of fixed nitrogen were obtained under filed conditions for soybean (24–30 kg N ha^{-1}) and pasture legume *Hedysarum coronarium* (26–53 kg N ha^{-1}) because of AM-fungi inoculation under field conditions (Ganry et al. 1982, 1985; Antunes et al. 2006b). Javaid (2005) conducted a comprehensive study to investigate the interactions of two AM species (*Glomus mosseae* and *G. fasciculatum*), and two strains of *Bradyrhizobium japonicum* (TAL-102 and MN-S) under field conditions, in soils amended with farmyard manure (FYM), green manure (GM), and recommended and half doses of NPK fertilizers using mungbean as test crop. The results showed that symbiotic efficiency was dependent on the particular combination of *Glomus* species and *Bradyrhizobium* strain and the type of soil amendment system. Grain yield was significantly enhanced when *G. mosseae* was used with MN-S in FYM and GM, and by *G. fasciculatum* with TAL-102 and *G. mosseae* with TAL-102 in NPK fertilizers amendments.

17.2.1.3 Mechanisms of AM Fungi–Rhizobial Interactions

Various mechanisms involved in growth promotion and yield of legumes following single or composite inoculation of AM fungi and nitrogen fixers have been suggested. Some of them are discussed in the following section.

Interaction of Microbes in the Rhizosphere

Mycorrhizal fungi and rhizobia could directly interact in the rhizosphere of their host plant before either organism forms a symbiotic relationship with the host (Linderman 1992). Generally, colonization of roots by AM-fungi favors nodulation by rhizobia (Smith et al. 1979) as also supported by Chalk et al. (2006), where dual inoculation of AM-fungi and rhizobia elicited a synergistic effect. When plants form symbiosis with AM-fungi, numerous physiological changes occur that could influence the formation and behavior of rhizobial nodules. Generally, it is believed that increased P level in the tissues of mycorrhized plants changes the quality and quantity of root exudates, which in turn induces quantitative changes in rhizosphere microbial populations. Such changes could influence the competition between rhizobia and other soil bacteria in the rhizosphere. If bacteria selectively favoured in the rhizosphere enhance rhizobial competitiveness, then nodulation would be favoured (Linderman 1992). For example, *Pseudomonas putida* markedly increased nodulation on bean (*Phaseolus vulgaris*), while *P. putida* strain R-20 enhanced growth and nodulation of subclover when applied in combination with AM-fungi (Grimes and Mount 1984; Meyer and Linderman 1986). In a follow up study, de Varennes and Goss (2007) tried to understand how the rate of mycorrhizal colonization could affect the interaction between AM fungi, *Sinorrhizobium meliloti*, and *Medicago truncatula* Gaertn using sieved or undisturbed soil before *M. truncatula* was sown. The AM fungi at early stage colonized the roots of *M. truncatula* faster when grown in undisturbed soil relative to the sieved soil, but by pod-fill stage the frequency of hyphae, arbuscules, and vesicles was similar in both treatments. At the latter stage, the dry matter accumulation in aboveground tissues *of M. truncatula* plants was greater when grown in undisturbed soil and showed a greater P and N contents compared to those observed for plants grown in disturbed soil, although soil compaction resulted in plants having a smaller root system than those from disturbed soil. This study clearly suggested that disturbances in soils led to variation in mycorrhizal colonization which in turn modified the interaction between indigenous AM fungi, rhizobia, and legumes leading to a reduced efficacy of the bacterial symbiont.

Enhanced Phosphorus Nutrition

Phosphorus is an essential nutrient for plant growth and development and next only to nitrogen in crop production. It is a key structural element of nucleic acids, phospholipids, and several enzymes and coenzymes and is involved in energy metabolism, activation of metabolic intermediates, signal transduction cascades, and enzyme regulation (Karandashov and Bucher 2005). A reliable source of P and maintenance of cellular P homeostasis is, therefore, essential for sustenance of life. Phosphorus is relatively immobile in soil and diffuses slowly to the plant roots leading to the formation of a depletion zone around the roots,

and consequently restricts the supply of P to the plant (Smith and Read 2008). To overcome such adverse conditions in soil, plants have evolved elaborate mechanisms to facilitate P uptake, by forming a symbiotic associations with AM-fungi. It is now a well-established fact that mycorrhizal colonization can enhance the uptake of P by plant roots (Karandashov and Bucher 2005; Medina et al. 2007). This symbioses improve P acquisition by plants because the extra-radical mycelium grows beyond the nutrient depletion zone of the root system (Cardoso and Kuyper 2006).

Increasing the available fertilizer P frequently can increase nitrogen-fixation, leading many to conclude that the role of AM-fungi in the enhancement of nitrogen-fixation is to enhance host P nutrition (Linderman 1992). Accordingly, Ianson and Linderma (1991) evaluated the nitrogen-fixation enhancement phenomenon on mycorrhized and non-mycorrhized pigeonpea [*Cajanus cajan* (L.) Millip] plants equally well nourished with P. They found that different mycorrhizal isolates enhanced nitrogen-fixation to varying degrees. Further, the P content of nodules of mycorrhizal plants is reported generally to be greater than that of non-mycorrhizal plants (Kawai and Yamamot 1986) that probably explain why mycorrhizal legume plants show a greater nitrogen-fixing activity. In contrast, Ames and Bethlenfalvay (1987) demonstrated a non-P-mediated influence of AM-fungi on root growth and nodule activity of cowpea [*Vigna unguiculata* (L.) Walp] plants. The hypothesis suggesting that the increased nitrogen-fixation by mycorrhizal inoculation is not P-mediated was proposed by Asimi et al. (1980). The apparent controversy regarding the P-mediated role of AM-fungi in nitrogen-fixation could be attributed to the variable soil P status in different experiments. From the available literature, one can conclude that in the absence of P fertilizers or in the presence of insoluble rock phosphate, the effect of AM inoculation was generally not much pronounced on yield and nitrogen-fixation of legumes (Ganry et al. 1985; Mortimer et al. 2008). On the other hand, significant increase in yield, P and N uptake as well as nitrogen fixation was obtained when AM inoculation was carried out along with superphosphate fertilizer (Ganry et al. 1985; Lekberg and Koide 2005; Chalk et al. 2006). These results suggest that the indirect function of the AM fungi is to facilitate the uptake of readily available P by the legume, but that AM has no direct role in increasing the size of the available P pool through solubilization (Chalk et al. 2006), as suggested by Lynch (1983). Moreover, soil pH also plays an important role in the effectiveness of AM inoculation in legumes. For example, Mosse (1977) reported that *Stylosanthes* plants were nodulated and grew best in acidic soils when inoculated with AM-fungi and were given rock phosphate. In neutral and alkaline soils, rock phosphate remained essentially unavailable to both mycorrhizal and non-mycorrhizal plants. Furthermore, it has been suggested in some studies that interendophyte compatibility may also play a role in the combined effect on plant growth (Bayne and Bethlenfalvay 1987). The growth and nitrogen-fixation response of legumes also varies with different species of AM-fungi (Xavier and Germida 2002; Antunes et al. 2006b; Meghvansi et al. 2008).

Enhanced Uptake of Micronutrients

AM-fungi may also play a significant role in the uptake of immobile micronutrients (Subramanian and Charest 1997; Chu 1999; Karagiannidis and Nikolaou 1999). Very few quantitative data is, however, available regarding the effect of AM inoculation on the acquisition of mineral nutrients other than P with respect to the performance of the legume-rhizobial symbiosis. However, AM-fungi was reported to be important in increasing yields of soybean and chickpea through improved uptake of Zn and Cu (Ross and Harper 1970; Thompson 1987). Similarly, Antunes et al. (2006a) reported increased Zn uptake by soybean plants co-inoculated with *Rhizobium* and AM-fungi than plants inoculated only with *Rhizobium*. The increase in nutrient uptake was accompanied by significant enhancement in hyphal colonization, nodule dry biomass, N and P uptake and nitrogen fixation. In other study, AM inoculation has also shown a substantially improved uptake of more mobile nutrient K in three varieties of common beans (Ibijbijen et al. 1996).

Enhanced Photosynthesis

In legumes, AM colonization is also reported to significantly increase the size and nitrogen-fixing activity of the nodules even when the numbers of nodules are not significantly increased (Pacovsky et al. 1986; Javaid et al. 1993, 1994a). The explanation for such increased nitrogen-fixing efficiency could be due to the partitioning of photosynthate into the roots supporting both symbiont and legumes (Linderman 1992). As reported earlier, the photosynthetic rate of mycorrhizal plants is generally greater than non-mycorrhizal plants (Kaschuk et al. 2009b), possibly because of enhanced P uptake (Cardoso et al. 2006). In photosynthesis, P is used for energy supply (ATP and NADPH), participates in the regeneration of the CO_2 acceptor, Ribulose bisphosphate (RUBP), and regulates the ratio of starch:sucrose biosynthesis (Cakmak and Engels 1999; de Groot et al. 2003; Rychter and Rao 2005). Owing to enhanced photosynthesis rate following mycorrhizal colonization, a greater percentage of the photosynthate goes to nodules of AM-colonized plants than to non-AM plants (Harris et al. 1985) accounting for the increment in size of the nodules as well as the energy for Nitrogen-fixation (Linderman 1992).

Alleviating Environmental Stresses

To circum the reduction in the performance of various agronomic crops including legumes mediated by various abiotic stresses, AM-fungi, when applied, have shown a substantial improvement in the growth and yield of plants (Burke et al. 2003; Tian et al. 2004; Farahani et al. 2008; Javaid 2007, 2008; Evelin et al. 2009; Porras-Soriano et al. 2009; Garg and Manchanda 2009). However, interactive effects of AM-fungi and environmental factors on the performance of the

legume-rhizobial symbiosis are inadequately explained (Chalk et al. 2006). Study conducted by Olesniewicz and Thomas (1999) revealed the possibility that AM colonization may play roles in the improvement of growth and nitrogen fixation under environmental stress factors. To explain this, they assessed the effects of mycorrhizal colonization on biomass production and nitrogen fixation of black locust (*Robinia pseudoaccacia*) seedlings grown under elevated atmospheric carbon dioxide. Their results indicated that plant dry biomass, nitrogen and phosphorus uptake, and nitrogen fixation were considerably higher in AM-colonized plants than in non-mycorrhizal plants. Rabie (2005), in his study, suggested that the AM-fungi protected the mung bean (*Vigna radiata*) against the detrimental effects of salt. Mycorhhized plants grown with different dilution of seawater had higher growth than the non-AM plants at all the levels of irrigation. The mycorrhizae-mediated improvement in legumes were due to protection against salt stress provided through better access to nutrients (Zandavalli et al. 2004), osmotic modifications (Rao and Tak 2002), and improved photosynthesis (Feng et al. 2002). In a recent study, Shokri and Maadi (2009), evaluated the effects of AM-fungus on the mineral nutrition and yield of *Trifolium alexandrinum* plants under a varying salinity regimes (2.2, 5, and 10 dS m^{-1}) in a pot trial experiment conducted under glasshouse conditions. The mycorrhizal inoculation enhanced total dry weight (5.29 times more than control plants), root length and nutrient uptake of the *T. alexandrinum* at high and low salinity levels. In shoot system of non-AM plants, Na^+ concentration was increased while the concentrations of K^+, Mg^{2+}, and Ca^{2+} were decreased with increasing salinity stress. The Na^+ level in shoots of AM plants showed slight increase with increasing salinity levels. This experiment showed that phosphorus levels in the plants were reduced with increasing salinity but the AM plants showed higher values of phosphorus at all salinity levels. Thus, it could be concluded that AM fungi increased phosphorus uptake, and saline stress in plants was thereby alleviated, suggesting that AM-fungi could serve as bio-ameliorators of saline soils (Yano-Melo et al. 2003; Tian et al. 2004).

Among the various stressors, salt stress is one of the major threats affecting 7% of the world's land area (Szabolcs 1994) and hence, cause food security problems due to gradual salinization of agricultural fields. At elevated concentrations, salt reduces water potential, causes ion imbalance or disturbance in ion homeostasis and toxicity. Such altered water status limits the growth and yield of plants including legumes (Turan et al. 2007) because of osmotic and ionic stress generated by high salt concentrations (Munns 2002; Katerji et al. 2005; Benlloch-Gonzalez et al. 2005). The lethal effects of salt leading to death of plants or decrease in productivity has been observed at every stage of plant growth and affects all the major physiological processes such as photosynthesis (Turan et al. 2007; Wang et al. 2009), protein synthesis (Behera et al. 2009), and nitrogenise activity (Ashraf and Bashir 2003). However, the effect of salts varies greatly among different plant species (Rabie and Almadini 2005). For example, in a pot trial experiment, biomass accumulation in fresh and dry organs (shoots and roots) of *Sesbania sesban* and root–shoot ratio decreased progressively with the increasing salinity levels. The impact of salinity was more pronounced on roots than on shoot. Though, nodules

were observed on the roots of plants growing at all salinity levels but they differed in size and shape. The number and size of the nodules per plant and their fresh and dry mass decreased as the concentration of salts increased. In addition, the percentage of tissue nitrogen decreased consistently with increasing salt concentrations (Mahmood et al. 2008).

Physiological and biochemical plant processes affected by salt stress, however, also trigger premature nodule senescence and decrease their ability to fix nitrogen. In order to assess the influence of AM-fungi on nitrogen-fixing ability of legumes grown under salt stressed environment, experiments were conducted using pigeonpea [*Cajanus cajan* (L.) Millsp.] exposed to salinity stress of 4, 6, and 8 dSm^{-1} raised in greenhouse. Various parameters linked to nodule senescence were assessed at 80 days after sowing. Nodulation, leghemoglobin content, and nitrogenase enzyme activity measured as acetylene-reducing activity (ARA) were evaluated. Two groups of antioxidant enzymes were studied (1) enzymes involved in the detoxification of O_2^- radicals and H_2O_2, namely, superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) and (2) enzymes that are important components of the ascorbate glutathione pathway responsible for the removal of H_2O_2, namely, glutathione reductase (GR) and ascorbate peroxidase (APOX). Exposure of plants to salinity stress enhanced nodule formation; however, nodule growth suffered remarkably and a marked decline in nodule biomass, relative permeability, and lipid peroxidation. Leghemoglobin content and ARA were reduced under saline conditions. In contrast, AM-fungi significantly improved nodulation, leghemoglobin content, and nitrogenase activity under salt stress. Activities of SOD, CAT, APOX, POX, and GR increased markedly in mycorrhizal-stressed plants. Furthermore, AM plants maintained higher K^+: Na^+ and Ca^{2+}: Na^+ ratios than non-AM plants under stressed and unstressed conditions. Salinity induced the accumulation of both proline and glycine betaine in AM and non-AM plants. The findings of these studies suggests a correlation between enhanced levels of antioxidant enzyme activities, reduced membrane permeability, reduced lipid peroxidation, and improved nitrogen-fixing efficiency of AM plants under stressed and unstressed conditions. These factors could be responsible for the protective effects of mycorrhiza against stress-induced premature nodule senescence (Garg and Manchanda 2008; Garg and Manchanda 2009).

17.2.2 Interaction of AM with Free-Living Nitrogen-Fixing Bacteria

Some free-living diazotropic species of bacteria belonging to genera *Azotobacter, Beijerinckia, Clostridium, Azospirillum*, *and Pseudomonas* fix atmospheric nitrogen non-symbiotically. Role of these bacteria in the improvement of crops, like, cereals and other members of the family Poaceae including sugarcane (*Saccharum officinarum)* is well documented (Kzlkaya 2008; Díaz-Zorita and

Fernández-Canigia 2009; Pedraza et al. 2009; Rosas et al. 2009). There are evidences that these diazotrophs also play role in the rhizosphere of many leguminous species especially soybean, where they not only fix atmospheric nitrogen but also interact with symbiotic rhizobia (Rodelas et al. 1999; Molla et al. 2009; Sharma et al. 2003; Cassán et al. 2009). However, reports on the dual co-inoculations of AM-fungi with both of the symbiotic nitrogen-fixing rhizobia and free-living diazotrophs are uncommon. Such bacteria when present in close proximity of AM-fungi, may affect AM colonization with their plant host through a variety of mechanisms – like (1) affects the receptivity of the root, (2) influence root-fungus recognition (3) affects fungal growth, (4) modify the chemistry of the rhizospheric soil, and (5) affects germination of the fungal propagules. Meyer and Linderman (1986) reported that root and shoot biomass, nodulation, and concentrations of Fe, Cu, Al, Zn, Co, and Ni were considerably greater in the shoots of subterranean clover (*Trifolium subterraneum* L.) plants inoculated with *Pseudomonas putida* and indigenous AM fungi than in plants inoculated with the *P. putida* or AM fungi alone. Biró et al. (2000) evaluated the effect of co-inoculations of the alfalfa (*Medicago sativa* L.) plants with *Azospirillum brasilense*, *Rhizobium meliloti*, and the AM-fungus *Glomus fasciculatum* in a pot experiment under controlled conditions. In the gamma-sterilised substrate, all of the mono-, dual- or multilevel co-inoculations with the selected cultures, *Glomus fasciculatum* strain were effective in improving plant growth, nutrient-uptake, and abundance of the microsymbionts in the rhizosphere of alfalfa. In contrast, a competition from the indigenous microflora in the non-sterilised soil greatly reduced the functioning of the applied mycorrhizal inoculum. Similarly, Tsimilli-Michael et al. (2000) investigated the synergistic and antagonistic effects of M-fungus *Glomus fasciculatum*, *A. brasilense*, and *R. meliloti* on the photosynthetic activity of alfalfa. The beneficial effect of AM-fungus was clearly revealed by the observed enhancement of the electron transport activity per leaf area. On the basis of the same criterion, an antagonism by both bacteria was detected. The antagonistic effect of *Azospirillum* was more pronounced than that of *Rhizobium*, though not strong enough to fully counterbalance for the beneficial effect of AM. However, in the case of co-inoculation with both diazotrophs and AM, the electron transport activity was found to be only slightly lower than in the case of single inoculation by AM, indicating that in the presence of each other, the diazotrophs are no longer antagonistic to AM. Recently, Bisht et al. (2009) studied the effect of arbuscular mycorrhizal fungi, *Pseudomonas fluorescens*, and *Rhizobium leguminosarum* on the growth and nutrient status of *Dalbergia sissoo* Roxb. Mollisol and Entisol were used to compare the effects of different soils. The tetrapartite interaction of AM, *P. fluorescens*, *R. leguminosarum*, and *D. sissoo* showed improved plant growth response in the Entisol compared to uninoculated plants. The interaction of AM with *R. leguminosarum* was found; however, AM did not show the same growth responses in combination with either *P. fluorescens* or *R. leguminosarum* and *P. fluorescens* regardless of soil type. AM and *P. fluorescens* showed decreased plant growth, suggesting that enhanced plant growth was dependent on the bacteria–AM combination used. They suggested that in the case of

D. sissoo, choice and testing of the combination of beneficial organisms is necessary to get desired plant growth promotion. In conclusion, the effectiveness of co-inoculation of AM, rhizobia and free-living diazotrophs depends upon the interactions among the participants in the rhizosphere that varies with soil types, test microorganisms, and host plant species. Similarly, bacterial-mycorrhizal-legume tripartite symbiosis in saline conditions, and the effects of dual inoculation of *A. brasilense* (NFB) and AM-fungus *Glomus clarum* on *Vicia faba* in pot cultures was investigated at five NaCl levels (0.0–6.0 dS m^{-1}) in irrigating water (Rabie and Almadini, 2005). AM inoculation significantly increased tolerance to salinity, mycorrhizal dependency, P level, phosphatase enzymes, nodule number, nitrogen level, protein content, and nitrogenase enzymes of all salinized faba plants in comparison with control and non-AM plants either in the absence and presence of *A. brasilense*. In shoot system of non-AM plants, Na^+ concentration was increased while the concentrations of K^+, Mg^+, and Ca^+ were decreased with increasing salinity stress. On the contrary, AM plants had K^+/Na^+ , Mg^+/Na^+, and Ca^+/Na^+ ratios higher than non-AM plants at all salinity levels. The Na^+ level in shoots of AM plants was increased marginally with raising salinity while, K^+ and Ca^+ increased considerably at higher salinity levels. This study thus provided the evidence that NFB aid AM-fungus in protecting the host plants against the lethal effects of salt. Considering the impact of mycorhizae–NFB association on legumes, it will be interesting to use this approach further to increase the salinity tolerance among legumes and hence, legumes could be cultivated in soils even contaminated with high salt concentrations

17.3 Conclusion

Arbuscular mycorrhizae have great potential to improve the crop growth, yield, nodulation, and nitrogen fixation in legumes. The effectiveness of the composite inoculation of symbiotic rhizobia or asymbiotic free-living nitrogen fixers and AM-fungi depends largely on the compatibility among interacting partners. Moreover, soil edaphic factors, especially the soil pH, and concentration and source of P significantly affect the legume-*Rhizobium*-AM-fungi tripartite symbiosis. In order to derive maximum benefits from the tripartite relationship, compatible *Rhizobium* and AM species should be selected for different leguminous plants under varying edaphic conditions. So far, most of the studies regarding tripartite interaction have been conducted under controlled pot conditions. However, few studies conducted under field conditions demonstrate that the dual inoculation of *Rhizobium* and AM can enhance nitrogen fixation and yield in the presence of indigenous AM and rhizobia. Moreover, AM-fungi can also play beneficial role in improving nitrogen-fixing efficacy of free-living diazotrophs in the rhizosphere of the legumes. Therefore, more field studies are required to achieve the maximum benefits of such natural inexpensive resources.

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Chapter 18
Symbiotic Nitrogen Fixation in Tropical Food Grain Legumes: Current Status

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Abstract In tropical regions, nitrogen (N) deficiency is frequently one of the major factors limiting the yield of grain crops which makes the contribution of symbiotic nitrogen fixation (SNF) of great importance, especially when legumes are involved in the cropping systems. Despite extensive research in the area of SNF worldwide, our knowledge on this subject and on how the research efforts could be translated and used to improve the productivity of legumes in different agro-ecological regions is still not sufficient. For these reasons and also considering the impacts that fossil fuels have upon global warming, the role of SNF in the twenty-first century will certainly increase. In this chapter, some aspects of the current status of SNF research are discussed focusing mainly on the four most important tropical food grain legumes, soybean, common bean, cowpea, and groundnut.

18.1 A Global View of the Nutrients Supply

According to Food and Agricultural Organization (FAO), by 2050, the world will have a population of 9.1 billion people, which represents a 34% increase over the next 40 years. During this period, the agricultural production would need to grow globally by 70%. Among plant nutrients, N and phosphorus (P) are often the most limiting nutrients worldwide whose continued supply as chemical fertilizers is necessary if world food needs are to be fulfilled. As a result of the green revolution,

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a huge increase in the application of fertilizers, particularly N, occurred during the late 1970s. Between 1960 and 2000, the world use of N increased from 12 to 81 million tones, a sevenfold increase in 40 years (Phillips 2003). However, improvements in the nutrient use efficiency and in the fertilizer technology are important, due in part to the increasing costs of fertilizers, environmental pollution, and the need for higher crop yields on sustainable basis. In this context, efforts must be directed to enhance the exploitation of beneficial soil microorganisms, of which the best-studied example is the use of atmospheric $N(N_2)$ fixers for crops including legumes. Legumes are cultivated on approximately 250 mha worldwide and the successful use of rhizobium-based inoculants has resulted in the partial or total replacement of N fertilizer for these crops around the world (Xavier et al. 2004). It is estimated that legumes provide almost half of all the N used in agriculture (Smil 1999; Graham and Vance 2003) and this contribution could further be increased by improving the nutrition of legumes, by attending to edaphic constraints such as soil acidity and drought, and by plant breeding programs (Graham et al. 2004). In relation to P nutrition, although the nutrient must be supplied as a fertilizer, several microbial mediated processes, such as the mycorrhizal associations, the solubilization of P, and the production of phosphatases can contribute to increase its availability and use efficiency. As tropical soils are usually very poor in N and P, the continuous addition of these nutrients is necessary to maintain soil fertility and plant nutrition. The production of fertilizers as those containing N requires a great consumption of fossil fuels and is thus subject of constant variations in prices. Smallholder farmers face numerous difficulties in developing countries and many times fertilizers are often too costly or not available. Consequently, the amounts of nutrients removed by the crops are not equaled by fertilizers inputs, resulting in nutrient deficient soils.

Considering the importance of soil microbes in the processes related to the decomposition of organic residues, mineralization/immobilization of plant nutrients and formation/degradation of soil organic matter, management practices that promote soil health, and keep it biologically active and productive should be a major goal for farmers. In this context, the use of microorganisms such as those capable of fixing N and promoting plant growth is likely to favor a modern agriculture, with optimum yields, better cost/benefit ratios and lower environmental impacts.

18.2 Symbiotic Nitrogen Fixation

In tropical regions, N deficiency is frequently one of the major factors limiting the yield of grain crops. In general, the mineralization of soil organic matter is the most important natural process to supply N to plants. The contribution of SNF is also of great importance especially when legumes are involved in the cropping systems. The extent to which N deficiency occurs depends on a number of factors related to the plant demand, to the soil (soil type, texture, organic matter content), to the type of management practices adopted by farmers, and to the presence or absence of

efficient rhizobial strains when legumes are cropped. Rhizobia is a general term used to describe a range of soil bacterial genera that elicit on the roots of specific legume hosts the formation of new organs, nodules. In these nodules, the bacteria proliferate, differentiate into bacteroids and fix N_2, which is transformed in ammonium by a prokaryote-exclusive enzyme nitrogenase. Ammonium is made available to the plants, which in turn provides C sources to the bacteria. These gram-negative bacteria can be present in soil or applied to seed or soil. In some places, the presence of compatible rhizobia in soil occurs naturally, while in others this availability does not exist. Biological N_2 fixation is the cheapest and the most environmentally correct form to provide N to plants and the most intensively studied model of beneficial plant–microbe interaction. The microorganisms able to fix N_2 were first isolated in 1888 by Beijerinck, a Dutch microbiologist, who named them as *Bacillus radicicola*. Subsequently, they were called *Rhizobium leguminosarum* by Frank (1889) (for detailed taxonomy, see Chap. 1). Previously, the term bacteroids was coined by Brunschorst in 1885 for the bacteria-like bodies found in root nodules and by the end of that century the first pure cultures of rhizobia were on sale to farmers in Europe under the trade name "Nitragin" (Fred et al. 1932).

The symbiosis establishment requires the coordination of plant and bacterial gene expression, which are regulated through the mutual exchange of molecular signals. The specific interaction between the two symbionts partners is initiated by the plant which produces flavonoids or related compounds, belonging to a relatively diverse family of aromatics compounds. Flavonoids induce genes (*nod, nol, noe*) involved in nodulation and vary according to the host species (Cooper 2004; Taurian et al. 2008). Flavonoid activation of *nod* genes results in the synthesis and secretion of a family of lipo-chito-oligosaccharidic (LCOs), *N*-acylated oligomers of *N*-acetyl-D-glucosamine (Lerouge et al. 1990) Nod factors (for details see Chap. 2). The symbiotic characteristics of the *nod* genes vary from one species to another. For example, some strains have only one *nodD* gene, while others such as *Bradyrhizobium japonicum* and *Rhizobium tropici* possess two to five copies of *nodD* (Göttfert et al. 1992; van Brussel et al. 1992) and amazingly, some strains of *Bradyrhizobium* sp. do not have *nodD* genes (Giraud et al. 2007). Likewise, two rhizobia species that nodulate the same plant may secrete different Nod factors. *Rhizobium tropici* and *R. etli* produce sulfated and acetylfucosylated Nod factors, respectively, although both effectively nodulate *Phaseolus vulgaris* (Poupot et al. 1993, 1995). The pre-activation of strains with flavonoid inducers has emerged as a promising technique to enhance the rhizobia effect in leguminous plants. Flavonoid pre-activated *B. japonicum* increased soybean nodule number and weight in about 30% and the yields in about 10–40%, when compared to conventional inoculants (Zhang and Smith 1996) and this positive response to the pre-activation has also been obtained for other legumes (Begum et al. 2001).

During root infection and invasion, at least under two circumstances, the plant cell wall must be breached: during infection, thread initiation and exit (Brewi 2004). On the basis of this fact, it has long been suggested that bacterial enzymes have a preponderant role in the *Rhizobium*–legume symbiosis establishment (Fauvart et al. 2009). Recently, it was demonstrated for the first time that a cellulase

(Celc2) from *R. leguminosarum* can erode the cell wall of the host root tips, besides being essential during the first steps of the symbiosis (Robledo et al. 2008). In the same way, it has been demonstrated that rhizobia are also capable of producing pectinolytic enzymes, as shown with *R. etli* Hrp W (Fauvart et al. 2009). Other species-specific sensor-activator systems are involved in the control of bacterial host range (Perret et al. 2000), as the *nodV* and *nod W* genes of *B. japonicum* in *Vigna unguiculata*, although they contribute marginally to the symbiosis with *Glycine max* (Göttfert et al. 1990). Rhizobia also use additional molecular signals, such as secreted proteins or surface polysaccharides (Deakin and Broughton 2009). Best studied in rhizobia is the transport of proteins via the type III secretion system (T3SS) (Hempel et al. 2009) that are essential for the virulence of many animal and plant pathogenic bacteria (Okazaki et al. 2009). Symbiotic and pathogenic bacteria use the T3SS to deliver proteins into the eukaryotic host cell (Pallen et al. 2003). The T3SS has been identified in *Rhizobium* sp. NGR234 (Freiberg et al. 1997), *Sinorhizobium fredii* USDA257 (Krishnan et al. 2003), *S. fredii* HH103 (de Lyra et al. 2006), *M. loti* MAFF303099 (Kaneko et al. 2000), and *Bradyrhizobium japonicum* strains USDA110 (Göttfert et al. 2001), and CPAC 15 (Godoy et al. 2008). Secreted proteins of rhizobia are designated as Nops (nodulation outer proteins) (Marie et al. 2001) and depending on the host, the T3SS may positively or negatively affect the symbiosis. In the following sections, some aspects of the current status of SNF research focusing mainly on four of the most important tropical food grain legumes, like, soybean, common bean, cowpea, and groundnut are discussed.

18.3 Soybean

Soybean is the most important cropped legume in the world. The high protein content of the soybean grains, about 40%, represents an important protein source for human and animal diet (Table 18.1). In addition, soybean seed products are widely used in industrial and pharmaceutical applications and more recently soybean biodiesel has been recognized as an alternative to fossil fuels. In 2008/2009, soybean world cropped area was of 96.3 million ha with a total production of 210.6 million of metric tons (http://www.cnpso.embrapa.br, verified December 02, 2009). United States, Brazil, and Argentina are, in this order, the three leading producers, accounting for over 85% of total world production. In 2007, average world yield was of 2,445 kg ha^{-1} (http://www.faostat.fao.org, verified December 02, 2009). In the past 15 years, soybean is also gaining popularity in west and southern Africa (in 2007, it was cropped in 1.2 million ha, according to http://www./faostat.fao.org/ site, verified December 02, 2009) where, in addition to its importance as a source of protein for human nutrition and fodder, its lower susceptibility to pests and diseases, better grain storage quality, huge leaf biomass yield (benefiting soil fertility to subsequent crops), and secure commercial market make its cultivation more advantageous over other grain legumes commonly grown in that continent by smallholder farmers (Mafongoya et al. 2009).

Table 18.1 Nutritional value of some important legumes

Nutritive properties	Soybean	Beans		Groundnut (all types)
		Kidney bean	Cowpeas	
Carbohydrates	30.16 g	60.01 g	60.03 g	21 g
Sugars	7.33 g	2.23 g	6.90 g	3.97 g
Dietary fibre	9.3 g	24.9 g	10.6 g	8.5 g
Fat	19.94 g	0.83 g	1.26 g	49.24 g
Water	8.54 g	11.75 g	11.95 g	6.50 g
Energy	1,866 kJ (446 kcal)	1,393 kJ (333 kcal)	1,406 kJ (336 kcal)	2,374 kJ (567 kcal)
Protein	36.49 g	23.58 g	23.52 g	25.80 g
Vitamin A equiv	1 µg (0%)	–	3 µg	–
Vitamin C	6 mg (10%)	4.5 mg	1.5 mg	–
Viamin K	47 µg (45%)	19 µg	5µg	–
Thiamine (Vit B1)	0.874 mg	0.529 mg	0.853 mg	0.640 mg (46%)
Riboflavin (Vit B2)	0.870 mg	0.219 mg	0.226 mg	0.135 mg
Niacin (Vitamin B3)	1.623 mg	2.060 mg	2.075 mg	12.066 mg
Vitamin B6	0.377 mg (29%)	0.397 mg	0.357 mg	0.348 mg (23%)
Pantothenic acid (B5)	0.793 mg	0.780 mg	1.496 mg	1.767 mg (36%)
Folate (VitB9)	375 µg	394 µg (99%)	633 µg	246 µg (62%)
Calcium	277 mg (28%)	143 mg	110 mg	92 mg
Iron	15.70 mg (126%)	8.20 mg (64%)	8.27 mg	4.58 mg
Magnesium	280 mg (76%)	140 mg (38%)	184 mg	168 mg
Zinc	4.89 mg (49%)	2.79 mg (30%)	3.37 mg	3.27 mg
Phosphorus	704 mg (101%)	407 mg	424 mg	376 mg
Potassium	1,797 mg (38%)	1,406 mg	1,112 mg	705 mg
Sodium	2 mg	24 mg	16 mg	18 mg
Tryptophan	0.591 g	0.279 g	0.290 g	0.250 g
Threonine	1.766 g	0.992 g	0.895 g	0.883 g
Isoleucine	1.971 g	1.041 g	0.956	0.907 g
Leucine	3.309 g	1.882 g	1.802 g	1.672 g
Lysine	2.706 g	1.618 g	1.591 g	0.926 g
Methionine	0.547 g	0.355 g	0.335 g	0.317 g
Cystine	0.655 g	0.256 g	0.260 g	0.331 g
Phenylalanine	2.122 g	1.275 g	1.373 g	1.337 g
Tyrosine	1.539 g	0.664 g	0.760 g	1.049 g
Valine	2.029 g	1.233 g	1.121 g	1.082 g
Arginine	3.153 g	1.460 g	1.629 g	3.085 g
Histidine	1.097 g	0.656 g	0.730 g	0.652 g
Alanine	1.915 g	0.988 g	1.072 g	1.025 g
Aspartic acid	5.112 g	2.852 g	2.840 g	3.146 g
Glutamic acid	7.874 g	3.595 g	4.454 g	5.390 g
Glycine	1.880 g	0.920 g	0.971 g	1.554 g
Proline	2.379 g	1.000 g	1.057 g	1.138 g
Serine	2.357 g	1.282 g	1.178 g	1.271 g

Source: USDA nutrient database; Percentages are relative to US recommendations for adults; values are per 100 g (35 oz)

18.3.1 Rhizobial Strains Nodulating Soybean

Rhizobial strains nodulating soybean were collectively known as *Rhizobium japonicum* until 1982, when Jordan (1982, 1984) reclassified this species into a new genus, *Bradyrhizobium*, with the species *Bradyrhizobium japonicum*, denominating strains which nodulate the soybean and with USDA 6 (=ATCC 10324) as the type strain. In the 1980s and beginning of the 1990s, studies evidenced a high morpho-physiological and genetic variability among the *B. japonicum* strains. On the basis of these studies, Kuykendall et al. (1992) suggested the division of the genus *Bradyrhizobium* in two species *B. japonicum* and *B. elkanii*. USDA 76 (=ATCC 49852) was defined as the type strain for *B. elkanii*. While *B. japonicum* fix more N_2, *B. elkanii* strains are more competitive and show high resistance to some antibiotics. Subsequently, other species able to nodulate soybean have been described: *B. liaoningense*, extra-slowly growing rhizobia that nodulate primitive and modern soybean genotypes (Xu et al. 1995) and *Rhizobium tianshanense*, later reclassified as *Mesorhizobium tianshanense* (Jarvis et al. 1997).

Soybean is also nodulated by fast-growing rhizobia, first reported in 1982, isolated from nodules and soil of the People's Republic of China, within the center of origin and diversity of the legume (Keyser et al. 1982). Other fast-growing rhizobia were isolated from different Asian regions (Xu and Ge 1984; Dowdle and Bohlool 1985); however, nodulation was reported only on primitive genotypes, as Peking (PI17852.B) and Malaya, and with *Glycine soja* Sieb. and Zucc., the probable ancestor of the modern soybean (Keyser et al. 1982; Devine 1984, 1985). These bacteria were initially classified as *Rhizobium fredii* (Scholla and Elkan 1984) and the type strain was defined as being USDA 205 (=ATCC 35423). Owing to several differences, two chemovars were proposed, *fredii*, with strain USDA 205 (=ATCC 35423, =LMG 6217, =PRC 205), and *siensis*, with strain USDA 201 (Scholla and Elkan 1984) as type strains. Following, bacteria were reclassified into a new genus, *Sinorhizobium* gen. nov. (Latin *sinae*, meaning China), with two species, *S. fredii* and *S. xinjiangensis* (from Xinjiang, China). The type strain of *S. fredii* continued to be USDA 205 and the type strain of *S. xinjiangensis* was defined as being CCBAU 110 (=RX 42). Strain USDA 201 was classified as *S. fredii* (Chen et al. 1988). More recently, it was proposed that *Sinorhizobium* and *Ensifer* should be combined as the genus *Ensifer* (Young 2003). In China, the diversification center of soybean and where it has been cultivated for more than 5,000 years at least six putative species in the *Sinorhizobium* and *Bradyrhizobium* genera were identified (Yang et al. 2006; Man et al. 2008) supporting the hypothesis that the original center of a legume is also the diversification center of compatible rhizobia (Lie et al. 1987). On the basis of the phylogenies of ribosomal/housekeeping genes, Man et al. (2008) identified four genomic groups: the *B. japonicum* complex (including *B. liaoningense* and a *B. japonicum* related genomic species) and *B. elkanii* were the major groups and *B. yuanmingense* and *S. fredii* were the minor groups. In contrast, in the saline–alkaline soils of Xinjiang, *B. liaoningense* and *S. fredii* were found as dominant groups nodulating soybean,

whereas other *Rhizobium* genomic species, *B. yuanmingense* and *B. japonicum* were minor groups (Han et al. 2009). The symbiotic gene phylogenies (*nif*H, *nod*C and *nod*Z) were coherent with those of the housekeeping genes in the four genomic groups proposed by Man et al. (2008), indicating that symbiotic genes were mainly maintained by vertical transfer. In North and Northeast China, soybean bradyrhizobia are mainly related to *B. canariense* (Yang and Zhou 2008). This species was first isolated from Canary Island, Spain (Vinuesa et al., 2005). In five ecological regions of India, all with alkaline soils, *B. liaoningense*, *B. yuanmingense*, and a novel *Bradyrhizobium* group were the dominant soybean rhizobia (Appunu et al. 2008). The worldwide distribution of *Bradyrhizobium* species able to nodulate soybean is shown in Table 18.2.

18.3.2 Current Status of SNF in Soybeans: Agronomic and Ecological Perspectives

Soybean is native to Asia and both the crop and the bacterial symbiont were introduced to the Western Hemisphere. For this reason, soils which were never cropped with inoculated soybean, lack appropriate rhizobia and show a significant response to inoculation with selected strains (McLoughlin et al. 1990). However, because rhizobia are able to survive saprophytically for prolonged periods in soil, the repeated cultivation of soybean in a same area promotes the establishment of soil populations of naturalized rhizobial strains. The increase in the bradyrhizobia soil populations over time has been reported by several authors (Brockwell et al. 1987; McLoughlin et al. 1990; Mendes et al. 2004) and is mostly associated with the release of rhizobia from nodules as they disintegrate. The so-called

Table 18.2 Worldwide geographical distribution of rhizobial species nodulating soybean

Country	Specie or genus	References
Japan	*B. japonicum*, *B. elkanii*, *S. fredii*	Suzuki et al. (2008), Saeki et al. (2006)
Vietnam	*B. japonicum*, *S. fredii*, *B. liaoningense*, *B. yuanmingense*	Vinuesa et al. (2008), Saeki et al. (2005)
Myanmar	*B. elkanii*, *B. liaoningense*, *B. yuanmingense*	Vinuesa et al. (2008)
India	*B. yuanmingense*, *B. liaoningense*	Vinuesa et al. (2008), Appunu et al. (2008)
Nepal	*B. japonicum*	Vinuesa et al. (2008)
China	*S. fredii*, *S. xinjiangense*, *B. elkanii*, *B. yuanmingense*, *B. japonicum*, *B. liaoningense*	Peng et al. (2002), Chen et al. (2005) and Man et al. (2008)
Paraguay	*B. japonicum*, *B. elkanii*	Chen et al. (2000)
Argentina	*B. japonicum*	Hungria et al. (2006a, b)
Brazil	*B. japonicum*, *B. elkanii*, *Rhizobium* spp.	Hungria et al. (2006a, b)
USA	*B. japonicum, B. elkanii*	Ramirez et al. (1997), Kuykendall et al. (1992), Keyser and Cregan (1987)

"competition problem" between established and inoculant strains has, however, been one of the major constraints to the introduction of new and more efficient strains, contributing for inconsistent responses to reinoculation. This phenomenon, occurring in areas cropped with soybeans all over the world, is site-specific and results from a combination of factors related to the environment (soil and climate), to the host plant, to the inoculant strains (competitive ability), to the size and composition of the indigenous rhizobium soil populations, and to the type of inoculant and inoculation technology (Singleton et al. 1992; Kvien et al. 1981; Lopez-Garcia et al. 2009).

Regarding the composition of established soybean rhizobial populations, strains serologically related to the 123 serocluster deserve special attention. Strain USDA 123 was isolated in 1960 from a soybean nodule in Iowa (Keyser and Cregan 1987) and since then strains serologically related to this serogroup have been reported as the most competitive of the *B. japonicum* strains from Midwestern United States, characteristically occupying 60–80% of the nodules formed (Kvien et al. 1981). The occurrence of this serogroup has also been reported in Canada (Semu and Hume 1979) and Korea (Kang et al. 1991). Strains from serogroup 123 also have important implications in Brazilian soils, where they occur in up to 70% of the soybean nodules (in 2008, an area equivalent to approximately 22 million ha), even in areas where it had never been inoculated, including the Amazon forest (Vargas et al. 1994; Ferreira and Hungria 2002; Mendes et al. 2004; Hungria et al. 2006a, b). The occurrence of strains from serogroup 123 in these areas confirmed its high saprophytic capacity and was attributed to contamination by wind, seeds, and agricultural machinery originating from the older soybean growing areas in southern Brazil, where inoculants containing strain SEMIA 566 (serologically related to the 123 serogroup) were used until 1978. Some studies have reported that in Brazil SEMIA 566 survives poorly in soil in the first 2 years, but become established and highly competitive thereafter (Freire et al. 1983), and similar results were reported for USDA 123 in USA (Streeter 1992). What makes strains serologically related to the 123 serocluster so competitive has not been established yet. The tenacious competitive ability of this serogroup does not appear to be related to the size of its population in the rhizosphere (Moawad et al. 1984), its lectin-binding ability (Robert and Schmidt 1985a), and its ability to reach the rhizosphere earlier or grow more rapidly than competitor strains (Robert and Schmidt 1985b). In Brazil, the strategy used to prevent the dispersal of highly competitive 123 serogroup strains, with lower N_2-fixing efficiency, in newly grown soybean areas, included the isolation of *B. japonicum* strain CPAC 15 (=SEMIA 5079) from the soil populations in 1986 (Peres et al. 1993). The selected strain CPAC 15 was released for the production of commercial inoculants in 1993 and is characterized by higher rates of N_2 fixation (Hungria et al. 1998) and higher competitive ability than the parental strain SEMIA 566 (Hungria et al. 1996; 1998; Scotti et al. 1997). The idea behind the isolation of CPAC 15 was to select from the 123 serogroup soil population a strain with good N_2-fixation capacity, associated with better competitive and saprophytic abilities. In fact, Mendes

et al. (2004) reported that in a cerrado soil, free of *Bradyrhizobium* populations, inoculation with CPAC 15 established an extremely unfavorable situation for the introduction of new strains. By the fourth and sixth years, after the introduction of several strains, serogroup 123 dominated the nodulation, occurring, on average, in more than 50% of the nodules of the treatments where it had never been inoculated.

A genomic panorama of CPAC 15 covering approximately 13% of the genome was generated by Godoy et al. (2008) and revealed that this strain was surprisingly different from *B. japonicum* strain USDA 110. At least 35% of the coding DNA sequences (CDS) of CPAC 15 showed higher similarity to microorganisms other than strain USDA 110. It was found some CDS that might help explain the strong competitiveness of CPAC 15 in the soybean rhizosphere such as genes related to the catabolism of rhizopines (mocR), which are inositol derivates synthesized in legumes in response to rhizobia (Murphy et al. 1995) and an opine oxidase gene (ooxA). Several genes that may facilitate the invasion of the host and the nodulation process also were identified.

In the United States, there are studies showing absence in the response of soybean to inoculation when the numbers of naturalized bradyrhizobia are as low as 10–20 cells g^{-1} soil (Thies et al. 1991a, b). It has also been often shown that in a soil harboring rhizobia the introduction of a new strain may fail or that the strain persists only for few years (McLoughlin et al. 1990) and hence yield increases are not obtained. More recently, yield responses to reinoculation of up to 12% have been reported in northern and eastern soybean production states within the Midwest, whereas states from the Great Plains region still do not have consistent yield responses to reinoculation of "old" fields (Abendroth et al. 2006). It seems that in northern states these responses are more related to cold spring temperatures which limit the growth and multiplication of *B. japonicum* and in eastern production states the extensive use of seed-applied fungicides could be partly responsible for the observed yield responses to reinoculation in the "old" soybean fields. In Brazil, soybean yield responses to reinoculation have been reported quite frequently even in areas with established populations of rhizobia (Hungria et al. 1998, 1996; Mendes et al. 1994b; Vargas et al. 1994, 2002). For example, Hungria et al. (2006a, b) carried out forty experiments over a 3-year period in oxisols containing at least 10^3 cells of *Bradyrhizobium* g^{-1} in the State of Paraná, southern Brazil. Compared with the noninoculated control, reinoculation significantly increased the contribution of SNF estimated by the N-ureide technique (on average from 79 to 84%), grain yield (on an average, 127 kg ha^{-1} or 4.7%), and total N in grains (on average 6.6%). In Table 18.3, we present data from six experiments carried out in cerrado oxisols with established rhizobial populations. Although significant differences were not observed in the three experiments, yield gains with reinoculation were of 227, 636, and 345 kg ha^{-1}. The lack of statistical significance for field data is a major limitation while measuring yield response to reinoculation (Singleton et al. 1992). For this reason, as pointed out by these authors, given the low cost of the inoculant and the small investment required to inoculate legumes, measuring economical relevance of legume inoculation should be considered when evaluating

Table 18.3 Yield grains of soybean in Brazilian oxisols as a function of reinoculation with different mixtures of *Bradyrhizobium* strains and nitrogen fertilizer (Mendes et al. unpublished)

Treatments	1993/94 (kg ha^{-1})	1996/97	1997/98	1998/99	1999/00	2000/01
Control	2,661 b	2,130 b	2,483	4,207	4,647	4,102
200 kg N ha^{-1}	3,657 a	2,818 a	2,660	4,192	4,691	4,326
B. elkanii 29W + *B. elkanii* 587	2,822 b	2,058 b	2,875	4,102	4,583	4,363
B. japonicum CPAC 7 + *B. japonicum* CPAC 15	2,888 b	2,218 ab	3,119	4,193	4,524	4,447
CV(%)	11	17	13 (ns)	7 (ns)	4,5 (ns)	6,0 (ns)

ns Nonsignificant
Values followed by the same letters in colums are not statistically different

these responses, as well as other benefits such as, better seed quality, total N increases, or conservation of soil N.

Several strategies have been reported to overcome the competition problem mainly at the early plant growth stages. In Argentina, soil placement (in-furrow inoculation) and selection of *B. japonicum* strains with enhanced motility have been suggested (Althabegoiti et al. 2008; Lopez-Garcia et al. 2009). Furrow inoculation with selected ELP 3008 strain significantly increased nodule occupancy, although grain yield and N content were unaffected. Pre-activation of the plant partner through root and seed treatments with Nod factors (Macchiavelli and Brelles-Marino 2004) and of rhizobial strains with flavonoid compounds such as genistein and jasmonate (Zhang and Smith 1996; Leibovitch et al. 2001) also have been suggested. For agricultural purposes, the use of HPLC-purified Nod factors are economically prohibitive, whereas methyl jasmonate is a promising alternative for inoculant production, because it is easier to produce and less expensive than inoculants produced based solely on genistein (Mabood et al. 2006).

Despite the ability of soybean to derive N from SNF, many studies have been conducted to compare the effects of SNF with supplemental N as starter and later N applications. Generally, yield responses have been extremely variable, depending on the efficiency of *Bradyrhizobium* strains (Simanungkalit et al. 1995), soybean cultivars and yield potential (Starling et al. 1998; Wesley et al. 1998), soil NO_3–N content (Lamb et al. 1990), N rates and time and modes of application. The combination of these factors makes responses to supplemental N and SNF site-specific. In the United States, around 20% of the soybean cropped area receives, on an average, 22 kg N ha^{-1} (http://www.ers.usda.gov/Data/FertilizerUse/, verified 24 November 2009) whereas in Brazil and Argentina soybean is grown only by using inoculation with N_2-fixing bacteria. In Brazil, the recent expansion of no-tillage (NT) cropping systems promoted the revival of the idea that it is necessary to use small N rates at sowing stage to overcome problems related with N immobilization, mainly when soybean is cultivated after a non-legume crop. Also, reports obtained in US on yield responses to late N applications under high-yielding soybean environments (Wesley et al. 1998) raised

concerns regarding whether SNF would be capable of reaching increased N needs of newly released more productive cultivars. Recent studies have, however, shown that the use of N fertilizer either at the start or late stage is not advantageous in Brazilian conditions (Mendes et al. 2003; Mendes et al. 2008). Salvagiotti et al. (2008) conducted a meta-analysis of a data set of 627 experiments dealing with the soybean response to N fertilization and found that 50–60% of the soybean N was met by SNF across a wide range of yield levels and environments and the proportion of N derived from fixation decreased with increasing inputs of N fertilizers. The authors pointed out that more studies on the partitioning of N and the contribution of N fixation in above and belowground parts for soybean need to be conducted, as this might be one of the reasons to explain why in most situations the amount of N fixed by soybean was not enough to replace N export from the field with grain, or was at best close to neutral if N from belowground parts is included. They also suggested that the capacity of the symbiotic N supply from soybean nodules to meet crop N demand in high yielding environments (grains yields above 5,000 Kg ha^{-1}) remains particularly uncertain and that more studies regarding the optimization of SNF and supplementation with N fertilizer must be carried out.

The release of genetically engineered (GE) soybean cultivars also has raised concerns whether the SNF process would be affected or not. Therefore, it is important that the biosafety studies, that usually follow the release of these GE soybean cultivars, also include aspects related to the SNF process. The first generation of GE soybean cultivars encompasses those with enhanced input traits such as herbicide tolerance and tolerance to environmental stresses like drought. The commercial use glyphosate-tolerant (Round up Ready RR) soybean cultivars was initiated in 1996 in the United States and nowadays it is estimated that 70 million ha worldwide are cropped with these cultivars (http://www.isaaa.org/resources/publications, verified 14 December 2009). Studies evaluating the effect of glyphosate on the *B. japonicum*/*B. elkanii* symbiosis with RR soybeans cultivars have been conducted under greenhouse and field conditions and some deleterious effects of glyphosate on the nodulation and/or N_2-fixation process have been reported (Reddy et al. 2000; King et al. 2001; Reddy and Zablotowicz 2003; Zablotowicz and Reddy 2004; Dvoranen et al. 2008; Bohm et al. 2009), although yield reductions have not been demonstrated. The magnitude of these responses changes with the glyphosate rates, salts, and time of application, soybean varieties, geographical and environmental conditions since they are more accentuated under water stress and sandy soils. It is possible, as suggested by Bohm et al (2009), that the reductions of nodule mass and SNF on GE_{RR} soybeans could lead to an increase in N scavenging from soils and consequently an eventual decrease of soil organic matter reserves; however, further studies are required to prove that.

In a scenario of global climatic changes that will affect rain distribution and considering the importance of legume-based agricultural systems that likely will not have adequate rainfall or irrigation during their cropping cycle, there is a great need for research in drought-tolerant N_2 fixation. With the exception of soybean, there has been little directed research to breed for improved N_2-fixation tolerance to

drought. Drought has a negative effect on SNF by causing O_2 limitation (which in turn inhibits nitrogenase activity because of lower nodule respiratory activity), shortage of C substrates for bacteroid, and higher accumulation of nitrogen compounds that provide a feedback loop to decrease nodule activity (Purcell 2009). Although GE soybean cultivars drought-tolerant are still in the pipe line (Chen et al. 2007), traditional plant breeding has provided soybean progeny lines (R01-416F and R01-518F) with decreased sensitivity of N_2 fixation to water deficits, which are publicly available as breeding material (Sinclair et al. 2007).

18.3.3 "Omics" Approaches in Soybean-Bradyrhizobium *symbiosis*

Despite the importance of the SNF, the great majority of the genomes of prokaryotes refer to pathogens and very few rhizobial strains have been completely sequenced: *Mesorhizobium loti* MAFF303099 (Kaneko et al. 2000), *Sinorhizobium* (=*Ensifer*) *meliloti* 1021 (Galibert et al. 2001), *B. japonicum* USDA 110 (Kaneko et al. 2002), *Rhizobium etli* bv. phaseoli CFN 42 (Gonzalez et al. 2006), *R. leguminosarum* biovar viciae 3841 (Young et al. 2006), *Bradyrhizobium* sp. ORS278 and BTAi1 (Giraud et al. 2007), *Azorhizobium caulinodans* ORS 571 (Lee et al. 2008), and *Cupriavidus taiwanensis* LMG19424 (Amadou et al. 2008), thus representing only 1.3% of all complete genomes of prokaryotes available today. By means of the complete genome sequencing, comparative genomics provide not only the information needed to perform functional analysis of the genes but also new insights into gene function, gene evolution, and genome evolution by comparing the gene components and organization in the genomes among species (Weidner et al. 2003). However, considering that the costs of sequencing are still high, creative low-cost initiatives to obtain genomic drafts based on partial sequencing of the genome also have been reported (Viprey et al. 2000; Godoy et al. 2008). The complete nucleotide sequence of the genome of *B. japonicum* USDA 110 was determined by Kaneko et al. (2002). This genome is composed of a single circular chromosome of approximately 9.2 Mb and contains 8,317 genes encoding for proteins, in addition to the RNAs and genes related to nodulation (*nod, nol*, and *noe),* N_2 fixation (*fix* genes*)*, and synthesis of nitrogenase (*nif* genes). Of the putative genes, 52% showed sequence similarity to genes of known function, 30% to hypothetical genes and the remaining 18% had no apparent similarity to reported genes. In 14 different positions of the genome, there are DNA fragments ranging from 4 to 97 kb inserted in the tRNA, with the capacity of generating partial duplication of the target genes of the tRNA. Thirty-four percent of *B. japonicum* USDA 110 genes showed similarity with *M. loti* and *S. meliloti* and 23% were unique to this species. A fragment of 681 kb was identified as a possible symbiotic island and includes the 410 kb where the nodulation and N_2-fixation genes are grouped, as previously identified by Göttfert et al. (2001). In this region, 655 putative genes were assigned, 301 of which are related to the transmission of

DNA and to the N_2 fixation. Out of 167 genes for transposases, 60% were located at the symbiotic island (which comprises only 7.5% of the entire genome) evidencing the accessory nature of these genes and their ability to be acquired via horizontal gene transfer (HGT).

The complete genome sequencing of strain USDA 110 suggested a high plasticity of the genome of *B. japonicum*, probably related to complex genomic rearrangements, including HGT and the insertion of several DNA elements and homologous recombination. In fact, Barcellos et al. (2007) showed evidence of horizontal transfer of symbiotic genes from a *B. japonicum* inoculant strain to indigenous diazotrophs *Sinorhizobium* (Ensifer) *fredii* and *B. elkanii* in a Brazilian savannah soil. The *B. elkanii* strain (S127) acquired a *nod*C gene from the inoculant *B. japonicum*, whereas the indigenous *S. fredii* strain (CPAC 402) received the whole symbiotic island form the *B. japonicum* strain and maintained an extra copy of the original *nif*H gene. The authors suggested that the acquisition of the *B. japoniucm* genes may have enabled indigenous rhizobia to effectively nodulate the exotic soybean host legume which is, under field conditions, an ecological advantage. Loureiro et al. (2007) also reported evidence for HGT in cerrado soils originally devoid of rhizobia capable of effectively nodulating soybean. After 18 years of the introduction of a maximum of four strains, up to 13 *rep*-PCR profiles were identified, some sharing many identical bands with the inoculant strains, but other quite distinct from the putative parental strain. Two uncommon combinations of ribosomal and symbiotic genes in *B. japonicum* and B. *yuanmingense* suggesting the occurrence of HGT also were reported in China by Man et al. (2008).

The term "nodulin" was coined to describe nodule-specific host polypeptides, and nodulin genes were those specifically induced during nodulation in plants, mostly existing as orthologues in different legumes. According to the pattern of expression, the nodulins may be classified as early or late nodulins. Early nodulins (ENODs) are induced within minutes of rhizobial binding and/or Nod factor perception. One example is GS52 apyrase which is induced within 3 h after soybean inoculation with *B. japoncium* (Day et al. 2000). The induction of late nodulin genes can be observed within days after inoculation of the plants with rhizobia (Ott et al. 2005). The most abundant late nodulin in active nodules is the leghemoglobin, and others are the glutamine synthase (GS) and the glutamate synthase (GOGAT). Although nodulins were originally considered to be either nodule-specific, it has been shown that their products can be found in other plant organs such as flowers (Crespi et al. 1994). Nodulin homologs were also identified in nonlegumes species (Kouchi et al. 1999), suggesting that many nodulins are normal plant proteins that have been recruited to carry out specific functions during the nodulation process (Brechenmacher et al. 2008). Regardless of whether these genes are nodule-specific, or upregulated, they play an important role in SNF. Large scale genomics approaches to study SNF which resulted in the discovery of key genes were accelerated after the adoption of *Medicago truncatula* (Barker et al. 1990) and *Lotus japonicum* (Handberg and Stougaard 1992) as the two model legumes (Vance 2009). *Medicago* forms indeterminate nodules while *Lotus* plants develop determinate nodule on their root systems. These two species are diploid ($n = 6$), posses an

autogomous nature, can be genetically transformed stably and transiently with *Agrobacterium,* and have small genomes, short generation times and prolific seed production.

Although soybean is characterized by a large genome (approximately 975 Mb distributed in 20 chromosomes) and ancient polyploidy, owing to its significance for plant biotechnology, a project to sequence its genome has recently begun (http://www.phytozome.net/soybean, verified 24 Nov 2009), providing an outstanding opportunity to gain new insights into several regulatory networks related to the infection process and symbiosis. The use of expressed sequence tags (ESTs), which are random sequences of gene transcripts, have been an invaluable tool for the identification of plant genes involved in nodule formation and N_2 fixation. EST sequencing involves making a cDNA library with RNA isolated from an organ or treatment regime, with inserts directionally cloned into a phage vector (Vance 2009). The frequency of occurrence of a given EST within an organ or treatment may give an idea of the level of expression of the gene represented by that EST (Fedorova et al. 2002; Journet et al. 2002). For soybean, around 1.279.502 ESTs have been released into the public domain (http://compbio.dfci.harvard.edu/tgi/plant.html; verified 17 November 2009). Of these, 18,169 are derived from nodules.

The progressing efforts in legume genome sequencing and the collection of ESTs, provided the sequences needed to make oligonucleotide and/or cDNA probe sets for whole genome studies. These probes are immobilized on either nylon membranes (macroarrays) or glass slides (microarrays) with each spot visualized by image analysis. Bulk RNA from the treatment or organ being studied which binds to the probe sets is then quantified by radioactivity or fluorescence detection. By using cDNA microarrays having 36,760 different soybean cDNA clones, Brechenmacher et al (2008) monitored genes that were differentially expressed in soybean roots at 4, 8, and 16 days after inoculation with *B. japonicum* USDA 110. They identified 6,555 genes that were significantly differentially expressed during nodulation, showing the profound effects on plant during the nodulation process. The results showed that *B. japonicum* reduces plant defense responses during nodule development and the presence of a high level of regulatory complexity (transcriptional, post-transcriptional, translational, and post-translational) essential for the development of the symbiosis and adjustment to an altered nutritional status. Genomic-wide transcriptional studies of the microsymbiont *B. japonicum* also have been conducted and include its chemoautotrophic growth (Franck et al. 2008), growth in bacteroid state and in minimal and rich media (Chang et al. 2007; Pessi et al. 2007), its response to osmotic stress and desiccation (Chang et al. 2007; Cytryn et al. 2007) and to genistein (Lang et al. 2008).

Proteomics is yet another important and powerful tool used in genetic analysis focusing on the functionally translated portion of the genome (Komatsu and Ahsan 2009). Elegant proteomic studies on the initial root hair interaction between soybean and *B. japonicum* strain USDA 110 were carried out by Wan et al. (2005). Four-day-old seedlings roots were treated either with the wild-type

and/or a NodC- mutant *B. japonicum*, unable to synthesize the signal required to initiate the nodulation process. After the treatment, roots and root hairs were collected for proteomic analysis. Seedlings treated with water served as control. The comparison of the protein profiles of the root hairs and roots showed 96 differentially expressed proteins, of which 12 proteins were unique to root hairs, confirming that root hairs are not only morphologically but also biochemically different from the remaining epidermal cells. A set of 27 candidate proteins out of 37 spots, derived from inoculated root hairs over different time points, were identified by data base comparisons. Among these proteins, some were previously known to respond to inoculation (e.g., peroxidase and phenylalanine ammonia-lyase) and some were novel proteins (e.g., phospholipase D and phosphoglucomutase). Furthermore, ten proteins were identified that were differentially expressed in response to the wild type and to the NodC-mutant *B. japonicum*. In another study, the most abundant proteins in soybean root nodule cytosol were analysed by Oehrle et al. (2008) who showed that 28% and 12% of all proteins identified were involved in C and N metabolism. A total of 12% was involved in O_2 supply, maintenance, and protection and 11% with vesicular transport suggesting that large macromolecules are actively transported in addition to small C and N metabolites. One anomaly found in this study was the paucity of proteins associated with fatty acid and lipid metabolism, despite the fact that the abundance of membranes in soybean nodules would imply an active and continuous need for their biosynthesis, repair and maintenance. Proteome studies on the microsymbiont *B. japonicum* have also compared the protein expression of free-living and nodule residing bacteria (Sarma and Emerich 2006), analyzed its growth under acidic conditions (Puranamaneewiwat et al. 2006) and its extracellular (secreted) proteome, which plays an important role in the interaction of this bacteria and its soybean host (Hempel et al. 2009).

Although transcriptomic and proteomic analysis provide a vast amount of genomic information, metabolite profiling is essential for gaining a better fundamental understanding of how changes at the transcription and translation level affect cellular function and to reveal the metabolic shifts that underlie nodule development (Stacey et al. 2006). Metabolomics, or the study of the profile of metabolites, gives an instantaneous snapshot of the physiology of the cell, providing a major tool for the characterization of post-genomic processes. Metabolite analysis on soybean nodules and its relation with abiotic stress have been carried out for many decades and has tended to be targeted on a few well-defined metabolites, which were often part of well-characterized metabolic pathways (Streeter 1980, 1987; Copeland et al. 1989; King and Purcell 2005; van Heerden et al. 2008). The recent development/refinement of a variety of analytical platforms, including GC-mass spectrometry and software, enabled the creation of tag libraries representing known and unknown metabolites in nodules, roots, leaves, and flowers and the identification of marker metabolites for various plant organs, such as octadecanoic acid and glutamine for nodules (Desbrosses et al. 2005) in *Lotus japonicus*. By allowing the discovery of novel metabolites, studies like this in *Lotus* also reveal opportunities to discover previously unknown aspects of metabolism

and to gain a more holistic picture of the nodule metabolism that underpins SNF. Global changes in the transcript and metabolic profiles during SNF under different growth conditions (e.g., P supply, water stress, etc.) will help identify candidate genes and metabolic pathways that will be useful to develop better N_2-fixing legume genotypes. As pointed out by Vance (2009), these studies are in infancy stage, and in the twenty-first century the integration of the knowledge gained from the legume genomes with biological and agronomical questions of importance will be a big challenge for molecular biologists, crop physiologists, agronomists, geneticists, and plant breeders.

18.4 Common Bean

The origin of common bean (*Phaseolus vulgaris* L.) is thought to be Latin America and the first areas of its domestication were the highlands of Mesoamerica and the Andean South America, some 5,000 years ago (Kaplan 1980), from where it was introduced to rest of the world thereafter (Martinez-Romero 2003). Common bean, from here onward referred to as bean, is the world's most important grain legume for direct human consumption as it is an important source of protein for the poorer populations of Central and South America and West Africa, with Brazil being the largest grower and consumer of the legume worldwide followed by Mexico. It serves as a perfect food, being an important source of fibers and complex carbohydrates (Table 18.1). According to FAO (http://www.faostat.fao.org, verified 02 December 2009), in 2007, Asia accounted for 43% of world production of common bean, followed by Americas (38%), Africa (17%), Europe (2%), and Oceania (0.1%). Developing countries account for 87% of the world consumption. Dry beans were grown on 26.5 million ha in 148 countries in 2007 and the total production was 18.3 million metric tons while 6.6 million tons of green beans were grown worldwide in 2007. Despite being grown in large areas, the bean crop is characterized by low productivity worldwide (691.2 kg ha^{-1}), especially because of poor cropping practices, such as pests and diseases control and the inadequate supply of fertilizers.

18.4.1 Rhizobial Strains Nodulating Bean

Currently, five species have been recognized as microsymbionts of *Phaseolus vulgaris*: *Rhizobium leguminosarum* bv. *phaseoli* (Jordan 1984), *R. tropici* (Martinez-Romero et al. 1991), *R. etli* bv. *phaseoli* (Segovia et al. 1993), *R. gallicum,* and *R. giardinii* (Amarger et al. 1997). As *P. vulgaris* is a relatively promiscuous host, new rhizobia nodulating beans in different parts of the world are frequently reported (Table 18.4), which means that their classification is always under review (Mouhsine et al. 2007). *R. leguminosarum* bv. *phaseoli* was first divided into types I and II

Table 18.4 Worldwide geographical distribution of rhizobial species nodulating common bean

Local	Rhizobial species	References
France, Spain	*R. giardinii* *R. gallicum* *R. leguminosarum* bv. *phaseoli*	Amarger et al. (1997); Jordan (1984)
Spain, Mexico, Colombia, Austria, USA	*R. etli*	Segovia et al. (1993)
West Africa (Senegal and Gambia) and Kenya	*R. leguminosarum* *R. tropici* *R etli*	Odee et al. (2002), Diouf et al. (2000), Anyango et al. (1995)
Tunisia	*R. etli* bv. *phaseoli* *R.gallicum* bv. *phaseoli* *R. giardini* bv. *giardini* *R. leguminosarum* bv. *phaseoli* *R. leguminosarum* bv. *viciae*	Mhamdi et al. (2002)
Mexico	*R. gallicum* bv. *gallicum* *Rhizobium etli* bv. *phaseoli*	Silva et al. (2003)
Ethiopia	*R. etli*	Beyene et al. (2004)
Jordan	*R. etli* *R. tropici*	Tamimi and Young (2004)
Tunisia	*Ensifer* (=*Sinorhizobium*) *meliloti* bv. *Mediterranense*	Mnasri et al. (2007a, b)
Egypts	*Rhizobium etli* bv. *phaseoli* *R. gallicum* bv. *gallicum*	Shamseldin and Werner (2007)
Morocco	*S. meliloti* *R. leguminosarum* bv. *viciae* *R. tropici* IIB *R. etli* *R. gallicum* bv. *gallicum*	Mouhsine et al. (2007)
Brazil	*R. tropici* (type A, B, and others) *R. etli* *R. leguminosarum* *R. giardinii* *Mesorhizobium* *Ensifer* (=*Sinorhizobium*)	Stocco et al. (2008), Kaschuk et al. (2006), Grange and Hungria (2004), Mostasso et al. (2002), Martinez-Romero et al. (1991)

(Martínez-Romero et al. 1998). *R. tropici* types A and B have been proposed for type II strains, carrying a single copy of *nifH*. Types A and B differ by the values of DNA-DNA hybridization, phenotypic characteristics and by the presence of specific megaplasmids (Martinez-Romero et al. 1991; Géniaux et al. 1995). *R. etli* was then proposed for the *R. leguminosarum* bv. *phaseoli* type I strains (Segovia et al. 1993). They have multiple copies of the structural gene for nitrogenase in their symbiotic plasmids. R. *tropici* type A and type B and *R. etli* bv. *phaseoli* have been isolated from *Gliricidia sepium*, a tropical tree native of the Americas (Acosta-Duran and

Martinez-Romero 2002) that is proposed to be the natural host of *R. tropici*. Later work carried out with isolates from *Mimosa affinis* root nodules led to the proposition of a new biovar, bv. *mimosae*, within the species *R. etli* (Wang et al. 1999). Although both biovars *phaseoli* and *mimosae* may nodulate *P. vulgaris*, only the biovar *mimosae* can form N_2-fixing nodules on *Leucaena leucocephala.* Two additional rhizobia, *R. gallicum* and *R. giardinii* (Amarger et al. 1997), able to form symbiosis with bean were characterized in France. Each species was subdivided into two biovars, as follows: *R. gallicum* biovar *gallicum* and *R. gallicum* biovar *phaseoli*; and *R. giardinii* biovar *giardinii* and *R. giardinii* biovar *phaseoli*. *R. gallicum* bv. *phaseoli* and *R. giardinii* bv. *phaseoli* showed the same variation relative to the host of *R. leguminosarum* bv. *phaseoli*, the same number of copies of the *nifH* gene and the same homology in terms of the *nodB* gene of *R. etli*. *R. gallicum* bv. *gallicum* and *R. giardinii* bv. *giardinii* are not homologous to *R. etli* with respect to *nodB* gene, as they are with *R. tropici*. They have single copies of *nifH* gene and no homology was detected in relation to structural genes *nifK*, *D,* and *H*, which are highly conserved between the N_2-fixing microorganisms. The biovar *gallicum* was reported only for *R. gallicum* strains isolated in Europe, America, and North Africa nodulating legumes in the genera *Phaseolus*, *Leucaena*, *Macroptilium*, *Onobrychis*, *Sesbania*, *Caliandra*, *Gliricidia,* and *Piptadenia* (Amarger et al. 1997; Herrera-Cervera et al. 1999; Silva et al. 1999; Rodriguez-Navarro et al. 2000; Zurdo-Piñeiro et al. 2004). The biovar *phaseoli* strains were isolated exclusively from *P. vulgaris* nodules and do not establish symbioses with *Leucaena*, *Macroptilium,* and *Onobrychis* (Silva et al. 2003). Recently, it was hypothesized that *R. gallicum* is a cosmopolite species that has a wide geographical distribution and a long history of adaptation to different environments and host plants (Silva et al. 2005). *R. yanglingense*, a rhizobial species isolated from wild legumes in China is also able to form nodules on *P. vulgaris*, although inefficiently (Tan et al. 2001). A new biovar, able to form nodules in bean, (*Ensifer* (=*Sinorhizobium*) *meliloti)* characterized to tolerate salinity was isolated from a Tunisian oasis and termed biovar *mediterranense*. This strain is more competitive and more effective under water deficiency than strain *R. tropici* CIAT 899 (Mnasri et al. 2007a). For all these reasons, bean is considered a promiscuous host nodulated by a variety of rhizobia. Among other rhizobia, *Bradyrhizobium* and *R. mongolense* (Michiels et al. 1998; van Berkum et al. 1998) and *S. meliloti* and *S. fredii* (Bromfield and Barran, 1990; Sadowsky 1988) have also been reported to form nodules on beans. The distribution of bean rhizobia able to nodulate *P. vulgaris* varies between geographical locations (Table 18.4), although *R. etli* and *R. tropici* appear to be widely distributed (Young et al. 2004; Amarger 2001). *R. etli* has been indicated as the dominant microsymbiont in both the Mesoamerican and the Andean centers of genetic diversification (Segovia et al. 1993; Aguilar et al. 1998, 2004) and it was introduced into Europe probably by seeds. In Brazil, there are reports of symbioses with *Rhizobium tropici*, *R. etli*, *R. leguminosarum,* and *R. giardinii* and also with other genera such as *Mesorhizobium* and *Ensifer* (=*Sinorhizobium*), and with other bacteria that may well represent new species (Mercante et al. 1998; Mostasso et al. 2002; Grange and Hungria 2004; Kaschuk et al. 2006; Ribeiro et al. 2009).

18.4.2 Current Status of SNF in Common Bean

The grain yield responses to bean inoculation under field conditions are variable, ranging from no responses to substantial increases (Table 18.5). Poor nodulation or lack of response to inoculation in field experiments have been attributed to the- (1) presence of high but inefficient population of indigenous common bean rhizobia in soil and in seeds (Andrade and Hungria 2002; Vargas et al. 2000) (2) genetic instability of selected strains and (3) sensitivity of the symbiosis to environmental stresses, such as high temperatures, soil dryness and low soil fertility (Graham 1981; Hungria and Vargas 2000). However, selected strains are able to increase yields even in the absence of N fertilizers (Mendes et al. 1994a; Peres et al. 1994; Hungria et al. 2000). In an oxisol of the Cerrado region of Brazil, without previous bean-cropping history, yields of 3,000 kg ha^{-1} were achieved following seed inoculation, which could probably be due to low or no indigenous soil rhizobial populations, allowing nodule formation by the inoculated strain (Vargas et al. 2000). Optimum bean yields can also be obtained by inoculating superior strains in association with low levels of N fertilizer (Hungria et al. 2003) or associated to the foliar application of molybdenum and cobalt (Berton et al. 2008). In Ethiopia, starter N use resulted in greater bean yields suggesting that although soil had enough rhizobial populations specific to beans, the plants needed a bit of N to get established before switching to SNF (Daba and Haile 2000). Strains selection programs for high temperature tolerance coupled with genetic stability began in 1991 with the isolation and characterization of strains able to nodulate leucaena and

Table 18.5 Bean yield increases as a function of field inoculation with selected rhizobial strains in Brazil

Local	Yield increase	Strain	References
Sandy clay loam Ultisols with low indigenus populations and under irrigation	200–1,776 kg ha^{-1}	Strains isolated from Federal District, Strains from University of Minnestoa and and *Rhizobium tropici* CIAT 899	Mendes et al. (1994)
Well-drained oxisols of cerrado region without irrigation	63–290 kg ha^{-1}	Several strains isolated from Federal District and *Rhizobium tropici* CIAT 899	Peres et al. (1994)
Well-drained oxisol of cerrado region with low indigenous populations and under irrigation.	248 kg ha^{-}	*Rhizobium tropici* CIAT 899	Vargas et al. (2000)
Cerrados region, Brazil	747 kg ha^{-1} in average	Several strains isolated from Federal District and *Rhizobium tropici* CIAT 899, and *Rhizobium tropici* PRF 81	Mostasso et al. (2002)
Parana state, Brazil	437–465 kg ha^{-1}	*Rhizobium tropici* H12 and H20 strains	Hungria et al. (2003)

fix large amounts of N_2 when in symbiosis with common bean (Martinez-Romero et al. 1991). These cross-inoculation experiments resulted in the selection of *R. tropici* CIAT 899. One important feature of *R. tropici* is its higher symbiotic stability, probably due to the presence of a unique copy of the *nifH* gene (Geniaux et al. 1993; Palacios et al. 1995). Based on this information, since 1994, the Brazilian strain-selection program for beans, aims at selecting strains belonging to *R. tropici* that are genetically stable and efficiently fix N (Hungria and Araujo 1995). Subsequently, three strains (CIAT 899, PRF 81, and H 12) identified during this program were officially recommended for commercial use in Brazil as inoculants against bean crops. They are capable of supplying sufficient N and produced grain yields of 2,500 kg ha^{-1} or more.

Despite the inoculation effects of *R. tropici* observed under certain soil and climate conditions, other species have been found more effective in promoting bean yields. For example, 4H41 strain (*Ensifer meliloti* bv. *mediterranense*), caused a significant increase in common bean grain yield in water-limited soils compared to *R. tropici* (Mnasri et al. 2007b). Strain 4H41 is also highly salt-tolerant indicating that under water stress this feature may be important for selection of effective rhizobia in bean cultivation. Likewise, *Rhizobium* spp. strains isolated from Andalusian soils (Southern Spain) were more effective than the reference strains *R.leguminosarum* bv. *phaseoli* TAL1121, *R. etli* strain CNF 42 and *R. tropici* type strain CIAT 899 (Rodriguez-Navarro et al. 2000). Since the agronomic selection of beans rarely considers the N_2-fixing abilities, it is important to improve *Rhizobium* strains (Martínez-Romero et al. 1998). Several bean-rhizobia have been genetically modified and some of the resulting mutant strains have been found to acquire increased nodulation or N_2-fixing abilities. This was observed in *R. tropici* strain CFN299 and in *R. etli* strain CFN42. The first strain received additional *nod* genes (*nodP* and *nodO*) (Martínez et al. 1993) and the later strain received additional citrate synthase genes (Pardo et al. 1994). The variation in nodulation obtained with the modified *Rhizobium* strains, were however, not reflected in any differences in plant dry weight of beans with high capacity to fix N (Martínez-Romero et al. 1998). Mutants derived from *R. etli* CFN42 had increased N_2-fixation ability (Cevallos et al. 1996; Peralta et al. 2004). The *nodH*, encoding a sulfotransferase causes the transfer of sulfate to the Nod factor backbone in *Sinorhizobium* sp BR816 whose inactivation results in increased N_2-fixation in *P. vulgaris* (Remans et al. 2007). Similarly, an increase in bean nodulation with *R. etli* was obtained through the inhibition of the constitutive expression of foreign glutamate dehydrogenase (GDH) (Mendoza et al. 1995). This inhibition is suppressed when *gdhA* expression by NifA is controlled, leading to a delay on the onset of GDH activity after nodule establishment. The expression of *gdhA* by bacteroids makes that the newly synthesized ammonia be preferentially incorporated into the amino acid pool and not transferred to the infected cells. It is believed that the reduction of the basal N in the bacteroid might stimulate the nitrogenase activity increasing the N supply to the plant (Mendoza et al. 1998). Increased yield of bean also was obtained through inoculation with a *R. etli* mutant (CFN037) having increased respiratory activity. In this case it was suggested that inoculation with special selected mutant

strains of *R. etli* could modulate nodule N assimilation and N transport compounds (Silvente et al. 2002). Other genes have been identified as symbiotically relevant. In *R. etli*, the inactivation of *relA* gene severely affected symbiotic phenotype at the late stage of the interaction. This gene catalyzes the synthesis of nucleotide alarmones which play an important role in the regulation of gene expression in *R. etli* bacteroids and in the adaptation of bacteroid physiology since it mediates the stringent response in bacteria which is triggered by various forms of nutritional stress (Moris et al. 2005). In the same way, inoculation of *P. vulgaris* with an *R. etli fnrN* mutant strain resulted in a severe reduction of the bacteroid N_2-fixation capacity compared to the wild type, demonstrating the importance of f*nr*N during symbiosis (Moris et al. 2004). This gene controls the expression of FnrN, which is a third N_2-fixation regulatory protein, operational in *R. etli* CNPAF512, involved in sensing a low-oxygen signal and in transducing this signal into a regulation cascade of a specific subset of N_2-fixation genes.

The ability of *Rhizobium* spp. to adapt to adverse conditions, such as low pH, is fundamental for the establishment of an efficient symbiosis. More than one-quarter of the world's arable land are acidic, and hence to understand how rhizoba survive under acid-stressed environment is of great agricultural relevance (Tiwari et al. 1996). For example, rhizobial species differ in the levels of tolerance to acidity (Graham et al. 1994) and the genetic and physiological bases of acid tolerance have only recently become clearer. The ability of *R. tropici* to grow under acidic conditions is attributed to its ability to produce glutathione (Riccillo et al. 2000; Muglia et al. 2008). Under conditions of low pH, the glutathione intracellular level is increased by the transcriptional activation of the *gshB* gene. Despite *R. tropici* being the most acid-tolerant *Rhizobium* species described to date, it has been found as predominant species in soils submitted or not to lime, while larger numbers of *R. leguminosarum* types also have been found in soils with lower pH. In these same soils, only a small proportion of the *R. tropici* isolates were able to nodulate *Leucaena*, characteristic that distinguished *R. tropici* from other *Phaseolus*-nodulating rhizobia (Andrade et al. 2002).

Soil P-deficiency also can affect the symbiotic partners. In order to improve the understanding of the molecular mechanisms for the adaptation to P deficiency of bean plants under SNF conditions, Hernández et al. (2009) evaluated the global changes in the transcript and metabolic profiles during SNF in P-stressed bean plants. Thirty-seven transcription factor genes were differentially expressed in P-deficient nodules and one gene was repressed. Glycolysis and glycerolipid metabolism, and starch and sucrose metabolism were among the pathways significantly induced or repressed in P-deficient nodules. These findings are important as they allow the identification of candidate genes and metabolic pathways that may be used for the selection of P-efficient N_2-fixing bean genotypes.

Drought stress is another limiting factor for growth and yield of crops. Anhydrobiotic organisms are rare but when present possess the ability to withstand long dried periods, having the capacity to rehydrate and restart their metabolic functions after being in contact with water (Crowe et al. 1992). The organisms growing under such stressed environment have the ability to synthesize and accumulate large

concentrations of the nonreducing disaccharide trehalose which helps legumes to grow better under drought-stressed conditions. Suárez et al. (2008) reported exciting results regarding the improvement of bean grain yield and tolerance to drought stress through the inoculation with a *R. etli* strain overexpressing the trehalose-6-phospahte synthase (TPS). Common bean (*P. vulgaris)* plants inoculated with *R. etli* overexpressing TPS gene had more nodules with increased nitrogenase activity (NA) and higher biomass relative to plants inoculated with wild-type *R. etli*. On the contrary, plants inoculated with an *R. etli* mutant in TPS gene had fewer nodules and decreased NA and biomass. Three-week-old plants subjected to drought stress and inoculated with the *R. etli* overexpressing TPS fully recovered whereas plants inoculated with a wild-type or mutant strain wilted and died. Increases of 50% in the yield of bean plants with constant irrigation also were obtained evidencing that the overexpression of trehalose was not a burden in terms of the energetic cost for the plant. Macroarray analysis of 7,200 expressed sequence tags from nodules of plants inoculated with the strain overexpressing TPS gene revealed upregulation of genes involved in stress tolerance and C and N metabolism, suggesting a signaling mechanism for trehalose. These results provided evidence that trehalose metabolism in rhizobia is key for signaling the transcription of plant genes involved with yield and adaptation to drought stress. This was the first report of the improvement to drought stress of a legume species by a different method than plant breeding or plant transformation.

The effect of different soil and climate stress factors on the biosynthesis of Nod factors from rhizobia may also explain how nodulation proceed under such conditions. For example, the *R. tropici* strain CIAT899 is highly stress resistant and forms an enormous number of different Nod factors structures that varies with the growth conditions. Under acid conditions, 52 Nod factor structures were formed and of these 37 differed from the 29 formed under neutral conditions (Morón et al. 2005). Under salt-stress conditions, the number of Nod factors produced by *R. tropici* CIAT 899 strain was almost 1.6 times greater than that produced under control conditions and this effect was sodium-specific, because high potassium or chloride concentrations did not change nod gene expression (Estévez et al. 2009).

Soil microorganisms, other than rhizobia, have been isolated from common bean nodules but their function in the N_2-fixing processes is still not fully understood. For example, *Herbaspirillum lusitanum* and arbuscular mycorrhizal fungi (AMF) were found colonizing root nodules of *P. vulgaris* (Valverde et al. 2003; Scheublin et al. 2004). The nodule colonization by *Streptomyces lydicus* was reported by Tokala et al. (2002) and led to an increase in the nodule size and enhanced iron assimilation by nodules leading to improved vigor of bacteroids.

18.5 Cowpea

Cowpeas (*Vigna unguiculata* L. Walp) are one of the most important food legume crops in the semi-arid tropics covering Asia, Africa, Southern Europe, and Central and South America. Drought-tolerant and warm-weather crops, cowpeas, are well-

adapted to the drier regions of the tropics, where other food legumes do not perform well. Cowpea is widely cultivated in West and Central Africa to feed people, livestock, and improve soil fertility by its N_2-fixation ability (Agbicodo et al. 2009). Africa is considered the origin of this crop (Appunu et al. 2009) and in 2007, of the 11.2 million ha cropped with cowpea in the world, 10.4 million ha were grown in Western and Central Africa with an average yield of 248.5 kg ha^{-1} (http://www.faostat.fao.org, verified 02 December 2009). Nigeria and Republic of Niger were the two world's largest producers. In Brazil, cowpea is a very important subsistence crop for small farmers in the North and Northeast regions and recently it is also being cultivated in big farms with high technological inputs in the Cerrado areas (Freire Filho et al. 2005). Cowpea serves as an important source of proteins and essential amino acids for populations with low income in developing countries, where it is consumed either as green or dry beans (Martins et al. 2003; Rumjanek et al. 2005). Generally, the yield rates of cowpea are low because of several biotic (pests and diseases) and abiotic (water deficiency, cultivation in marginal environments, low input of fertilizers, prostrate growth habit, and late maturity) constraints and problems related to the low adoption of proper crop management strategies and improved cultivars (Singh et al. 2003). Although this crop can be benefited from SNF, the efficiency of native rhizobial strains is variable raising doubts on whether indigenous nodulating rhizobial populations can fulfill its optimum N demand (Appunu et al. 2009).

18.5.1 Rhizobial Strains Nodulating Cowpea

Cowpea is known to form nodules in a symbiotic relation with slow-growing rhizobia known as "cowpea-miscellany", which is now classified in the genus *Bradyrhizobium*. Cowpea bradyrhizobial species from Africa, China, and Brazil were identified as *Bradyrhizobium elkanii*, *B. japonicum*, *B. liaoningense*, unnamed *Bradyrhizobium* genospecies, or as novel *Bradyrhizobium* lineages (Krasova-Wade et al. 2003; Steenkamp et al. 2005; Zhang et al. 2008; Zilli et al. 2004). However, fast-growing rhizobia have also been isolated from nodules of cowpea and grouped in the genera *Rhizobium*, *Sinorhizobium,* and *Mesorhizobium* (Germano et al. 2006; Martins et al. 1997). Cowpea nodule isolates from several Zimbabwean soils included similar proportions of both fast (49%) and slow-growing (51%) rhizobia (Mpepereki et al. 1997). On the contrary, in nine Chinese subtropical provinces, *Bradyrhizobium* spp. occupied 90% of the cowpea nodules, whereas fast-growing rhizobia occupied only 10% of the nodules (Zhang et al. 2008). Some slow-growth rhizobia collected in different regions of China were, however, phylogenetically distinct from the reference strains *B. japonicum*, *B. liaoningense,* and *B. elkanii* and only 7 of the 55 isolates were fast-growing and belonged to *S. fredii* and *R. leguminosarum* (Zhang et al. 2008). On the other hand, 99% of rhizobial strains isolated from V*igna* spp. grown in India were phylogenetically related to a single species, *B. yanmingense* (Appunu et al. 2009). Considering that India is the center

of diversification of the *Vigna*, the presence of a one single *Bradyrhizobium* species might reflect a long history of co-evolution between both partners.

18.5.2 Current Status of SNF in Cowpea

Several studies conducted in the 1960s and 1970s reported that the performance of cowpea in tropical soils was not improved by inoculation with bradyrhizobia (Doku 1969; Ezedinma 1963; Kang et al. 1977) which was suggested to be due to two reasons – (1) indigenous cowpea bradyrhizobia outcompeting the inoculated strains and (2) the low host specificity of cowpea (Awonaike et al. 1990; Neves and Rumjanek 1997). Since the indigenous soil populations may act as a barrier to the introduced strains, the understanding of their abundance and effectiveness is fundamental for improving the contribution of SNF to cowpea. For instance, Fening and Danso (2002) conducted a survey on the cowpea bradyrhizobia in 20 soils in Ghana and observed that out of 100 isolates examined most of them (68%) were ranked as moderately effective and only 26% were ranked as highly effective. The enumeration of indigenous bradyrhizobial populations capable of nodulating cowpea showed that most of the soils in Ghana contained large populations (more than 1,000 cells g^{-1} soil) that could nodulate cowpea, with the highest numbers occurring in areas where cowpea was commonly cultivated. Opposite results were reported by Law et al. (2007) in soils sampled in Botswana and South Africa, which contained effective soil populations at moderate levels (76–570 cells g^{-1}). These results evidenced different strategies to approach the field inoculation of cowpea. In the study by Fening and Danso (2002), with predominance of sub-effective populations and where some of the field isolates in Ghanaian soils had high N_2-fixing capabilities, native populations could be a potentially useful source of strains for preparing highly effective cowpea inoculants since the superior strains would be already adapted to environmental stresses, such as drought. As an example, in a study conducted by Krasova-Wade et al. (2006) in Senegal, differences in nodulation of two strains (ORS 3260 and ORS 3257) were associated to their different adaptation to soil water status. Strain ORS 3260, originally isolated from a soil submitted to water stress, proved to be the best competitor under these conditions when compared to strain ORS 3257, originally isolated from a soil under favorable water condition. More recently, another study to identify indigenous rhizobia with potential as inoculants for increasing cowpea yields in Ghanaian soils was reported by Ampomah et al. (2008). The effective field isolate AII-5-2 was the most competitive on cowpea and possessed the better potential for use as inoculant. On the other hand, in the study by Law et al. (2007), in Botswana and South Africa soils, where efficient populations predominate, management practices that encourage effective nodulation by indigenous populations could be of more value than inoculation alone (Thies et al. 1995; Van Kesse and Hartley 2000). These practices might include the use of cowpea varieties able to efficiently nodulate with these indigenous soil populations, liming to correct soil acidity, and the addition of

organic/inorganic fertilizers. As an example, in West Africa, Bado et al (2006) reported that noninoculated cowpea fixed more atmospheric N when NPK fertilizer was associated with dolomite or manure. In the same way, Onduru et al. (2008), in eastern Kenya, observed a yield increase of 22.5% when cowpea plants were inoculated and fertilized with P as compared to the treatment which received P only, evidencing the importance of soil fertility to enhance the benefits from SNF. In the Brazilian semi-arid region, Martins et al. (2003) reported that the soil populations of cowpea rhizobia were very low and incapable of promoting proper root nodulation. The rhizobial populations, however, consistently increased following introduction of cowpea in the same soil (10^4 cells g^{-1} soil after the second crop). Local studies aiming at the selection of more efficient strains resulted in the selection of *Bradyrhizobium sp*. strain BR 3267 (=SEMIA 6462) which, under field conditions, was capable of establishing an efficient nodulation and improving both yield and total N accumulated in grain. Cowpea inoculated with strain BR 3267 showed grain productivity similar to plants receiving 50 kg N ha^{-1}. Further studies with this strain showed yield increases of up to 40% under experimental conditions and of 52% under farmer conditions, which supported its recommendation for the production of cowpea inoculant in Brazil (Rumjanek et al. 2005; Zilli et al. 2006). The results obtained with strain BR 3267 highlight the importance of local studies for the selection of superior rhizobial strains for inoculant production, because of the better competitive ability and better adaptation to the environment displayed by the indigenous populations, as observed by Fening and Danso (2002) and Krasova-Wade et al. (2006). Today, local studies in Brazil also led to the recommendation of three other strains for cowpea inoculant: SEMIA 6461 (= UFLA 384); SEMIA 6463 (INPA 3-11B); and SEMIA 6464 (BR 3262) (Soares et al. 2006; Mello and Zilli 2009).

In spite of cowpea being nodulated by selected or indigenous rhizobia, indirect benefits of cowpea to other crops have also been reported in both intercropping and crop rotational systems. For example, Eaglesham et al. (1981) reported a positive balance of 53 kg N ha^{-1} by cowpea to the cropping system when it was intercropped with maize (*Zea mays*). In the northern Guinea Savanna of Ghana, estimates of benefits of rotational systems including cowpea to soil N were around 50 kg N ha^{-1} when residues from two crops were left in the field (Horst and Härdter 1994) and 60 kg N ha^{-1} when residues from one cowpea crop were incorporated in the soil (Dakora et al. 1987), showing the ability of this legume for improving the sustainability of low-input farming systems. The ability of cowpea as pre-crop for the amelioration of the inherent low fertility of the degraded soils of the Guinea Savanna zone of northern Burkina Faso and Ghana was also reported by Bado et al. (2006) and Ahiabor et al (2009). Regardless of whether the cowpea plants in these experiments were inoculated or not, these results are significant considering that soil constraints play a major role in limiting crop yields in these low-input subsistence cropping systems. The importance of crop rotational systems involving cereals after sole cropped legumes in Africa was emphasized by Dakora and Keya (1997), who considered rotational systems with legumes more suitable for increased crop yields than intercropping systems, the most dominant cultural practice in that continent.

Recent successes in cowpea genetic transformation will pave the way for the introduction of important agronomic traits to cowpea such as insect resistance and increased drought tolerance (Solleti et al. 2008; Obembe 2009). Therefore, in the same way as mentioned for soybeans, the biosafety studies that usually follow the release of GE cultivars also must include aspects related to the SNF process, to prevent negative effects on this important nitrogen input.

18.6 Groundnut

Groundnut or peanut (*Arachis hypogaea* L.) is an ancient crop of the New World which was widely grown in Mexico, Central America, and South America in pre-Colombian times (Stalker, 1997). It is generally accepted that groundnut has its origin center in South America, probably in the region of Gran Chaco (Paraguay), including basins of Paraná and Paraguay rivers, and it has been proposed that its domestication took place in a region located between the north of Argentina and the south of Bolivia and Paraguay (Kochert et al. 1996; Krapovickas 1969). Two-seeded types originating from Brazil were taken to Africa whereas three-seeded types originated from Peru were transported from the west coast of South America to China and islands in the western Pacific (Hammons 1982). Spanish types were introduced to Europe in the late 1700s from Brazil and grown for oil and for human consumption as chocolate-covered peanuts (Hammons 1982). Groundnut is currently grown on nearly 22.2 million ha worldwide being the third legume widely cultivated in the world after soybean and common bean (http://www.icrisat.org/newsite/crop-groundnut.htm, verified at December 02, 2009). Asia and Africa account for 56% and 40% of global groundnut area, respectively, where the crop is grown mostly by smallholder farmers under rain-fed conditions with limited inputs. Major groundnut producers in the world are: China (35% of world production in 2007, http://faostat.fao.org/, verified in December 06, 2009), India, Nigeria, USA, Indonesia, Sudan, and Argentina. Average yields on a global scale are 1,520 kg ha^{-1} but when diseases are controlled and good management practices are used, yields $\geq$3,000 kg ha^{-1} can be achieved (US average is ca. 2,700 kg ha^{-1}). Groundnut is an important oil seed legume crop as its seeds contain 44–56% of oil and 22–30% of proteins (Table 18.1) on a dry seed basis (Reddy et al. 2003). Whereas in the U.S. and South America most of the production is sold as edible seeds, in other countries the primary use of groundnut is for the oil market. In addition to seeds, the foliage and the meal remaining after oil extraction are important source of animal feed (Stalker 1997).

18.6.1 Rhizobial Strains Nodulating Groundnut

Most rhizobial isolates nodulating groundnut belong to the genus *Bradyrhizobium* (Zhang et al. 1999; Chen et al. 2003; Urtz and Elkan 1996; van Rossum et al.

1995; Yang et al. 2005), although some effective fast-growing rhizobia have also been described. *Bradyrhizobium* species nodulating groundnut have, however, not yet been clearly defined and are all classified as *Bradyrhizobium* (*Arachis*) sp., although several studies indicate that groundnut bradyrhizobia are phenotypically and genetically diverse (Li et al. 1999; Zhang et al. 1999). Several reports describe the isolation and characterization of groundnut rhizobial strains such as NC92 (Gillette and Elkan 1996), MAR 411 (van Rossum et al. 1995), and Spr3–7 (Urtz and Elkan), that were assigned to the genus *Bradyrhizobium*. In different geographical regions of China, although fast-growing rhizobia have been discovered, it was found that the predominant groundnut bradyrhizobia are related to *B. yuamingense* which was isolated from *Lespereza* (Yang and Zhou 2008). In Argentina, the fast growing rhizobia isolated from groundnut were closely related to *R. giardini* and *R. tropici* species, which are symbionts of common bean (Taurian et al. 2006; Ibanez et al. 2009). In Morocos, rhizobia isolates obtained from groundnut belonged to genera *Bradyrhizobium* and *Rhizobium* and varied in their ability to fix N_2 (El-Akhala et al. 2008). In the semiarid region of Brazil, greater diversity among groundnut-nodulating rhizobia has been found (Santos et al. 2007). Recently, there have been reports regarding the presence of several opportunistic bacterial isolates including *Pseudomonas* spp., *Enterobacter* spp. and *Klebsiella* spp from surface-sterilized groundnut root nodules of plants growing in soils from Córdoba, Argentina. This finding provides evidence that the presence of other bacteria in nodules, such as the Gammaproteobacteria, is common in nature (Ibáñez et al. 2009), and that more studies are required to find out the real benefits of these bacteria to plant growth.

18.6.2 Current Status of SNF in Groundnut

In relation to the other food grain legumes discussed earlier, one aspect that makes the symbiosis between rhizobia and groundnut peculiar, is that the infection site is at the junction of the root-hair cells with epidermal and cortical cells and penetration occurs by a crack-entry mechanism followed by intracellular spreading (Boogerd and van Rossum 1997). Rhizobia penetration during symbiosis formation occurs into the root by breaching the epidermal barrier instead of entering through curled root hairs and hence infection thread is not formed (Azpilicueta et al. 2004; Taurian et al. 2008). Considering the close relationship between symbiosis and pathogenesis, it was suggested and later confirmed that exopolysaccharides (EPS) are a critical factor in the symbiotic interactions between rhizobia and legumes infected by crack entry (such as *Arachis, Sesbania, Stylosanthes*, *Neptunia,* and *Aeschynomene*) participating in the protection against the host defense system (Morgante et al. 2005; Morgante et al. 2007). Subsequently, Azpilicueta et al. (2004) reported that the symbiotic N_2 fixation in groundnut triggers biochemical changes which tend to enhance the production of natural defenses against pathogens such as phytoalexins (identified

as medicarpin and formononnetin) in leaf extracts of *Bradyrhizobium*-inoculated plants.

Like other legumes, the knowledge of naturally occurring populations of rhizobia able to nodulate groundnut is also a significant factor for the establishment of superior inoculant strains under field conditions. For *A. hypogaea,* the naturally occurring populations can be either indigenous or from other legume species, because groundnut is considered a "promiscuous" host legume. As a consequence of this promiscuity, groundnut responses to inoculation have been inconsistent, with positive responses occurring mostly in soils new to this crop (Toomsan et al. 1988; Alwi et al. 1989). In China (world largest producer), there are no indigenous groundnut bradyrhizobia but groundnut plants form nodules with bradyrhizobia from other indigenous legume hosts (Chen et al. 2003) and yield responses to inoculation with selected rhizobial strains have been reported (Huang 1987, 1988; Chen et al. 2003). In India, (second largest producer) groundnut is nodulated by *Bradyhizobium* species of cowpea and the use of rhizobium inoculant is a common practice (Anandham et al. 2007). In Argentina (one of *A. hypogaea* origin center and important world producer), nodulation of groundnut by indigenous population is usually assumed to be adequate and inoculation is rarely practiced by farmers (Bogino et al. 2006; Castro et al. 1999).

Despite being the second legume in terms of world production, long-term and comprehensive studies on SNF in groundnut under field conditions are still lacking. In a series of field trials in North Carolina, USA, Lanier et al. (2005) observed that groundnut does not always respond to inoculation. Groundnut pod yield was higher following inoculation in seven of 20 experiments, and out of these seven, six were carried out where groundnut had not been planted previously. In the experiments where groundnut responded positively to inoculation, pod yield was higher when inoculant was applied in the seed furrow rather than to seed before planting. Groundnut pod yield of inoculated plants increased with increasing N rates (up to 210 kg N ha^{-1}, applied at the soil surface 40 days after planting). The authors concluded that, under those conditions, groundnut had a high N demand to produce high yields (up to 5,000 kg ha^{-1}) and could not fix enough N through SNF. In Argentina, in a series of greenhouse experiments using pots with vermiculite, *Bradyrhizobium* spp USDA 4438 and USDA 3180 (used as inoculants in the USA) and C-145 (recommended by INTA, Instituto Nacional de Tecnologia Agropecuaria) were evaluated for N_2-fixing efficiency and subsequently in field trials conducted at four locations (with and without prior groundnut cultivation) in Cordoba province. Seed and in-furrow inoculation with strain C-145 was compared to seed inoculation with USDA 4438 and a noninoculated control (Bogino et al. 2006). Strain C-145 was the most effective in the greenhouse experiments and increased nodule numbers under field conditions when in-furrow inoculation was used. However, pod yield was not increased significantly by either type of inoculation or strain. The high degree of nodulation and N_2 fixation by indigenous rhizobia were sufficient for maximal yield (ranging from 2,913 up to 4,875 kg ha^{-1}) under field conditions. It should be pointed out that in all field experiments seeds were treated with a commercial fungicide. In another field experiment, in-furrow

inoculation increased nodule occupancy by the inoculant strain and also increased nodule numbers and plant dry biomass (Bogino et al. 2008). These results supported the recommendation of in-furrow inoculation of selected strains as the method of choice when indigenous rhizobial populations of groundnut crops inhabit soil (Bogino et al. 2008). An additional benefit of in-furrow inoculation for groundnut is that since its seeds are too fragile, the conventional seed inoculation method may cause damages that will affect germination, which is prevented when the inoculant is applied directly into the seed bed.

Rhizobia nodulating groundnut exhibit great genetic diversity and some strains have been found excellent nodulators and N_2 fixers (El-Akhala et al. 2008; Taurian et al. 2008). However, in order to establish an efficient symbiosis under field conditions, compatible plant cultivar bradyrhizobia combination should be selected either by finding inoculant strains compatible with the plants used or plants compatible with the indigenous bradyrhizobia in different soils and regions (Chen et al. 2003). Studies with *A. hypogaea* (Ngo-Nkot et al. 2008) and other legumes (Coutinho et al. 1999; Zilli et al. 2004) have found a decrease in rhizobia diversity associated with the host plant suggesting that legumes promote the selection of particular *Rhizobium* taxa.

In the same way as described for cowpea, by increasing N inputs, groundnut also has an important role for improving small farmer's production systems besides providing grains for human consumption. In a 2-year study in the Guinean savannah of Burkina Faso (West Africa), NPK fertilizer significantly increased groundnut grain yields, indicating that in spite of its capability to fix N_2 a low quantity of starter chemical N fertilizer (14 kg N ha^{-1}) was necessary to improve the SNF with naturally occurring soil populations. Under those conditions, groundnut was found to fix between 8 and 23 kg of N ha^{-1}, the proportion of N derived from SNF (% Ndfa) varied from 27 to 34% and rotation effects of groundnut with sorghum resulted in yield increases of 285% in relation to continuous sorghum (Bado et al. 2006). Similar results were also obtained in the smallholders cropping systems of subhumid Zimbabwe, where maize yields (with and without N fertilizer) were improved in rotation with groundnut (Jeranyama et al. 2007). In Brazil, Okito et al. (2004), using the natural ^{15}N abundance, estimated the contribution of SNF for groundnut inoculated with strain BR 423 of 40.9 kg ha^{-1} and the proportion of N derived from SNF (% Ndfa) ranged between 49 and 58%. They also reported that although crop rotational systems, where groundnut was followed by maize, had a better economical performance; in the long term, they could lead to a depletion of soil N reserves caused by the negative soil N balance. This observation is very important and shows that SNF achieved by many crops in the field may be high, but are not always sufficient to offset the N removed by the harvested seed. One option to prevent this problem is to use in the crop rotations late-maturing varieties of groundnut. These late-maturing varieties, compared with early maturing ones, have lower nitrogen harvest indices (ratio between the N exported in grains and the total plant N), and their contribution to soil fertility can therefore be expected to be more pronounced than that of short-duration crops (Mafongoya et al. 2009).

18.7 Conclusion

Despite extensive research in the area of SNF worldwide, our knowledge and how this research could be translated and used to improve the productivity of legumes in different agro-ecological regions is not sufficient. Efforts are, therefore, needed to identify host and region-specific rhizobial strains besides the delivery systems for inoculants so that maximum benefits of this technology could be achieved by agrarian communities engaged in legume production. To achieve this, farmers should be involved in the entire research process from problem identification to technology development and adoption (Mafongoya et al. 2009). So far as the legume technologies are concerned, the selection and production of high-quality commercial rhizobium inoculants deserves special attention as poor quality inoculants may destroy all the efforts put in for selecting effective strains and successfully introducing them into the soil (Hungria et al. 2005). Furthermore, the seed treatments with agrochemicals like, fungicides and insecticides should be avoided while using rhizobia as inoculants for legume inoculation because such synthetic chemicals are reported to adversely affect the survival and colonization of rhizobia onto the root systems of legumes. Hence, research is required to find strategies as to how the survival of rhizobia could be improved when applied along the agrochemicals. More recently, the benefits of plant growth promoting rhizobacteria (PGPR) and rhizobia co-inoculation have also been reported and might play a critical role especially in situations where plants are cultivated in stressed soils (Spaepen et al. 2007; Dardanelli et al. 2008; Remans et al. 2008). The production of inoculants with pre-activated *Rhizobium* strains and the more extensive use of in-furrow inoculation are other promising alternatives to overcome the competition problem between inoculated and indigenous (established) populations. Unfortunately, these facts are still far from reality in many countries, where inoculants are often not available to end users and their storage is still a problem. Therefore, inoculant formulations with prolonged shelf life and/or strategies such as breeding higher-yielding varieties for nodulation with indigenous population should be pursued.

The constraints to the use of nonrenewable fossil fuels will increase in the coming years, since they have a direct influence upon global warming. The energetic basis of N_2 fixation in legume versus fertilizer-based systems is the greatest factor that differentiates the sustainability of these two N sources and will be a major driving force toward the increased adoption of legume-based systems. As an example, in 2009, biological N_2 fixation was selected by the Brazilian government as one of the nationally appropriate mitigation actions (NAMAs) to reduce greenhouse gases emission in agriculture, along with the recovery of degraded pastures and the adoption of no-tillage and lay farming systems (where crops and pastures alternate). It is important to further realize that legumes are not miracle crops that will solve the complex problems involving increasing food production with environmental sustainability. It is imperative, however, to recognize that the principle of limiting factors is also applicable to legume N_2 fixation. To explain this further, Brockwell et al. (1995) suggested that whatever biotechnological approaches are

employed to improve either legumes or to engineer rhizobial strains, they will not be realized unless sound crop and soil management practices such as no-tillage/minimum tillage, improved germplasm, pests and disease control, and the judicious use of organic and inorganic fertilizers are practiced. Therefore, issues related to soil chemical, physical and biological degradation also should be taken into account, as only healthy legumes will be able to fully express their potential for SNF. In this regard, research efforts toward maximizing the contribution of N_2 fixation should also be accompanied by the efficient use of the fixed N, preventing problems related to leaching, denitrification, and gaseous losses. On global basis, where lands are degrading very fast, costs of fertilizers are rising and human populations are increasing alarmingly, greater emphasis should be placed on sustainable production of legumes involving use of SNF. Research challenges for the next decades include the effects of climatic changes (increased CO_2 concentrations and raising temperatures) on SNF in food tropical grain legumes, breeding for drought tolerance and how the knowledge generated by the omics approaches could be translated from labs to lands.

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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

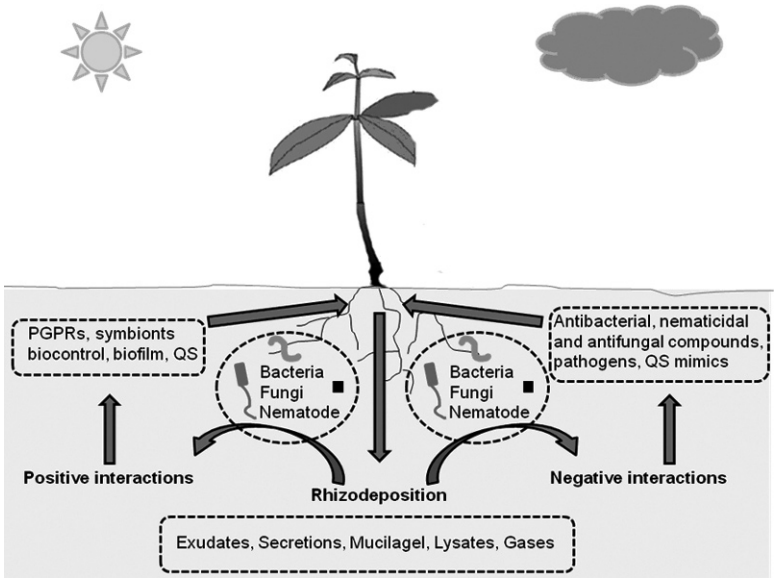

Fig. 19.1 Rhizodeposition and active rhizospherics interactions; *QS* quorum sensing

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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Chapter 20
The Potential Use of *Rhizobium*–Legume Symbiosis for Enhancing Plant Growth and Management of Plant Diseases

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Abstract Legumes are able to establish a symbiotic interaction with soil bacteria collectively termed rhizobia. These bacteria can enhance growth and development of associated crops by transferring atmospheric nitrogen into a form that is available for plant growth or by improving nutrient uptake through modulation of hormone-linked phenomena in inoculated plants. The anticipated benefits of the nitrogen-fixing bacteria may be positive or negative depending on *Rhizobium* species and its interaction with the environment. Selection of effective *Rhizobium* strain is the most critical aspect to achieve maximum benefits from this technology. In addition to their direct role in growth promotion, various rhizobacteria can also protect plants from infection caused by pathogens. Studies on numerous plant–microbe interactions have shown that such antagonistic rhizobacteria could function by competition and antibiosis but also indirectly by induction of systemic resistance against plant diseases. This chapter aims to focus on recent findings highlighting the enhancement in plant growth, nitrogen uptake, and plant protection against pathogen, during the symbiosis between rhizobia and leguminous plants. The potential use of such microorganisms due to their multifaceted beneficial activities is likely to play an important role in modern high-intensive agricultural practices.

20.1 Introduction

An essential element of agricultural sustainability involves the effective management of N in the soil environment. This usually implicates at least some use of biologically fixed N (BNF), because N from this source is absorbed directly by the

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plants and so is less susceptible to volatilization, denitrification, and leaching. In agricultural settings, about 80% of BNF come from symbioses formed between leguminous plants and species of *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium*, and *Allorhizobium* (Vance 1998).

Legumes and rhizobia together fix atmospheric N and because of this feature they are often introduced to manage agricultural ecosystems to improve organic fertility, N economy, or farming system flexibility (Brockwell et al. 1995). Optimal performance of the nitrogen-fixing symbiosis depends upon preselection of both symbiotic partners for adaptation to the target environment, which may in some form present a challenge to rhizobial survival or nodulation (Sessitsch et al. 2002). Inoculation of stress tolerant strains of rhizobia may enhance the nodulation and N_2 fixation ability of legumes under stressed conditions. For example, the ability of legumes to grow and survive in saline conditions is improved when they are inoculated with salt tolerant strains of rhizobia (Zou et al. 1995). Rhizobial populations, however, vary in their tolerance ability to major environmental factors (Ulrich and Zaspel 2000; Wei et al. 2008; Biswas et al. 2008). In addition to N_2 fixation, recent work has shown that these beneficial microorganisms exhibit biocontrol activity as well. The use of rhizobia as biofertilizers under severe conditions and biological control of pathogens and pests are discussed.

20.2 Biological Nitrogen Fixation

Nitrogen is an essential plant nutrient and, in agriculture, fertilization with N products is widely and increasingly practiced to increase the yields of crop plants (Reinhold-Hurek and Hurek 2003). The earth's atmosphere contains about 10^{15} tons of N_2 gas, which cannot be used in this form by most living organisms unless it is reduced to ammonia. The nitrogen cycle involves the transformation of some 3×10^9 tons of N_2 per year on a global basis (Postgate 1982). Biological fixation is the principal process of N entry into natural ecosystems and the fixation associated with vascular plants usually contributes greatest quantities of added N (Cleveland et al. 1999). Nitrogen fixation also occurs as a result of nonbiological processes (such as, Haber-Bosch, and combustion) where lightning alone accounts for about 10% of the world's supply of fixed N (Sprent and Sprent 1990). Furthermore, World production of fixed N from dinitrogen for chemical fertilizer accounts for about 25% of the Earth's newly fixed N_2, while biological processes account for about 60%. Significant growth in fertilizer-N usage has occurred in both developed and developing countries (Peoples et al. 1995). The requirements for fertilizer-N are predicted to increase further in future (Subba-Rao 1980). However, the use of elevated doses of fertilizers may have negative and unpredictable effects on the environment and may cause the contamination of soil, water, and natural areas (Sprent and Sprent 1990). Such impacts, in turn, pose a serious threat to human and animal health. In addition, developing countries have to face the demand of high costs for such technology and chemical utilization. To circumvent such problems,

the use of BNF provides an interesting option for decreasing the reliance on the use of chemical fertilizers. For more than 100 years, BNF has commanded the attention of scientists concerned with plant mineral nutrition, and it has been exploited extensively in agricultural practices (Dixon and Wheeler 1986). However, its importance as a primary source of N for agriculture has declined in recent times probably due to slow effect of BNF on crops (Peoples et al. 1995). However, the use of renewable resources in sustaining the crop development following low input BNF has renewed the interest of agrarian communities (Dixon and Wheeler 1986). The expanded interest in its use is due to the fact that BNF is ecologically benign, could reduce the use of fossil fuels, and may be helpful in reforestation and in restoration of misused lands (Sprent and Sprent 1990).

20.2.1 Nitrogen-Fixing Organisms

Nitrogen fixation is carried out by numerous prokaryotes including bacteria, actinobacteria, and certain types of anaerobic bacteria. Microorganisms that fix N are called diazotrophs. All organisms reduce N_2 to NH_3 by an enzyme called nitrogenase. The nitrogenase enzymes are irreversibly damaged by exposure to atmospheric levels of oxygen (Giller and Wilson 1991). Nitrogenase activity is usually measured by the acetylene reduction assay, which is cheap and sensitive (Sprent and Sprent 1990). The ^{15}N isotopic method, which is also used to measure N_2 fixation, is accurate but expensive. A wide range of organisms have the ability to fix N. However, only a very small proportion of species are able to do so; about 87 species in 2 genera of archaea, 38 genera of bacteria, and 20 genera of cyanobacteria have been identified as diazotrophs or organisms that can fix N (Sprent and Sprent 1990). This wide range of diazotrophs ensures that most ecological niches will contain one or two representatives and that lost N can be replenished.

20.2.2 Importance of Biological Nitrogen Fixation

Most of the N added naturally to soils is from biological fixation that is symbiotic or nonsymbiotic in nature. It has been estimated that about 100 Tg N, valued at $US 40 billion, is required annually for the production of the world's grain and oilseed crops (David and Ian 2000). The other sources are mainly from lightning discharges, burning of fossil fuels and forest, and from the emission of magmatic gases. Much land has been degraded worldwide, and it is time to stop the destructive uses of land and to start a serious reversal program of land degradation. BNF, however, can be effective in land remediation. The success of legumes is largely due to their symbiotic relationship with specific nitrogen-fixing bacteria, the rhizobia, a name that portray root and stem nodulating bacteria. Phylogenetically, rhizobia are very diverse representing several lineages. Rhizobia currently includes

12 genera and more than 70 species of α- and β-proteobacteria (Sawada et al. 2003). A tremendous potential for contribution of fixed N to soil ecosystems exists among the legumes. There are approximately 700 genera and about 13,000 species of legumes, only a portion of which have been examined for nodulation and shown to have the ability to fix N (Sprent and Sprent 1990). Estimates suggest that the rhizobial symbioses with the somewhat greater than 100 agriculturally important legumes contribute nearly half the annual quantity of BNF entering soil ecosystems (Tate 1995). Legume symbioses contribute at least 70 million tons of N per year, approximately half deriving from the cool and warm temperature zones and the remainder deriving from the tropics (Brockwell et al. 1995). Increased plant protein levels and reduced depletion of soil N reserves are obvious consequences of legume N_2 fixation. The deficiency in mineral N often limits plant growth, and so the symbiotic relationships between plants and a variety of nitrogen-fixing organisms (Freiberg et al. 1997) fulfil the N demands of crop plants. Yield increases of crops planted after harvesting legumes are often equivalent to those expected from application of 30–80 kg N ha^{-1}. For example, inputs of fixed N for alfalfa, red clover, pea, soybean, cowpea, and vetch were estimated to be about 65–335 kg N ha^{-1} $year^{-1}$ (Tate 1995) or 23–300 kg N ha^{-1} $year^{-1}$ (Wani et al. 1995). The behavior of these symbioses under severe environmental conditions and their applications in arid regions is discussed in the following section.

20.3 Factors Limiting Biological Nitrogen Fixation in Arid Climate

In the *Rhizobium*–legume symbiosis, the process of N_2 fixation strongly depends on the physiological state of the host plant. Therefore, a competitive and persistent rhizobial strain is not expected to express its full N_2-fixation activity if limiting factors impose limitations on the vigor of the host legume. Several environmental conditions acts as limiting factors to the growth and activity of the nitrogen-fixing plants in Mediterranean region such as Tunisia, mostly located in the semi-arid, arid, and Saharan climatic zones, where the annual rainfall varies from 300 to less than 100 mm (Ben Romdhane et al. 2009). In Tunisian farming systems, yields of leguminous crops are generally more affected by drought than those of cereal crops. As a consequence, over the past 20 years, grain legumes cultivation surfaces, have decreased in favor of cereal monoculture. In addition to drought, legumes are also affected by salinity, unfavorable soil pH, nutrient deficiency, mineral toxicity, high temperature, insufficient or excessive soil moisture, inadequate photosynthesis, and plant diseases (Mabrouk et al. 2007c).

Water deficiency and drought directly affect persistence and survival of rhizobia in the soil, nodule activity, and function (Davey and Simpson 1990) and also limits nodulation through its effects on root-hair colonization and infection by rhizobia (Graham 1992). Like drought, salinity is a serious threat to agriculture in arid and

semi-arid regions. Plant responses to salt and water stress have much in common. Salinity reduces the ability of plants to take up water, which in turn reduces growth rate along with several metabolic changes identical to those caused by water stress (Munns 2002). An essential aspect of the strategy to improve the yield of arid legumes in stressed environments must involve a combination of stress-tolerant cultivars and stress-tolerant rhizobia. Breeding and selection of genotypes tolerant to water stress were extensively studied for different leguminous plants (Turner et al. 2001). BNF capable of improving agricultural productivity while minimizing soil loss and ameliorating adverse edaphic conditions are essential.

20.3.1 Soil Salinity

Nearly 40% of the world's land surface can be categorized as having potential salinity problems. Most of these areas are confined to the tropics and Mediterranean regions (World Resources 1987). High concentrations of sodium chloride lead to marked changes in the growth pattern of plants. Salinity is known to reduce the growth of glycophytes (salt-sensitive species). This effect may result from changes in dry matter allocation, ion relations, water status, physiological processes, biochemical reactions, or a combination of these. Salt tolerance in plants is a complex phenomenon that involves morphological and developmental changes as well as physiological and biochemical processes (Greenway and Munns 1980). Survival and growth in saline environments are the result of adaptive processes such as ion transport and compartmentation, osmotic solute synthesis, and accumulation, which lead to osmotic adjustment and protein turnover for cellular repair (Munns and Termaat 1986; Paul and Cockburn 1989). Legumes have been suggested as appropriate crops for the enhancement of bio-productivity and the reclamation of marginal lands, because these plants not only yield nutritious fodder, protein-rich seeds and fruits, but also enrich soil N in symbiotic association with *Rhizobium* (Alexander 1984). Nodulation and nitrogen fixation in legume–*Rhizobium* associations are, however, adversely affected by salinity, which can preclude legume establishment and growth, or reduce crop yields (Mohammad et al. 1991). Unlike their host legumes, rhizobia can survive in the presence of extremely high levels of salt and show marked variation in salt tolerance. Some strains are inhibited by 100 mM of NaCI (Singleton et al. 1982; Yelton et al. 1983), whereas strains of *R. meliloti* and *R. fredii* grew at salt concentrations above 300 mM (Sauvage et al. 1983; Kassem et al. 1985; Yelton et al. 1983). Some *Acacia* and *Prosopis* strains can tolerate up to 500 mM NaCI (Hua et al. 1982; Zeghari et al. 2000). Osmotolerant *Rhizobium* strains can support large modifications in the osmolarity without decrease in the number of viable cells (Singleton et al. 1982). Consequently, their multiplication in the rhizosphere of the plant host will not be affected in saline soils, as is the case in sensitive strains.

The osmoadaptation of most microorganisms involves the accumulation of K ions and one or more of a restricted range of low molecular mass organic solutes, collectively termed "compatible solutes" (Welsh 2000). These solutes are accumulated to high intracellular concentrations, in order to balance the osmotic pressure of the growth medium and maintain cell turgor pressure, which provides the driving force for cell extension. Other investigators demonstrated that during the earlier stages of plant–rhizobia interaction, the host plant reacts to the invasion of the bacteria by the overproduction of reactive oxygen species (ROS) to initiate the hypersensitive reaction (Santos et al. 2001). The excess of ROS production can drastically damage bacteria and plant tissues. In this process, catalase (CAT) and superoxide dismutase (SOD) antioxidant enzymes were reported to play a key role in the establishment and the protection of the symbiosis (Jamet et al. 2003; Santos et al. 2000). Moreover, ROS generation can be enhanced under environmental stresses as salinity, which is considered as a major constraint of symbiotic nitrogen fixation (SNF) and plant production (Zahran and Sprent 1986). Salt-stressed plants display complex oxidative defense strategy, mainly the overexpression of antioxidant enzymes. The SOD (E.C. 1.15.1.1) is the first enzyme involved in the antioxidative process. The increase of its activity in plant tissues under salt stress was signaled in several reports (Lee et al. 2001; Rubio et al. 2002). This enzyme converts superoxide radical to H_2O_2 and molecular oxygen (O_2). However, H_2O_2 is itself a cellular toxic product that is scavenged by other antioxidant enzymes, mainly CAT (E.C. 1.11.1.6), which cleaves H_2O_2 to H_2O and O_2 without consuming reductants and, thus, may provide plant cells with an energy-efficient mechanism to remove H_2O_2 (Scandalios et al. 1997). Hydrogen peroxide can be removed also by "nonspecific" peroxidases (POX, E.C. 1.11.1.7) which use H_2O_2 as electron donor to metabolize phenolic compounds. These latter enzymes are ubiquitous and are involved in various processes such as cell growth regulation and tolerance to environmental stress (Quiroga et al. 2000).

20.3.2 Water Deficiency and Drought

Water deficiency is a major limiting factor of plant productivity and SNF in many arid regions of the Mediterranean basin. One of the immediate responses of rhizobia to water deficiency concerns the morphological changes (Shoushtari and Pepper 1985; Busse and Bottomley 1989). The modification of rhizobial cells by water stress eventually leads to a reduction in infection and nodulation of legumes (Hunt et al. 1981). 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 occurrence of rhizobial populations in desert soils and the effective nodulation of legumes growing therein (Jenkins et al. 1989; Waldon et al. 1989) emphasize the fact that rhizobia can exist in soils with limiting moisture levels; however, population densities tend to be lowest under the most desiccated conditions and to increase

as the moisture stress is relieved. It is well known that some free-living rhizobia are capable of survival under drought stress or low water potential (Fuhrmann et al. 1986). The wide range of moisture levels characteristic of ecosystems where legumes have been shown to fix N suggests that rhizobial strains with different sensitivities to soil moisture can be selected. In vitro studies have, however, shown that sensitivity to moisture stress varies among rhizobial strains (Fuhrmann et al. 1986; Busse and Bottomley 1989; Osa-Afina and Alexander 1982) while nodulation and N_2-fixation in alfalfa (*Medicago Satina*) was improved by inoculating plants with competitive and drought tolerant rhizobia (Zahran 1999) suggesting that rhizobial strains can be selected with moisture stress tolerance within the range of their legume host which is generally more sensitive to moisture stress than bacteria. Several mechanisms have been suggested to explain the varied physiological responses of legumes to water stress. Under osmotic stress, a balance between internal and external water potentials can be reached if the cells accumulate compatible solutes or osmoprotectants. These include potassium ions, glutamate, glutamine, proline, quaternary amines (glycine betaine) and the sugars trehalose, sucrose, and glucosylglycerol. Compatible solutes help maintain the stability of proteins during osmotic stress via a "preferential exclusion mechanism" (Potts 1994).

20.3.3 High Temperature and Heat Stress

In arid regions, high soil temperature affects both the free-living and symbiotic life of rhizobia. For most rhizobia, the optimum temperature for growth ranged between 28 and 31°C, and many are unable to grow at 38°C (Graham 1992). Some strains of the rhizobia surviving under heat stress may lose their infectivity, due to plasmid curing or to alterations in cellular polysaccharides necessary for infection (Zahran 1999). High-soil temperature (35–40°C) usually result in the formation of ineffective nodules; however, some strains of rhizobia (e.g., *R. leguminosarum* bv. *phaseoli*) were reported to be heat tolerant and formed effective symbioses with their host legumes (Hungria and Franco 1993 and Michiels et al. 1994). These associations will be more relevant for growing inoculated legumes in arid climates.

20.3.4 Acid Soils and Soil Acidification

Acid soils constrain agricultural production in more than 1.5 Gha worldwide (Edwards et al. 1991), this damage is related to air pollution and other factors. Despite much controversy, there exists no doubt that air pollution stresses like "acid rain" and enhanced N deposition affect soils, for example, by accelerating soil

acidification. Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they perform symbiotic N_2 fixation (Bordeleau and Prevost 1994; Brockwell et al. 1991). Acidity is reported to limit both survival and persistence of nodule bacteria in soil and the process of nodulation (Correa and Barneix 1997). And hence, the failure of legumes to nodulate under acid-soil conditions is common, especially in soils of pH less than 5. The inability of some rhizobia to persist under such conditions is one cause of nodulation failure (Bayoumi et al. 1995; Carter et al. 1994; Graham et al. 1982), but poor nodulation can occur even where a viable *Rhizobium* population persist. In a study, Evans et al. (1980) found that nodulation of *P. sativum* was ten times more susceptible to acidity than was either rhizobial multiplication or plant growth. Some legumes (e.g., *Trifolium subterranean*, *T. balansae*, *Medicago murex*, and *M. Truncatula*) showed tolerance to soil acidity as indicated by dry-matter yield; however, the establishment of nodules was more sensitive to soil acidity in most of these plants than was indicated by the relative yields of dry matter (Evans et al. 1990). Despite this, elevated inoculation levels have enhanced the nodulation response under acidic conditions in some studies (Pijnenborg et al. 1991). For example, the growth, nodulation, and yield of *V. faba* was improved after inoculation with strains of *R. leguminosarum* bv. *viciae* in acid soils (Carter et al. 1994). It appears that the pH-sensitive stage in nodulation occurs early in the infection process and that *Rhizobium* attachment to root hairs is one of the stages affected by acidic conditions in soils (Caetano-Anolles et al. 1989; Vargas and Graham 1988). In other report, Taylor et al. (1991) concluded that acidity had more severe effects on rhizobial multiplication than did Al stress and low P conditions. They suggested that colonization of soils and soybean (*Glycine max*) roots by *B. japonicum* may be adversely affected by acidity, which may lead to poor nodulation on root systems of tested legumes. By selecting acid-soil tolerant symbiotic partners, annual medics such as *M. murex* was grown in acidic soils (Cheng et al. 2002). Further, they reported that the acid-sensitive species *M. sativa* exhibited delayed nodulation under acid stress relative to the acid-tolerant species *M. murex*, but those nodules were eventually formed on both species in the same section of the root. While the genetic control of acid tolerance in *Sinorhizobium* is becoming increasingly understood (Dilworth et al. 2001), there is little information on the mechanisms contributing to enhanced nodulation at low pH in host species such as, *M. murex* in comparison to *M. sativa* (D'Haeze and Holsters 2002). The establishment of legume symbioses requires the interaction of specific recognition signal molecules produced by both bacterial and plant partners (Denarie et al. 1996). It has been shown that pH affects the exchange or recognition of these signal molecules by both plant and bacterial partners in both the medic symbiosis and the clover (*Trifolium pratense*) symbiosis (Richardson et al. 1988). As an example, Howieson et al. (1992) noted that root exudates collected at decreasing pH from acid-tolerant species of *Medicago* resulted in increased *nod* gene induction up to a critical pH, which drastically reduced thereafter. The root exudates of acid-sensitive species of *Medicago* demonstrated a general reduction in *nod* gene-inducing capacity with decreasing pH.

20.4 Role of Biotechnologies in Improving Nitrogen Fixation in Severe Conditions

Symbiotic nitrogen-fixing organisms possess the ability to reduce the dependence on man-made forms of N fertilizer. There is a considerable economic incentive to explore ways to increase the efficiency of BNF as biofertilizer resource. Although, usually the microsymbionts are modified by recombinant or classic genetic manipulation, the host plant can also be engineered for increased N_2-fixation efficiency. Identification and analysis of host plant genes involved in nodule morphogenesis and functioning has lagged behind that of *Rhizobium* (Morris et al. 1999). A wide array of new and explosive molecular tools, like, genomics and proteomics, are currently available that provides plant breeders several new opportunities to create and manipulate genetic variation to produce improved plants more easily (Morris et al. 1999). In this context, McCardell et al. (1999) reviewed several strategies to improve SNF suggesting that either structure or regulatory genes could be altered to enhance N_2 fixation. In a study, transgenic alfalfa plants transformed with the soybean or pea lectin genes became susceptible to infection by *B. japonicum* (Van Rhijn et al. 2001). Because *Rhizobium* strains differ in their ability to use opines (Murphy et al. 1995), genetic engineering of legumes or other plants for opine synthesis may result in the enhanced growth of rhizosphere organisms with the ability to utilize this substrate (Oger et al. 1997; Savka and Farrand 1997). Malate is the primary plant C source used by bacteroids, and is also a factor in plant adaptation to P and Al stress (Driscoll and Finan 1993; Johnson et al. 1996).

The modification of bacterial strains for synthesis of the peptide antibiotic trifolitoxin is another area of interest. Trifolitoxin produced by some strains of *R. leguminosarum* bv. *trifolii* is toxic to a wide range of Gram-negative bacteria including most rhizobia. While a strain of *R. etli* expressing trifolitoxin (*tfx*) genes was more competitive for nodulation in unsterilized soil than a near isogenic tfx^- strain (Robleto et al. 1997), a concern could be the transfer of such genes to other less-effective rhizobia in the soil population. Transfer of a chromosomal DNA from the salt-tolerant (grow at 30% NaCl) *Bacillus* species into a strain of *R. leguminosarum* was successful (El-Saidi and Ali 1993). This rhizobia strain became salt tolerant and grew at about 10–15% NaCl. Furthermore, the lentil plants inoculated with salt tolerant strain of *Rhizobium* grown in reclaimed desert soil demonstrated improved plant yield and N content.

One of the most important and interesting strategy dealing with salt tolerance in leguminous plants could be the identification and cloning of genes regulating salt stress. However, some of these genes have been cloned in the model legume *Arabidopsis thaliana* (Shi et al. 2000; Venema et al 2002). These genes encode putative Na^+/H^+ antiporters. In saline environments, plants accumulate Na^+ in vacuoles through the activity of tonoplast Na^+/H^+ antiporters. The first gene for a putative plant vacular N^+/H^+ antiporter, AtNHX1, was isolated from *Arabidopsis* and shown to increase plant tolerance to NaCl (Venema et al 2002). Other species are highly specific in their requirements. If nodules do not occur, an appropriate

strain of *Rhizobium* inoculum is frequently added to the seed, sometimes as a fine cap or pelleting. It has been reported that the introduction of target indigenous species of plants, associated with a managed community of microbial symbionts, is a successful biotechnological tool to aid the recovery of desertified ecosystems (Requena et al. 2001).

20.5 Management of Phytopathogens Using Rhizobia

Soil-borne pathogens are among the major factors restricting the growth and yield of crops including legumes in many countries around the world. For example, root rot caused by *Fusarium* spp., *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Aphanomyces euteiches* is the most destructive soil-borne disease of pea (*Pisum sativum*), chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), faba bean (*Vicia faba*), and lupine (*Lupinus perennis*) (Abou-Zeid et al. 1997; Abdel-Kader et al. 2002; Infantin et al. 2006). In intensive agricultural practices, synthetic pesticides are therefore, frequently used to offset the pathogens inflicting severe losses to legumes. However, due to the adverse impact of excessive and injudicious application of such chemical substances on soil fertility, there is a need to reduce the use of chemical pesticides and to optimize the use of alternative management strategies to control soil-borne pathogens in sustainable farming systems. In this context, though rhizobia, in general, have largely been exploited for their nitrogen-fixing ability, but many species of rhizobia are also reported to promote legume yields by suppressing the growth of pathogenic fungi (Estevez de Jensen et al. 2002; Bardin et al. 2004; Essalmani and lahlou 2003; Elbadry et al. 2006; Huang et al. 2007; Huang and Erickson 2007; Mazen et al. 2008) as presented in Table 20.1. Generally, the microorganisms including species of *Rhizobium* serving as biocontrol agents have evolved various mechanisms for suppressing the population of phytopathogens. Such mechanisms include (1) inhibition of the pathogen by synthesizing antimicrobial substances (antibiosis) (Krishnan et al. 2007) (2) synthesis of microbial metabolites, like, siderophore (Sridevi and Mallaiah 2008a, b) and rhizobiotoxin (Deshwal et al. 2003) (3) competition for nutrients (4) induction of plant-resistant mechanisms, and (5) production of cell wall degrading enzymes, like, chitinase (Sridevi and Mallaiah 2008a). Rhizobia utilize one or combination of such mechanisms to suppress plant disease at one time. The potential of rhizobia in the management of diseases are discussed in the following section.

20.5.1 Antagonistic Effects of Rhizobia to Pathogens and Pest

Field and glasshouse studies show that inoculating plants with rhizobia can be a cheap and effective method of controlling soil-borne pathogens in cropping systems. For example, inoculating soybean and common bean plants with their

Table 20.1 Examples of *Rhizobium* strains used as agents to control diseases

Rhizobium strains	Pathogen	Host–plant	References
R. etli	*Globodera pallid*	Potato	Reitz et al. (2000)
	Meloidogyne incognita	Potato	Hallmann et al. (2001)
R. leguminosarum bv. *phaseoli*	*Fusarium solani*	Bean	Dar et al. (1997)
	F. solani	Okra, soybean, sunflower	Omar and Abd-Alla (1998)
	Macrophomina phaseolina	Okra, soybean, sunflower	Omar and Abd-Alla (1998)
	Rhizoctonia solani	Okra, soybean, sunflower	Omar and Abd-Alla (1998)
R. leguminosarum bv. *viceae*	*Curtobacterium flaccumfaciens*	Bean	Huang et al. (2007)
	Pythium spp.	Bean	Huang and Erickson (2007)
	Yellow mosaic virus	Bean	Elbadry et al. (2006)
	Orobanche crenata	Pea	Mabrouk et al. (2007c)
	Root rot fungal diseases	Faba Bean	Mazen et al. (2008)
R. tropici	*Fusarium oxysporum* f. sp. *phaseoli*	Bean	Estevez de Jensen et al. (2002)
	F. solani	Bean	Estevez de Jensen et al. (2002)
	R. solani	Bean	Estevez de Jensen et al. (2002)
	F. oxysporum f. sp. *ciceris.*	Chickpea	Arfaoui et al. (2006)
	Fusarium oxysporum f. sp. *lentis*	Lentil	Essalmani and Lahlou (2003)
R. leguminosarum bv. *phaseoli*	*Fusarium solani*	Bean	Dar et al. (1997)
	F. solani	Okra, soybean, sunflower	Omar and Abd-Alla (1998)
	Macrophomina phaseolina	Okra, soybean, sunflower	Omar and Abd-Alla (1998)
	Rhizoctonia solani	Okra, soybean, sunflower	Omar and Abd-Alla (1998)

respective microsymbionts significantly decreased the severity of *Phytophthora megasperma*, *Fusarium oxysporum, Pythium ultimum*, and *Ascochyta imperfecta* by 75, 47, 65, and 35%, respectively (Tu 1978). The fungal populations decreased substantially with increase in rhizobial inoculum. In a similar study, different rhizobial strains applied either as seed dressing or soil drench, successfully protected field-grown soybean, mungbean [*Vigna radiata* (L.) wilczek], sunflower (*Helianthus annuus*), and okra (*Abelmoschus esculentus*) plants from infection by the root-borne pathogens, *Macrophomina phaseolina*, *R. solani*, and *Fusarium* species (Ehteshamul-Haque and Ghaffar 1993). Moreover, rhizobia isolated from root nodules of *Acacia pulchella* declined the survival of the zoospores of *Phytophthora cinnamoni* in vitro (Malajczuk et al. 1984) and hence, provided protection to the host plant. It is evident from these findings that rhizobia show a great

potential against plant diseases and, therefore, deserve more attention to be considered as biocontrol agent.

Many workers have tried to understand the mechanisms as to how rhizobia inhibit the growth and development of pathogen, thereby leading to increase in crop productivity. In this context, early work revealed that *Rhizobium* spp. have the potential to produce extracellular compounds (such as trifolitoxin) with direct antimicrobial activities (Breil et al. 1996) indicating that antibiosis may be part of their reported biocontrol efficacy. However, these compounds seem to be rather specific to other *Rhizobium* spp. suggesting that they would be more involved in limiting nodule formation by competing *Rhizobium* strains rather than biocontrol (Triplett 1990; Robleto et al. 1998). Another trait by which *Rhizobium* spp. could limit the growth of phytopathogen is their ability to produce iron-chelating siderophores. These compounds reduce or eliminate the available iron for other microorganisms in the same ecological niche and thus produce their antagonistic activity through competition. Antoun et al. (1998) determined that 181 of 196 tested *Rhizobium* spp. produced siderophores while Arora et al. (2001) suggested that only siderophore producing strains of *S. meliloti* were able to inhibit *M. phaseolina* in vitro. These strains were equally capable of increasing groundnut (*Arachis hypogaea*) seed germination in the presence of *M. phaseolina*. Although siderophore production by PGPR other than rhizobia has been extensively linked to biocontrol capabilities, there is very little evidence on siderophore synthesis by rhizobia and its role as a component of antagonism toward plant pathogens. For example, Kumar et al. (2006) in a study isolated five sinorhizobia from medicinal legume *Mucuna pruriens* and determined their genetic diversity using ARDRA. All five isolates solubilized inorganic insoluble P, produced IAA but did not produce HCN. Strains MPR3 and MPR4 produced siderophore and inhibited fungal pathogens, *M. phaseolina* and *F. oxysporum*. In yet another study, Mabrouk et al (2007c) have demonstrated that *Rhizobium* isolates significantly increased the growth and N_2 fixation efficiency of pea plants. In addition to compatibility with pea, inoculation with rhizobia also significantly decreased pea susceptibility to the parasite *O. crenata*. Induced resistance in inoculated peas occurred throughout the infection process, which was expressed at different developmental stages of *Orobanche* including germination, radicle growth, parasite attachment to pea roots, and finally tubercle growth on host roots.

20.5.2 Induction of Systemic Resistance by Rhizobium *spp. Against Pest and Diseases*

Rhizobial populations may also promote plant health by stimulating the plant host. The *Rhizobium* spp. indirectly stimulates the plant to activate its defense mechanisms when challenged with a pathogen through the production of plant defense compounds (e.g., phenolics, flavonoids, or other phytoalexins). For example,

Rabie (1998) demonstrated that total and free phenolics increased significantly in *Botrytis fabae*-infected broad bean when the plant was pre-inoculated with *R. leguminosarum* bv. *viceae*. Elicitation of isoflavonoid phytoalexins by *Rhizobium* spp. has been associated with disease control in alfalfa and common bean (Dakora et al. 1993a, b; Dakora 2003). *Rhizobium*-mediated induction of phenolics (particularly gallic, ferulic, tannic, and cinnamic acids) was correlated with reduced sheath blight (*R. solani*) of rice (Mishra et al. 2006). Induced resistance against broomrape in the nodulated pea was shown to be associated with significant changes in rates of oxidative lipoxygenase (Lox) and phenylpropanoid/isoflavonoid pathways and in accumulation of derived toxins, including phenolics and pisatin (pea phytoalexin). In parallel, the nodulated roots displayed high Lox activity related to the overexpression of the lox1 gene. Similarly, the expression of phenylalanine ammonia lyase (PAL) and 6a-hydroxymaackiain 3-O-methyltransferase (Hmm6a) genes were induced early during nodule development, suggesting the central role of the phenylpropanoid/isoflavonoid pathways in the elicited defense (Mabrouk et al. 2007a, b, c). Arfaoui et al (2006) have also identified *Rhizobium* spp. that induced defense responses and reduced disease severity in chickpea plants infected with *F. oxysporum* f.sp. *ciceris*. They have shown that pretreatment of germinated chickpea seeds with selected rhizobal isolates, a few days before inoculation with *F. oxysporum* f.sp. *ciceris*, reduced the wilt incidence and induced significant increases in the activity of several defense-related enzymes, such as peroxidases and polyphenoloxidases, in the accumulation of phenolic compounds and expression of phenylpropanoid defense-related genes (Arfaoui et al. 2005, 2007).

20.5.2.1 Role of Lipopolysaccharides in Induced Resistance

Rhizobacteria-mediated induced resistance has been demonstrated against fungi, bacteria, and viruses in *Arabidopsis*, bean, cucumber, and radish, under conditions in which the inducing bacteria and the challenging pathogen remained spatially separated. However, bacterial compounds that induce plant defense mechanisms are highly variable depending on bacterial strain and pathosystems. For example, salicylic acid (SA) production has been observed for several bacterial strains, and exogenously applied SA can induce resistance in many plant species. In beans, enhanced defense by *Pseudomonas aeruginosa* strain 7NSK2 toward the pathogenic fungus *Botrytis cinerea* was initiated by SA synthesized by bacteria. In tomato and radish, lipopolysaccharides (LPS) of nonpathogenic pseudomonads induced resistance against challenge inoculations by pathogenic bacteria. In case of *R. etli*, LPS have been implicated in triggering the induced systemic resistance (ISR). For instance, Reitz et al. (2000, 2002) showed that LPS from *R. etli* played a major role in the elicitation/triggering of ISR in potato against cyst nematode *Globodera pallid*. In pea induced systemic resistance to infection by *O. crenata* was triggered by heat-killed cells and purified LPS of *R. leguminosarum* (Mabrouk et al. 2008, 2009).

20.6 Conclusion

Biological nitrogen fixation is an important process for providing nitrogen inexpensively to legumes in farming system for increasing the productivity of crops. The BNF system such as rhizobia and legume adapts well under different ecological conditions and fixes considerable amounts of nitrogen. However, under certain adverse environmental situation, the actual impact of rhizobia on legumes is not realized. Therefore, there is an increasing demand for identifying rhizobial species that could also work under stressed soil environment so that the productivity of inoculated legumes does not suffer under derelict soils. Furthermore, not only the importance of rhizobia as a natural resource is restricted to their SNF activity or to several other activities in the soil, which eventually improve soil fertility and plant productivity, but also some strains of rhizobia may be used to suppress phytopathogens, capable of causing severe losses to legumes, and concomitantly enhance legume growth. However, further studies on exploring the precise mode of action and the eco-physiology of these microorganisms in relation to other soil borne inhabitants are required in order to better exploit this relationship under different agro-ecological niches.

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Chapter 21
Microbial Inoculants for Sustainable Legume Production

C. R. Patil and A. R. Alagawadi

Abstract The role of legumes in improving soil fertility is well known and hence is being introduced to newer areas to enrich the soil with plant nutrients, especially nitrogen. Since legumes are an important source of dietary protein, their production is linked to food security. Furthermore, identification of insecticidal components in legume extract and fiber in pea offers other interesting opportunities to legume growers. All these properties together have generated greater interest in growing legumes worldwide. Therefore, there is a need to enhance the productivity of legumes. To optimize the productivity of legumes, synthetic chemicals are used which are not only costly but also adversely affect the environment when used inadvertently. To circumvent both cost and environmental hazards and to increase productivity of legumes, rhizobial inoculation either alone or in combination with other plant growth promoting microorganisms (PGPMs), such as phosphate-solubilizing microbes and mycorrhizal fungi, have been practiced over the years. In a number of studies, inoculations of legumes with various PGPMs have shown enhanced production. Inoculation with PGPMs has been found to improve uptake of nutrients and protection against pests, pathogens and induce systemic acquired resistance to legumes. It may, however, be important to identify the combination of PGPMs that work synergistically to enhance productivity of various legume crops. In this review, the published literature on the use of PGPMs in legume cultivation has been compiled so as to identify microbial inoculants for sustainable production of legumes.

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21.1 Introduction

The nitrogen-fixing ability of legumes is being exploited in agriculture for more than a century. This ability of legumes is due to their capacity to establish symbiotic association with *Rhizobium*, a soil bacterium that forms root nodules (Vance 1997). The nodule-forming nitrogen-fixing bacteria is collectively called as rhizobia. It is reported that legume–*Rhizobium* symbiosis fixes 200–300 kg N ha^{-1} annually (Peoples et al. 1995a, b). Cultivation of legumes is generally known to maintain productivity of agricultural system (Graham and Vance 2000; Giller and Wilson 1991). Similarly, inclusion of legumes in crop rotation was found to improve soil physical and chemical properties (Biederbecke et al. 2005), and enhance yield and quality of subsequent crops (Gan et al. 2003; Mc Vicar et al. 2000; Miller et al. 1998). Legumes in crop rotation were also found to be useful in controlling pests, diseases, and weeds (Howieson et al. 2000). Fodder and grain legumes are also most important source of protein (Kannaiyan 2000). Therefore, the productivity of legumes is linked to the nutritional quality and food security. It is estimated that the rhizobial symbioses with over 100 agriculturally important legumes contribute nearly half the annual quantity of biological nitrogen fixation (BNF) entering soil ecosystems (Tate 1995). The diversity and adaptability of legumes is thus being explored to introduce them into newer areas. Efforts to isolate and identify effective strains of *Rhizobium* from wild legumes for their subsequent inoculation with other legume crops are a new strategy to harness the full benefits of the *Rhizobium*–legume symbiosis (Zahran 1999). Recently, insecticides comprising naturally occurring compounds such as PA1b-related peptides, terpenoids, and saponins in pulse crop extracts (Bodnaryk et al. 1999), dehydrosoyasaponin I in extracts of yellow pea (*Pisum sativum* L.) with anti-feedant and insecticidal properties (Taylor and Fields 2006), and other insecticides such as soyasaponins and lysolecithins in pea (Taylor et al. 2004a, b) have been identified. Similarly, use of pea fiber in the formulation of food products (Shand et al. 2008; Shand et al. 2007) is known. These findings could only increase the demand for pulses. Hence, there is a need to enhance pulse production using sustainable approaches.

Cultivation of legumes requires less or no external N fertilizer as compared to other crops. Application of chemical fertilizers to enhance legume production may not be a sustainable approach as the fertilizer consumption (from 7 to 17 kg ha^{-1} between 1973 and 1988; FAO 1990) and demand (Subba Rao 1980) are on the rise. The fertilizer N accounts only for 25% of the earth's newly fixed N. When fertilizer N is applied, it may leach out (Sprent and Sprent 1990) leading to increased levels of toxic nitrates in drinking water and eutrophication of water bodies. These have raised serious concerns for environment and consumers' health. Biological nitrogen fixation in legumes thus forms a renewable source in agriculture (Peoples et al. 1995a, b). Unlike chemical N fertilizers which are of high concentration and low use efficiency, the BNF can be tailor-made for crop uptake and can be easily combined with other PGPMs. Legumes are important in increasing the diversity of soil flora and fauna contributing to greater stability of total life in the soil (Vance 1997).

They enhance production of total biomass in the soil by providing additional N which the soil microbes can use to break down carbon-rich residues. Like rhizobia, several microorganisms such as fluorescent *Pseudomonas,* mycorrhizal fungi, and bacilli also enhance legume growth and yields, in addition to their role in suppressing diseases of crop plants (Recep et al. 2009; Dileep Kumar 1999; Duijff et al. 1993; Schippers 1993; Weller 1988). Such beneficial microorganisms have been used in sustainable crop production. An attempt has been made in this chapter to highlight the role of microbial inoculants in improving the sustainable production of legumes in different agroecological regions.

21.2 Legumes and Biological Nitrogen Fixation

Legumes include pulses, pastures, and grain legumes. Taxonomically, they are classified under 700 genera, covering nearly 18,000 species (Brockwell et al. 1995). Rhizobia can elicit nitrogen-fixing nodules on most of the 18,000 species of the leguminosae family (Raychaudhuri et al. 2007). This unique association is known to be mutually beneficial to *Rhizobium* (microsymbiont) and the legume (macrosymbiont). While *Rhizobium* gets protection, obtains its nutrients and energy from the legume, legume benefits from the nitrogen fixed by the *Rhizobium*. The nitrogen fixation by *Rhizobium* commences with the formation of root nodules. *Rhizobium* present in soil invades roots and multiplies within the cortical cells, eventually forming nodules that can be seen 2–3 weeks after planting. Generally, legumes such as alfalfa, chickpea, and clover have finger-shaped indeterminate type of nodules. Mature nodules often resemble a palm with protruding finger-like structures and the entire nodule (Fig. 21.1) is less than half an inch in diameter (Lindemann and Glover 2003). These nodules are 10–50 in number, mostly concentrated on the tap root, long-lived, and fix atmospheric nitrogen throughout the growing period. Annual legumes such as beans, peanut and soybean bear determinate type of nodules which are round in shape and can reach the size of a large pea. These nodules are short-lived and degenerate in about 40–45 days, and new nodules are formed continuously. At crop maturity stage, these nodules do not fix nitrogen. The number of nodules in annual legumes can vary from 100 to few hundred per plant. Only nodules with pink or red colored tissues are active in nitrogen fixation. In contrast, nodules with white or green tissues are ineffective and do not fix nitrogen. The nitrogen fixed within the nodule is subjected to availability of photosynthates and other nutrients to rhizobia within the nodule (O'Hara 2001). A number of other factors attributed to host, rhizobial strains, and soil can influence legume–*Rhizobium* symbiosis. Despite these constraints, the amount of nitrogen fixed by legume–*Rhizobium* symbiosis is a renewable source for agriculture (Peoples et al. 1995a, b) and this amount can vary among legume species (Table 21.1). Some legumes like common beans fix less N than their needs. Therefore, to obtain maximum economic yield of beans, additional fertilizer N is required. Contrarily, if beans are not nodulated their yields often remain low,

Fig. 21.1 Chick pea (*Cicer aerintium)* root showing nodule occupancy by *Rhizobium* (Courtesy P. Jones Nirmalnath)

Table 21.1 Estimates of nitrogen fixed by legumes

Crop	Nitrogen fixed (Kg ha^{-1})
Blackgram	119–140
Chickpea	23–97
Cluster bean	378–196
Cowpea	9–125
Greengram	50–66
Pigeonpea	4–200
Soybean	49–450
Peas	46

Adapted from (Peoples et al. 1995b)

regardless of the amount of N applied. Possibly, the nodule bacteria in these pulses help the plant to utilize applied N fertilizer efficiently. Other grain legumes, such as peanuts, cowpeas, soybeans, and faba beans are good N fixers and can meet all of their N needs through BNF (Lindemann and Glover 2003). Such of these legumes may not respond to applied N fertilizer. However, there is a need to apply small dose of fertilizer N in the initial stage of crop growth to meet the N requirement before N fixation starts. External N fertilizer application to legumes depends on the success and efficiency of symbioses with *Rhizobium*. The rhizobia from different legumes also differ in their abilities to form nodules and fix N. For example, rhizobial strains, which effectively nodulate soybean, do not form nodules on other legumes. This reflects a kind of host specificity being operated by the microsymbiont. Rhizobia can be grouped according to the legume host they nodulate.

Table 21.2 Cross-inoculation groups and associated *Rhizobium* species

S. no	Cross-inoculation group	Legumes included	*Rhizobium* species
1	Alfalfa	Alfalfa, Black medic, Burr clover (medic), Button clover (medic), Sweet clovers (yellow and white)	*Rhizobium meliloti*
2	Bean	Beans	*Rhizobium phaseoli*
3	Clover I	Berseem clover, Crimson clover, Lappa clover, Persian clover, Rose clover	*Rhizobium trifolii* strain
4	Clover II	Rose clover, Subterranean clover	*Rhizobium trifolii* strain
5	Clover III	Alsike clover, Ball clover, Hop clover, Ladino clover, Red clover, White clover	*Rhizobium trifolii* strain
6	Clover IV	Arrow leaf clover[a]	*Rhizobium trifolii* strain
7	Lupine	Lupines	*Rhizobium lupini*
8	Pea	Caley pea, Garden peas, Lentils, Vetches, Winter peas	*Rhizobium leguminosarum*
9	Soybean	Soybeans	*Bradyrhizobium japonicum* strain
10	Cowpea	Alyce clover, Cowpeas, Lespedeza, Lima bean, Peanut, Kudzu	*Bradyrhizobium japonicum* strain
11	Trefoil	Birds foot trefoil	*Rhizobium loti*

[a]Arrow leaf clover requires specific inoculum. It will not cross-inoculate with other clovers
Source: Jennings, http://www.uaex.edu

These are referred to as cross-inoculation groups. Leguminous plants nodulated by the same species of *Rhizobium* comprise a "cross-inoculation group" (Table 21.2).

Common forage legumes such as clover, alfalfa, vetch, trefoil, and lespedeza are important sources of fodder protein and do not require N fertilization. Similarly, grain legumes are the only source of protein in vegetarian diet. Newer roles for legumes in farming systems are envisaged as agriculture continues to develop. Some of them include the use of legumes in continued expansion of crops onto infertile and stressful soils. Similarly, explorations are on for new genera and species of pasture and forage legumes with deep rooting habits to combat soil moisture stress and salinity (Howieson et al. 2000). In this context, proven microbial inoculants useful for legume production need to be popularized.

21.3 Rhizobium

Rhizobia are gram-negative, aerobic, heterotrophic, non-sporulating rods with a generation time of 3–6 h. They grow on the surface of solid media and also in static liquid media with a large surface area. Growth in submerged culture in aerated fermentors provides maximum viable cell production. Optimum growth occurs at 29–30 °C. Rhizobia must be supplied with a source of energy, N, minerals, and

growth factors. As sucrose is universally available and inexpensive, it is the most commonly used carbon source for fast growers. Mannitol and glycerol are also used by some *Rhizobium* biofertilizer manufacturers to grow rhizobia. The slow-growing rhizobia are reported to prefer pentoses (and hexoses), such as arabinose or xylose. Most rhizobia are able to use ammonium or nitrate form of nitrogen, but growth is usually better in media which supply an adequate amount of low molecular weight amino acids. Earlier, rhizobia were characterized based on their ability to induce nodule formation on the roots of certain leguminous plants. Their grouping, based on leguminous plants they nodulate (Table 21.2), is found to have far more practical significance (Burton 1984).

21.3.1 Rhizobium Strains

Legumes plants must be inoculated with specific strains of rhizobia for maximizing N-fixation efficiency. Sometimes, a group of leguminous plants are nodulated by the same kind of rhizobia. For many years, bacteria isolated from the nodules on any of the plants in one of these groups were considered a single species of *Rhizobium*. Two distinct groups of rhizobia-like fast growers and slow growers have been recognized based on their relative growth on culture media. Of these, fast growers include *R. meliloti* nodulating *Medicago* spp., *Melilotus* spp., and *Trigonella* spp; *R. leguminosarum* biovars: *trifolii* nodulating *Trifolium* spp., *phaseoli* nodulating *Phaseolus vulgaris*, *P. coccineus, viceae* nodulating *Pisum, Lathyrus, Lens,* and *Vicia; R. loti* nodulating *Lotus* spp. (fast growers), *Lupinus* spp., and *R. japonicum* nodulating *Glycine max* (certain Chinese and Asian isolates). Slow growers include *Bradyrhizobium japonicum* biovars: *glycinae* nodulating *Glycine max, vignaea* nodulating *Vigna, lupinea* nodulating *Lupinus* and *Lotus pedunculatus*.

The rhizobia isolated from cowpeas and numerous other legumes were not given species names until recently. They were simply identified with the name of their host. Many of the *Rhizobium* strains isolated from legumes have not been adequately characterized with respect to their growth and the range of hosts with which they are able to form symbiosis (Burton 1984). Recently, attempts have been made to reclassify rhizobia based on the sequences of 16 S and 23 S ribosomal RNA genes. These attempts have clearly shown that the nodule-forming bacteria fall under four distinct groups under the α-subclass of proteobacteria (Sy et al. 2001) (Fig. 21.2): Group 1 includes *Rhizobium*, *Allorhizobium*, *Sinorhizobium,* and *Mesorhizobium*; Group 2, *Azorhizobium*; Group 3, *Bradyrhizobium*; and Group 4, *Methylobacterium nodulans*. Raychaudhuri et al. (2007), in their review on changing face of rhizobial taxonomy, have compiled various genera and species into these four taxonomic clads within the α-sub class of proteobacteria. Weir (2009) keeps updating the current taxonomy of rhizobia online. In this compilation, the author has 22 species listed under *Rhizobium,* 19 within *Mesorhizobium,* 14 under *Ensifer* (formerly *Sinorhizobium*), eight in *Bradyrhizobium*, two under *Azorhizobium*, one species each in *Methylobacterium, Cupriavidus* (formerly *Wautersia,* formerly *Ralstonia*), *Devosia* (*Devosia neptuniae), Shinella*, *Herbaspirillum*

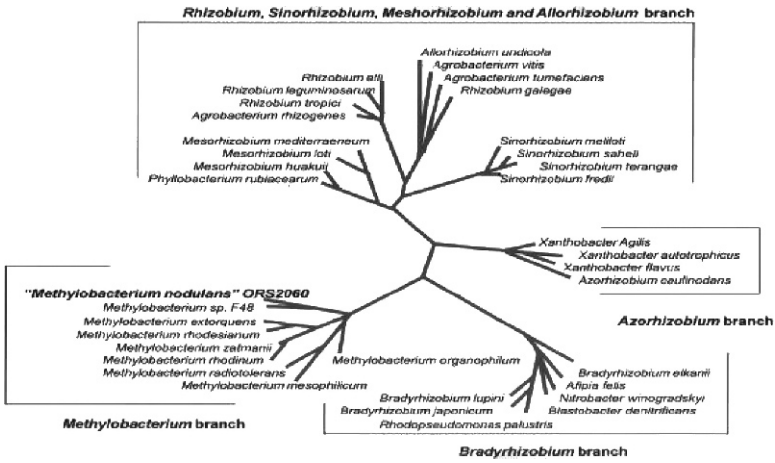

Fig. 21.2 Branching of rhizobial phylogenetic tree on alpha sub class of proteobacteria (Adapted-from Sy et al. 2001)

(*Herbaspirillum lusitanum;* Lin et al. 2008), and *Ochrobactrum* (*Ochrobactrum cytisi:* Zurdo-Pineiro et al. 2007), and three species in *Phyllobacterium*.

Exploring more legumes imply more and more new strains of *Rhizobium* to be classified. Thus revision of rhizobial classification is likely to be a continuous process. A competitive and highly efficient *Rhizobium* strain is a prerequisite for nitrogen fixation in legume–rhizobial symbiosis. However, a competitive and persistent rhizobial strain may not express its full capacity for N fixation due to other limiting factors. Some of these originating from soil (like salinity, soil pH, nutrient deficiency, mineral toxicity, heavy metals, biocides, temperature extremes, and insufficient or excessive soil moisture) and host plants (inadequate photosynthesis, plant diseases, and grazing) (Peoples et al. 1995a, b; Thies et al. 1995; Walsh 1995). These factors impose limitations on the vigor of the host legume. The most problematic environments for legume–*Rhizobium* symbiosis are marginal lands with low rainfall, extremes of temperature, acidic soils of low nutrient status, and soils with poor water-holding capacity (Bottomley 1991).

In recent days, a number of studies have shown that co-inoculation of legumes with rhizobia and plant growth-promoting rhizobacteria (PGPR) have shown increased nodulation and N fixation (Table 21.3) under normal growth conditions (Verma et al. 1986; Li and Alexander 1988; Dashti et al. 1997; 1998; Parmar and Dadarwal 1999). Principles and mechanisms of plant growth promotion by PGPMs have been well studied (Lugtenberg 2009; Compant et al. 2005). These need to be extended to legume–rhizobia symbioses. The best-known effect apart from nutrient mobilization is through the production of auxin and other phytohormones (Okon et al. 1998). Production of certain volatiles and cofactors such as pyrrolquinoline quinone (PQQ) by PGPMs are also known to stimulate plant growth (Lugtenberg 2009). An induced stimulation of root growth due to auxin production was found to enhance nodulation (Molla et al. 2001; Srinivasan et al. 1996; Vessey and Buss 2002).

Table 21.3 Plant growth-promoting rhizobacteria (PGPR) stimulating the rhizobia–legume symbioses and percentage change in characteristics of the symbioses

Legumes	PGPR	Nodule number	Nodule weight	N benefit	Reference
Alfalfa	*Pseudomonas syringae*	+122 to +141	123	+48 to +194	Knight and Langston-Unkefer (1988)
Common bean	*A. brasilense*	17	Not measured	Not measured	Burdman et al. (1996)
	Bacillus sp.	+37 to +87	+33 to +83	+76 to +115	Srinivasan et al. (1996)
Lentil	*P. putida*	0 to +46	Not measured	0 to +228	Chanway et al. (1989)
Pea	*Pseudomonas* sp.	0	0	0	Chanway et al. (1989)
	P. fluorescens	298	Not measured	Not measured	Andrade et al. (1998)
Peanut	*Azospirillum lipoferum*	+47	+31 to +70	Not measured	Raverkar and Konde (1988)
Red clover	*Pseudomonas* sp.	+210	Not measured	+124	Marek-Kozaczuk and Skorupska (2001)
Soybean	*Bacillus* sp. and *Pseudomonas* sp.	+55 to+57	Not measured	Not measured	Li and Alexander (1988)
	Aeromonas hydrophila	0 to +73	0 to 300	0 to 140	Zhang et al. (1996, 1997)
	Serratia proteamaculans	0 to +191	0 to +1351	0 to + 92	Dashti et al. (1997, 1998)
	Bacillus cereus	+16	None	12	Vessey and Buss (2002)

Adapted from Vessey (2003)

Stimulation of root growth possibly provided more sites for infection and nodulation by rhizobia. Similarly, inoculation of soybean with *Bradyrhizobium* and PGPR strain producing IAA and ACC deaminase improved shoot and/or root growth in soybean, but had no positive effects on nodulation (Cattelan et al. 1999). These workers also observed enhanced soybean–*Bradyrhizobium* symbioses by several rhizospheric isolates known to produce either β-glucoanase or cyanide. Another important mechanism by which PGPMs influence plant growth is by increasing the availability of nutrient for the plant in rhizosphere (Glick 1995; Rodriguez and Fraga 1999) by solubilization of unavailable forms of nutrients (Vassilev et al. 2006) and/or sideraphore production which facilitate transport of nutrient especially iron (Schippers et al. 1987). Apart from nitrogen-fixing bacteria, a number of nutrient mobilizers such as P-solubilizers (Podile 1995) and elemental S oxidizers have been found useful in crop production. Some of these are also found effective in legume production.

21.4 Microbial Inoculants for Legume Production

Microbial inoculants can be defined as a formulation containing one or more beneficial microbial strain (or species) in an easy-to-use and economical carrier material either organic, inorganic, or synthesized from defined molecules (Yoav 1998). An inoculant is the means of transporting beneficial microorganism from the factory to the living plant. The desired effects of the inoculants on plant growth can include N fixation, biocontrol of (mainly) soil-borne diseases, enhancement of mineral nutrient uptake, weathering of soil minerals, nutritional or hormonal effects. In some instances, microbial inoculants are referred to as biofertilizers. By contrast, biofertilizers refer to preparations of microorganism(s) that may be a partial or complete substitute for chemical fertilization. However, other beneficial effects on plant growth are largely ignored (Yoav 1998). Studies involving mixed inoculation with different functional groups of microorganisms have clearly exhibited synergistic effects with concurrent significant increase in growth and nutrient uptake of legumes (Lugtenberg and Kamilova 2009; Egamberdiyeva et al. 2008; Kamilova et al. 2008; Van Loon 2007; Haas and Defago 2005; Okon et al. 1998; Barea 1997; Isopi et al. 1995), as listed in Table 21.3. Soil bacteria that promote plant growth are also known to overcome stress in host plants by producing metabolites such as ACC deaminase (Glick et al. 2007).

21.4.1 Azospirillum

Azospirillum, a bacterium associated with many plants (Bashan and Holguin 1997a), is one of the most studied PGPR. Bashan and Holguin (1997a, b), in their extensive review, have highlighted the beneficial effects of inoculating *Azospirillum* along with other microorganisms. Co-inoculations, as compared to single inoculation with *Azospirillum*, provided the plants with more balanced nutrition, improved absorption of mineral nutrients, and significantly increased growth and yield. For example, dual inoculation of *Azospirillum* and P-solubilizing bacteria to sorghum (Alagawadi and Gaur 1992) and barley (Belimov et al. 1995) resulted in synergistic effect. *Azospirillum* inoculation can also help *Rhizobium* as it stimulates nodulation, nodule activity, and plant metabolism, which in turn improve many growth characteristics and resistance to unfavorable conditions in pea (Andreeva et al. 1993), chick-pea (Fabbri and Del Gallo 1995), and alfalfa (Itzigsohn et al. 1993). It has been observed that *Azospirillum* produces more phytoharmones when grown in mixed culture (Janzen et al. 1992) which also provides suitable conditions for N fixation (Holguin and Bashan 1996; Lippi et al. 1992). The efficiency of biocontrol microorganisms is known to be enhanced in the presence of *Azospirillum* (Frommel et al. 1991; Lemanceau and Alabouvette 1991). It is evident from these studies that microbial inoculants involving strains of *Rhizobium* and *Azospirillum* can easily be combined to inoculate legumes for enhancing legume production.

21.4.2 P-Solubilizers

Solubilization of insoluble nutrients in the rhizosphere is an important mode of increasing nutrient availability to host plants (Richardson 2001). Phosphorus, next only to N, is the most limiting mineral nutrient influencing the growth of plants. Many soils may have large reserves of total P, but most of them are unavailable for plant uptake due to its fixation with soil elements (Stevenson and Cole 1999). A number of PGPMs solubilize unavailable P (Khan et al. 2007; Vassilev et al. 2006) in the rhizosphere by secreting organic acids (Rodríguez et al. 2006) and phosphatase (Kim et al. 1998) and make it available to host plants. Beneficial effect of P- solubilizing microorganisms such as *Pseudomonas chlororaphis* and *P. putida* (Zaidi et al. 2009; Cattelan et al. 1999) on soybean, *Rhizobium* sp. and *Bradyrhizobium japonicum* (Antoun et al. 1998) on radish, and *R. leguminosarum* bv. *Phaseoli* (Chabot et al. 1998) on maize have been reported. Combined inoculation of P- solubilizing bacteria and *Rhizobium* to lentil and chickpea significantly improved aspects of symbiosis, growth, P uptake and yield (Kumar and Chandra 2008; Zaidi et al. 2003). Role of mycorrhizal fungi in improving uptake of P and other diffusion limited nutrients such as K, Cu, Mn, and Zn (Manjunath and Habte 1988; Pacovsky and Fuller 1986) in legumes such as green gram (*Vigna radiate* var. radiata) is well known. The use of arbuscualr mycorrhizal fungi (AMF) in legume production may be absolutely essential because of their synergistic interaction with rhizobium, phosphobacter (Poi et al. 1989) and other PGPMs possessing biocontrol activity.

21.4.3 Sulphur-Oxidizing Bacteria

Sulphur is one of the essential plant nutrients which contributes to yield and quality of pulses (Pasricha and Fox 1993; Vidyalakshmi et al. 2009). Plants take up sulphur in sulphate form and the transfer of sulphur between the inorganic and organic pool is entirely caused by the activity of the soil micro flora, dominated by *Thiobacillus thioxidans* (Kuenen and Beudeker 1982). Also, the acidity caused due to the formation of sulphate helps to solubilize other plant nutrients and to improve alkali soil (Wainwright 1984). In a greenhouse experiment conducted during January–May 2001, Stamford et al (2002) evaluated the effects of elemental S inoculated with *Thiobacillus* and compared with gypsum, in the amendment of two saline sodic soils (Neosol Fluvic Salic sodic) of the Brazilian semi-arid region. They studied the growth response of two tropical legumes cowpea and yam bean, along with inoculation of specific rhizobia strains. Sulphur was applied at 0.6, 1.2, and 1.8 t/ha and gypsum at 1.8 and 3.6 t/ha, and irrigation water had the salts, $NaHCO_3$, $MgCl_2$, $CaCl_2$, NaCl, and KCl. The treatment without S or gypsum served as control. When S was applied with *Thiobacillus,* it was more efficient than gypsum, reducing soil-exchangeable sodium. Sulphur with *Thiobacillus* in soil 1 reduced pH

(8.2–4.7) and electrical conductivity of the soil-saturation extract (15.3–1.7 mS/cm) to values below those used for classification as saline and sodic soil. The growth of cowpea and yam bean was increased by rhizobia inoculation when soil ameliorants were used, especially S in combination with *Thiobacillus*. In another study, co-inoculation of *Thiobacillus* sp. strain LCH (applied at 60 kg ha^{-1}) with *Rhizobium* under field condition significantly increased nodule numbers (136.9 $plant^{-1}$), dry matter accumulation in nodules (740 mg $plant^{-1}$), and plant biomass (15 g $plant^{-1}$) 80 days after sowing groundnut and enhanced the pod yield by 18%. Also, inoculation of S-oxidizing bacteria increased the soil-available S from 7.4 to 8.43 kg ha^{-1} (Anandham et al. 2007). These results suggest that mixtures of S-oxidizing bacteria and rhizobia had a synergistic effect and consequently enhanced the yield and oil content of groundnut when grown in S-deficient soils. Sulphur application in legumes has also been reported to facilitate nitrogenase enzyme, nitrogen fixation (Saraf 1988), and chlorophyll synthesis (Poorani 1992), as is observed in green gram. Considering the importance of sulphur in legume production, it would be necessary to include sulphur-oxidizing bacteria as a microbial inoculant in sustainable production of legumes.

21.4.4 Microbial Inoculants for Control of Pests and Diseases

Pulses are affected by insect pests and diseases at all stages of their growth, storage, and consumption. The use of chemical pesticides with escalating costs, toxicity to non-targeted species, development of resistance by the pest, and residual toxicity in food and environment are the major areas of concern. Microbial inoculants used as commercial biocontrol formulations that include, viruses, bacteria, and fungi are alternate eco-friendly approaches to reduce losses because of pest and diseases.

21.4.4.1 Bacterial Inoculants

The most widely used bacterial biocontrol agent is *Bacillus thuringiensis* with fast larvicidal activity and minimal to no effects on beneficial insects and other non-target organisms. Other advantages of this bacteria are: its production on relatively inexpensive media, it has a long shelf life and is applicable in field using conventional equipment. The crystal protein from *B. thuringiensis* (Bt) normally acts as a stomach toxin and hence is often referred to as a biorational insecticide or biopesticide (Lacey and Goettel 1995). The possibilities for integrating *B. thuringiensis* with other biological control agents, cultural practices, and conventional pesticides have made it a versatile biocontrol agent. Development of *Bt* toxins with broader host ranges, improved formulations, and options for their applications have resulted in greater demand for this biocontrol agent (Marrone 1994). Development of Bt toxin producing transgenic plants have given newer dimension to biocontrol strategies (Marrone 1993). Other bacterial agents include *Bacillus popilliae,*

B. sphaericus (Lacey and Goettel 1995), and *Serratia* (Lindsey and Belnavis 2009). *Bacillus licheniformis* with antagonistic activity is an endospore-forming bacterium known to tolerate unfavorable environmental conditions of drought, high temperature, and low O_2 which makes it a suitable candidate for use against fungal pathogens under field conditions (Neyra et al. 1996). Some bacteria with biocontrol potentiality such as *B. popillae* and *B. lentimorbus* cannot be grown in fermentation tanks. They are "cultivated" in laboratory-reared insect larvae. Products containing *B. popillae* and *B. lentimorbus* can be applied to control the larval (grub) stage of the Japanese beetle and, less effectively, some other beetle grubs. The symptoms develop slowly, often over a period of three to 4 weeks following initial infection. The internal organs of grubs are liquefied and turn milky white (hence, the name, milky spore disease). *Bacillus sphaericus* and *Serratia entomophila* are other bacteria that control Culicidae (Davidson 1985) and *Costelytra* spp. a scarabaeid (Jackson et al. 1992) insect pest, respectively.

Bacterial agents known to control soil-borne pathogens have been widely employed in biocontrol strategies. One genera *Pseudomonas* has been reported as the most common biocontrol agent for a number of bacterial, fungal, and viral diseases in wide host plants (Whipps 2001). Species of this bacterial genera including *aureofaciens, fluorescens, putida, and chlororaphis* are common soil bacteria. They can utilize a wide array of carbon source owing to their metabolic diversity. They also exhibit the greatest adaptability; as a result, they predominate soil microbial population. Many also possess biocontrol potentiality. They can be easily cultured and are amenable for genetic manipulation (Whipps 2001). Several species of *Pseudomonas* are also responsible for inducing resistance to plants referred to as "induced systemic resistance" (ISR' Lugtenberg and Leveau 2007; Kloepper et al. 2004) or "systemic acquired resistance" (Kloepper et al. 1999) as noticed in case of soyabean (Elbadry et al. 2006).

21.4.4.2 Fungal Inoculants

Fungi are important organisms which are known to regulate the populations of many insect pests and pathogens. They normally invade via the external cuticle of insects and need not be ingested to initiate disease. This ability of fungi makes them prime candidates for being used against plant-sucking insects. Accordingly, Lacey and Goettel (1995) reported different fungi having biocontrol potentiality against one or more insect pests. The fungi useful in controlling Lepidopteran, Coleopteran, and Dipteran insect pests that also occur on legumes include *Beauveria bassiana, Beauveria brongniartii, Entomophaga maimaiga, Hirsutella thompsonii* (on mites), *Metarhizium anisopliae, Metarhizium flavoviride, Nomuraea rileyi, Paecilomyces fumosoroseus*, and *Verticillium lecanii*. Species belonging to *Trichoderma* among potential biocontrol agents are reported for their ability to suppress a number of plant pathogens (Whipps 2001). Inoculation of AMF also helps in controlling diseases as it competes for space with pathogens and thus limits disease incidence (Whipps 2001). Induction of systemic resistance due to inoculation with *Trichoderma* and/or

AMF is well known. The most significant aspect of using these microbial inoculants in pest control is their ability to secrete compounds such as antibiotics, HCN, and extracellular enzymes, besides competing with the pest (Lugtenberg 2009).

21.4.4.3 Viral Inoculants

Viruses, obligate parasites, are of major concern as pathogens of legumes causing mosaic diseases. The host-specific characteristic of viruses is exploited against pests of crops to bring down the population of insect pests. Viruses classified as baculoviruses, consisting of an enveloped rod-shaped nucleocapsid containing circular, super coiled double-stranded DNA, are known for their action on insect pests (Lacey and Goettel 1995). The family is divided into two main groups: the Eubaculovirinae (nuclear polyhedrosis viruses [NPV] and granulosis viruses [GV]) and the Nudibaculovirinae (non-occluded viruses). The NPV against *Heliothis* (*Helicoverpa*) causing severe losses has been widely used in pulse cultivation (Lindsey and Belnavis 2009). The four strategies for using baculoviruses for pest control as suggested by Starnes et al (1993) are: inoculation of virus results in establishment and permanent regulation of the pest; application of virus that results in controlling epizootics, but not permanent regulation; manipulation of the habitat that results in activation of established or naturally occurring virus; and repeated applications of the virus to control pest insects due to little or no horizontal transmission of the virus. In a study, Cherry et al (2000) reported that treatment of chickpea with *Hear* NPV at 1.5×10^{12} p.i.b. per ha was as effective, or better, in controlling *Helicoverpa armigera* larvae, and increasing yield relative to control, than either a standard chemical insecticide, endosulfan, or *Bacillus thuringiensis*, in two successive years. Currently, high virus production costs make the viral treatments uncompetitive compared with the chemical treatment, but more economic than *B. thuringiensis* treatments. In this study, several formulations of viruses were tested, including an emulsifiable concentrate, an ultra-low volume (ULV) suspension, and a microencapsulated preparation, but none were consistently more effective than a filtered but unpurified aqueous suspension of *Hear* NPV. Yield and pod damage correlated with mid- and late-season number of medium-sized and large larvae. Persistence of all treatments including endosulfan and *B. thuringiensis* was short, and six treatments were necessary to provide adequate crop protection. *Hear*-NPV was the slowest-acting of the three control agents, with average survival times (AST) of 5.5 days, compared with 3.2 and 4.3 days for larvae fed leaves treated with *B. thuringiensis* and endosulfan, respectively.

The benefit of integrating baculoviruses into Integrated Pest Management (IPM) programs are presented by Huber (1986) and the interaction of baculoviruses and other components of IPM is presented by Harper (1986) and Groner (1990). Efforts are on to enhance bio-efficacy of these insecticidal viral particles by augmenting with formulations that confer protection against UV light. Recent improvements in commercial production of infective juveniles and formulations that have extended

shelf life and enable application using a broad range of equipment will boost their use in biological control (Lacey and Goettel 1995).

21.5 Formulations and Strategies of Microbial Inoculants Application

Formulation is the industrial "art" of converting a promising laboratory-proven microorganism into a commercial field product. It is crucial as it can determine the success or failure of a biological agent. Formulations with long shelf life and ease of use can only be effective. Microbial inoculant formulations are expected to match the above characteristics and overcome two major problems of living organisms: (1) loss of viability during short storage in the grower's warehouse (developing countries usually lack refrigeration), and (2) long shelf life and stability over the range of −5–30°C (Bashan 1998). Furthermore, Jones and Burges (2009) have elaborately explained various formulations of bacteria, viruses, and protozoa and their efficacy as microbial biocontrol agent. The most commonly employed strategies for developing microbial of formulations include:

(1) *Powders*: This is the most common formulation used in making inoculants and is applied as a seed coat before planting. The particle size of carrier varies from 0.075 to 0.25 mm, and the amount of inoculant used is around 200 to 300 g ha^{-1}.
(2) *Slurries:* This is based on powder-type inoculants suspended in liquid (usually water). The suspension is directly applied to the furrow or alternatively the seeds are dipped in the slurry of inoculum just prior to sowing.
(3) *Granulars:* The purpose of these is to apply directly to the furrow along with the seeds. Size of granules ranges from 0.35 to 1.18 mm and used at a rate of 5–30 Kg ha^{-1}. These inoculants are popular and have been successfully commercialized. Bead-like forms are synthetic variations of granular forms. These can be in macro sizes (1–3 mm in diameter) used as granules applied to soil, or in micro size (100–200 μm) used as a powder for seed coating.
(4) *Liquids*: Developed using broth cultures or liquid formulations, predominantly in water, but also in mineral or organic oils. The seeds are either dipped into the inoculant before sowing, or an applicator evenly sprays the liquid inoculant on the seeds. The treated seeds are dried before sowing. Using applicator ensures even coverage of the seeds with the microbial inoculant.

21.5.1 Encapsulated Formulations

The encapsulation of microorganisms into a polymer matrix is being attempted in bacterial-inoculation technology. The concept underlying immobilized microbial

cells is to entrap beneficial microorganisms into a matrix. The formulation (bacteria-matrix) is then fermented in a bacterial growth medium. These formulations can be stored intact for long time. However, these are not cost-effective and application methods do not go with conventional agricultural practices.

Microbial inoculants can be applied during three possible phases: (1) at the seed-processing stage as a seed coating, months before the actual sowing, (2) "on-site", as a seed application just before sowing, or by inoculant delivery directly onto the seeds in the furrow, and (3) after seedlings emergence (Bashan 1998). The most popular method to date with peat- and/or lignite-based inoculants is the "on-site" seed treatment method, primarily because of lower costs. However, seed and soil inoculations (Hegde and Brahmaprakash, 1992) are preferred method of inoculation owing to their ease of application and acceptance by the farmers.

21.6 Conclusion

Microbial inoculants involving diverse organisms are useful in legume production. The choice of the inoculants, however, depends primarily on their functional properties. One microorganism with all the desirable functional properties is the ultimate search of microbiologists/agronomists. Till that time, the cultures of microorganisms, each with one or more functional properties, will continue to be co-inoculated for achieving the highest benefit. Several microbial inoculants useful for sustainable legume production can, however, be combined and inoculated together. A preliminary experimental confirmation that all combined microbial inoculants work synergistically would ensure greater efficacy of microbial inoculants. The farmers can choose the method of inoculation that suits them the most but the only choice for enhanced legume production using sustainable approaches is to use microbial inoculants to overcome the cost and hazards of chemical fertilizers.

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