Chapter 14
Nitrogenase (a Key Enzyme): Structure and Function

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14.1 Introduction

Sustainable agriculture involves designing farm system employing nature as a model. Sustainable agriculture has currently to cope with serious threats that compromise the food security for a human population under continuous growth, all these exacerbated by climate change. Some of these include the loss of usable land through overuse, deforestation, and poor irrigation practices, which have led to desertification and salinization of soils, especially in dry lands. Approaches currently being taken to face this situation come from the development of stress-tolerant crops, e.g., by genetic modification or breeding traits from wild plants. Genetic engineering has been proposed as the solution to these problems through a rapid improvement of crops. Crop genetic modification has generated a great public concern regarding their potential threats to the environmental and public health. As a consequence, legislation of several countries has restricted their use in agriculture. On the other hand, exotic libraries from wild plants for clever plant breeding could overcome the problem of narrowed genetic variability of today's high-yield crops. Plant breeding driven by selection marker has also been a major breakthrough. However, these approaches have met limited success, probably because stress tolerance involves genetically complex processes and the ecological and evolutionary mechanisms responsible for stress tolerance in plants are poorly defined (Choudhary et al. 2011).

The present world population of seven billion is expected to reach ten billion by the middle of the twenty-first century due to the high growth rate, in developing countries. By 2050, there is a need to produce about 70% more food to feed world's

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population (Glick 2014). With more than 20,000 different plant species, legumes are the third largest family of higher plants. Legumes are a pivotal constituent of the ecosystem and sustainable agriculture worldwide and are of immense importance for providing food to the ever-growing world's population. Legumes are also a significant source of food and are grown on a large scale in the arid and semiarid area of the world; India ranks first in the world as legume producer and consumer.

Legumes are belonging to the genera and species of the family *Fabaceae* or *Leguminosae* (with about 700 genera and 18,000 species) in the order Fabales. Legume crops can be divided into four major classes, namely, (i) food legumes (*Vicia faba*, *Glycine max*, *Phaseolus*, *Cajanus*, *Vigna radiata, Cicer arietinum*, etc.), (ii) fodder legumes (*Lablab*, *Centrosema, Stylosanthes*, *Desmodium*, etc.), (iii) green manure legumes (*Mucuna*, *Crotalaria*, *Tephrosia*, *Canavalia*, etc.), and (iv) tree legumes/multipurpose trees (*Leucaena*, *Sesbania*, *Calliandra*, *Pterocarpus*, *Acacia*, *Gliricidia*, *Senna*, etc.), and divided into two groups on the basis of their habitat to grow in different seasons, namely, cool season food legumes and warm or tropical season food legumes. The cool season food legumes include faba bean (*V. faba*), lentil (*Lens culinaris*), lupins (*Lupinus* spp.), dry pea (*Pisum sativum*), chickpea (*C. arietinum*), grass pea (*Lathyrus sativus*), and common vetch (*Vicia sativa*) crops. On the other hand, the warm or tropical season food legumes include pigeon pea (*Cajanus cajan*), cowpea (*Vigna unguiculata*), soybean (*G. max* L.), mung bean (*V. radiata* L.), and urad bean (*Vigna mungo*) crops; these are mainly grown in hot and humid climatic environment. Tropical food legume crops are most popular in different parts of the world, such as soybean, cowpea, mung bean, and urad bean, and are mainly grown in India, the USA, and African countries, especially in different states of India. Legumes provide a sustainable agriculture and the maintenance of the adequate fertility of the soil. These include fixing atmospheric nitrogen (N_2); improving soil structural characteristics; encouraging beneficial microorganisms; deep-rooted perennial legumes reducing the risk of groundwater contamination by nitrate and the development of dry land salinity, due to their ability to grow and extract water all year round; and the reclamation and revegetation of degraded or cleared lands (Chaer et al. 2011). Based on these attributes, legumes are one of the most promising component of the Climate Smart Agriculture concept. Legumes rank third after cereals and oilseeds in world production and have major effects on the environment and animal and human health. Legumes are a primary source of protein and provide around one-third (20–40%) of all dietary protein and produce secondary metabolic compounds that can protect the plant against pathogens and pests (Kragt and Robertson 2014).

Nitrogen is one of the essential nutrients for plants, and it is also a growth limiting factor for agricultural ecosystem. Plants cannot use atmospheric N directly; hence, this form of N needs to change into another form to be available for the plants, such as nitrate (NO_3^-) and ammonium (NH_4^+). BNF through rhizobium legume symbiosis is a well-known mechanism employed by PGPB to fix atmospheric nitrogen. PGPB convert atmospheric nitrogen to ammonia, a form that can be used up by plants (Franche et al. 2009; Bhattacharyya and Jha 2012). These

bacteria contain enzyme complex nitrogenase that fixes atmospheric nitrogen to ammonia. Both endophytes and epiphytic bacteria are capable of increasing the nitrogen content of stressed soil, thus increasing the amount of the macronutrient available for plant uptake and possibly preventing or correcting nitrogen deficiency symptoms in plants under abiotic stresses (Franche et al. 2009). Hence, BNF is considered as an important trait of PGPB as it directly provides nitrogen to the plant for their growth.

Agriculture has a long history of research targeted at understanding how to improve the effectiveness of root symbionts, viz., rhizobia and mycorrhiza. A promising approach has been employed to understand how natural selection regulates changes in mutualistic interactions (Denison et al. 2003). A descriptive knowledge of basic evolutionary processes can be employed to develop agricultural management practices that favor the most effective symbionts. Mutually beneficial interactions between plant and associated rhizospheric microorganisms are ubiquitous which is important for ecosystem functioning. Symbiotic nitrogen fixation by bacteria, e.g., *Rhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium*, and *Azorhizobium* spp., which are collectively known as rhizobia, or by *Frankia* spp. is the major N input to many natural and agricultural ecosystems in the root nodules of legumes or actinorhizal plants, respectively. Among them, several PGPB have been commercialized, namely, *Agrobacterium radiobacter*, *A. lipoferum*, *A. brasilense*, *B. fimus*, *B. pumilus*, *B. subtilis* var. *amyloliquefaciens*, *Burkholderia cepacia*, *P. fluorescens*, *P. macerans*, *P. syringae*, *Serratia*, *Streptomyces lydicus*, various *Rhizobia* spp., etc. (Glick 2012).

In addition, mycorrhizal fungi supply their host plants with mineral nutrients, viz., P, and other benefits. Several rhizospheric microorganisms cause severe infection to roots and so-called root pathogens that can be suppressed by *Pseudomonas fluorescens* after colonization of the roots, thereby improving plant health (Denison et al. 2003). Plant-mediated mineralization for nutrient acquisition in agro-ecosystem would reduce the potential for nutrient losses because of tight coupling between net mineralization of N and P and plant uptake in the rhizosphere. Microorganisms and their products in the rhizosphere react to the many metabolites that are released by plant roots in a variety of positive, negative, and neutral ways. Such interactions can influence plant growth and development, change nutrient dynamics, and alter plant's susceptibility to disease and abiotic stresses. Overall, the general rhizosphere effect could help the plant by maintaining the recycling of nutrients through the production of hormones that help provide resistance to microbial diseases and to aid tolerance to toxic compounds. This benefit can either persist or be lost in well-fertilized agricultural soils where nutrients are readily available to plants and symbionts that reduce growth (Morgan et al. 2005).

Legumes are simultaneously one of the largest families of crop plants occupying nearly all terrestrial biomes (Table 14.1).

The unusual flower structure, podded fruits, and the ability of the 88.0% species to form root nodules with compatible rhizobacteria define the legumes (Graham and Vance 2003). The wide use of legumes as food crops, forages, and green manures is mainly associated with their ability to establish symbiotic associations

Table 14.1 Legumes with their common and scientific name along with their important uses

Common name	Scientific name	Uses
Garden pea	*Pisum sativum*	Pulse
Chick pea (Gram)	*Cicer arietinum*	Pulse
Pigeon Pea (Arhar)	*Cajanus cajan*	Pulse
Lentil (Masur)	*Lens culinaris*	Pulse
Soybean	*Glycine max*	Pulse, oil
Imli	*Tamarindus indica*	Fruit pulp is eaten
Babul	*Acacia nilotica subsp indica*	Good source of gum, fuel, and timber
Khejri	*Prosopis cineraria*	Good source of timber and fuel, pods are used as vegetable, leaves are used as fodder
Black gram (Urad)	*Vigna mungo*	Pulse, dietary protein with high protein content
Green gram (Mung bean)	*Vigna radiate*	Pulse, provide essential amino acids
Moth bean	*Vigna aconitifolia*	Pulse, source of dietary proteins
Jangali moth	*Vigna trilobata*	Seeds are good source of protein
Field bean (Bankla)	*Vicia faba*	Vegetable
Cowpea (Lobia)	*Vigna unguiculata*	Vegetable
Cluster bean (Guar)	*Cyamopsis tetragonoloba*	Vegetable
Jack bean	*Canavalia ensiformis*	Vegetable
Groundnut	*Arachis hypogaea*	Oil
Fenugreek	*Trigonella foenumgreacum*	Seeds are used in various medicines, given to cattle
Guar	*Cyamopsis tetragonoloba*	Plant is used as green manure, pods used as vegetables, grains are given to cattle and used against swellings
Shisham	*Dalbergia sissoo*	Valuable timber
Neel	*Indigofera argentea*	Yield dye
Neel	*Indigofera tinctoria*	Yield dye
Rijaco	*Medicago sativa*	Used as cattle feed

with stem- and root-nodulating nitrogen (N_2)-fixing bacteria, which are collectively referred to as rhizobia. Rhizobia are of particular interest due to their symbiotic association with members of Leguminosae (Saleena et al. 2001), which is the second largest family of flowering plants. Recent information indicates that about

3000 bacterial taxa are capable of nodulating 400 taxa, while information is lacking for more than 40% of the genera (http://www.ildis.org).

Nitrogen being an essential component of proteins, nucleic acids, and other nitrogen compounds is considered as one of the vital component of living system. PGPB have the capacity to fix atmospheric dinitrogen by forming nodule in roots and provide nitrogen to plant in the form of ammonia. The bacteria responsible for nitrogen fixation are called diazotrophs. The members of the alpha and beta subgroup of the phylum proteobacteria are the main rhizobial bacteria associated with legumes (Bomfeti et al. 2011). PGPB can fix nitrogen symbiotically or non-symbiotically. Symbiotic N_2 fixation accounts for nearly 65% of the total biologically fixed nitrogen (Rajwar et al. 2013). Symbiotic N_2 fixation occurs in *Azotobacter* spp., *Bacillus* spp., *Beijerinckia* spp., etc. (Bhattacharyya and Jha 2012), whereas non-symbiotic nitrogen fixation occurs in free-living diazotrophs, *Azospirillum* (Bashan and de-Bashan 2010), *Pseudomonas* (Mirza et al. 2006), and *Burkholderia* (Estrada-De Los Santos et al. 2001).

Rhizobia are known to suppress the population of soil pathogens in agricultural and natural ecosystem, viz., a strain of *Bradyrhizobium japonicum* can cause up to a 75% decrease in sporulation of *Phytophthora megasperma,* 65% in *Pythium ultimum*, 47% in *Fusarium oxysporum*, and 35% in *Ascochyta imperfecta*. From an agricultural point of view, the most significant interactions are those of the Fabaceae–*Rhizobium* spp./*Bradyrhizobium* spp. root nodule symbioses (Squartini 2003). Recent work on root nodule bacteria has demonstrated that this interaction is not restricted to *Rhizobium*/*Bradyrhizobium* but includes N_2-fixing strains of *Ralstonia, Burkholderia*, and *Methylobacterium* that have been recovered from the nodules of several tropical Fabaceae. The plant–bacteria association has been commercially exploited wherein seed and soil inoculants of rhizobia are employed for many crops that include soybean, bean, peanut, and clover (Deaker et al. 2004).

14.2 Nitrogenase: Structure and Function

Diazotrophs contain nitrogenase enzyme complex that is mainly involved in nitrogen fixation. This enzyme system consists of three subunits, and it is regulated by a complex system of multiple genes: Nitrogenase 1 (classic), encoded by *nif* gene that shows iron and molybdenum; Nitrogenase 2, which is encoded by *vnf* gene and exhibits vanadium; and Nitrogenase 3, which is encoded by *anf* gene and has an iron (Fig. 14.1) (Yang et al. 2014). Some novel nitrogenases had been discovered in *Streptomyces thermoautotrophicus* (Zhao et al. 2006) and in *Rhodopseudomonas palustris* (Li et al. 2005). PGPB accelerate nodulation and increase nitrogen fixation activity in soybean (Dashti et al. 1998), in *Phaseolus* (Figueiredo et al. 2008), and many other legumes (Divito and Sadras 2014).

Among biogeochemical cycles, nitrogen fixation has been considered an important process that helps in shaping the fertility of an ecosystem. Codispoti et al. (2001) described a need for suitable restraints on rates of nitrogen fixation that has

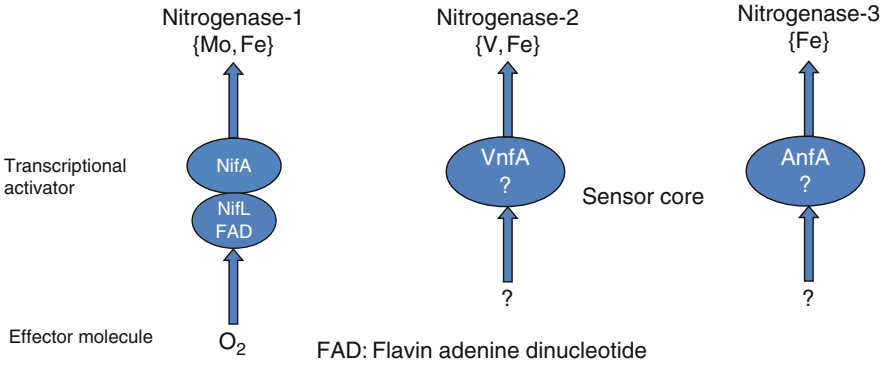

Fig. 14.1 A schematic representation of three-nitrogenase system

urged surveys into new destinations for nitrogen fixation along with novel nitrogen fixers. Based on previous reports, biologically mediated nitrogen fixation is catalyzed by an enzyme nitrogenase that exists in three different isozymes and deploy either Mo, Fe-only, or V at the active site (Robson et al. 1986). Besides having an established Mo-nitrogenase, some of the diazotrophs encode an additional Fe-only nitrogenase, V-nitrogenase, or both. Because of their low occurrence and productivity compared with established nitrogenases, these isoforms of nitrogenases are usually regarded as "backup" enzymes and used only when Mo is not accessible (Eady and Robson 1984).

tructurally nitrogenase is encoded by operons *nifHDK* (Mo-nitrogenases), *anfHDK* (Fe-only nitrogenases), and *vnfHDK* (V-nitrogenase) wherein alternative nitrogenases similarly involve *anf*/*vnfG* (Waugh et al. 1995). Gaby and Buckley (2011) described diversity of established Mo-nitrogenases that has been studied extensively by employing PCR primers targeting *nifH* wherein sequences detected that thought to belong to alternative nitrogenases (*anfH, vnfH*) (Farnelid et al. 2013). Young (2005) reported that *nif*/*anf*/*vnfH* genes inappropriately do not encode the region that harbors the metal center and not considered finally indicative for the type of isozyme. Recently, Tan et al. (2009) surveyed *nif*/*anf*/*vnfH* genes in the environment and concluded that alternative nitrogenase diversity has mainly been untouched. Upon sequencing, genomes of taxonomically diverse diazotrophs genes for alternative nitrogenases have been recognized (Oda et al. 2008; Dos Santos et al. 2012). In addition, microbial strains have been recovered from soils, wood chips, mangrove sediments, termite hindgut, and lichen cyanobionts with expression of alternative nitrogenases Nitrogenase:alternative nitrogenases (Betancourt et al. 2008; Noda et al. 1999; Hodkinson et al. 2014) together with mesocosm soil experiments amended with vanadium (Bellenger et al. 2014). The isotopic

acetylene reduction assay (ISARA) technique has been deployed to characterize alternative nitrogenases in Sippewissett Marsh that discriminates between established and alternative N_2 fixation by measuring ^{13}C isotopes (Zhang et al. 2016).

Based on published report, the distribution of nitrogen fixation genes in bacterial and archaeal genomes is sporadic and intricated by horizontal gene transfers (Boyd and Peters 2013). According to Dos Santos et al. (2012), microbes with alternative nitrogenases also encoded established Mo-nitrogenases and genes for alternate nitrogenase were reported in α-, γ-, and δ/ε-proteobacteria. Interestingly, no alternative nitrogenase sequences have been recovered from the β-proteobacteria. As such, genome sequencing efforts should continue to reveal organisms with alternative nitrogenases and provide a broader understanding of the taxonomic distribution of these enzymes (Noda et al. 1999; Dos Santos et al. 2012; Zhang et al. 2016).

14.3 PCR Amplification of N_2-Fixing bacteria

Genetic diversity of bacteria is being analyzed increasingly by PCR-based genomic fingerprinting methods. As more knowledge is acquired and isolates from unexplored legumes are studied, new species are discovered and former species rectified. Due to improved methods of characterization, the classification of rhizobia has undergone drastic changes and the phylogenetic analysis of the family Rhizobiaceae and related genera has been upgraded (Young et al. 2001). Molecular tools for the identification of bacteria were used and 16S rRNA gene analysis was intensively used to understand the phylogenetic relationships. Bacterial phylogenetic classification is based on sequence analysis of the SSU 16S rRNA molecule or its genes. Given the conservation of 16S rRNA gene, at least 99% similarity seems to be a commonly accepted score for identification (Drancourt et al. 2000). Homology tree based on sequence alignment of 16S rDNA of bacterial isolates permitted rapid phylogenetic analysis. However, strains isolated from different geographic locations shared similar DNA homology. Phylogenetic analysis on the basis of 16S rDNA sequences provided better understanding in evaluation of genetic diversity of rhizobacteria isolated from same and different ecological niche; phylogenetic analysis of 500 bp of terminal region of 16S rDNA from cultivated strain has been found to show existence of large bacterial diversity (Hunter-Cerva 1998). Researchers extensively applied the restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rRNA gene for identification of rhizobia, and thereby, several novel species have been reported during the last decade (Wang et al. 2002). Applying these techniques, *Allorhizobium undicola* and all species of *Agrobacterium* have been reassigned to the genus *Rhizobium* (Young et al. 2001). Pandey et al. (2005) characterized PGPR isolates as *Burkholderia* from root nodules of *Mimosa* species using 16S rDNA gene analysis. Similarly based on the sequencing of the 16S rRNA gene, *Bacillus thuringiensis* KR-1, *Enterobacter asburiae* KR-3, and *Serratia marcescens* KR-4 were characterized as non-rhizobial

PGPR isolates from Nodules of Kudzu (*Pueraria thunbergiana*) (Selvakumar et al. 2008). Partially identified diazotrophic PGPR isolates from traditional Indian rice cultivars through amplification of *nifH* gene and sequencing of 16S rDNA gene. Binde et al. (2009) used rep-PCR fingerprinting and sequencing of 16s rDNA for taxonomic identification of 54 elite commercial rhizobial strains used as rhizobial inoculants in Brazil. Taghavi et al. (2009) used restriction analysis and sequencing of amplified 16S rRNA gene for identification and characterization of the endophytic bacteria exhibiting beneficial effects on the growth and development of Poplar trees (a non-legume plant).

Pandey et al. (2004) applied rep-PCR fingerprinting along with amplified ribosomal DNA restriction analysis (ARDRA) and amplification of *nifH* gene for identification of genetic diversity of rhizobia from medicinal legumes growing in sub-Himalayan region of Uttarakhand (India). Solano et al. (2006) applied PCR-RAPD analysis and 16S rDNA sequencing for screening and identification of isolates to improve the growth of *Cistus ladanifer* seedlings for reforestation of degraded Mediterranean ecosystems. Santillana et al. (2008) characterized diversity of rhizobial isolates exhibiting different RAPD profiles from *V. faba* and *P. sativum* in Peru based on *rrs, atpD, recA* genes and 16S–23S intergenic sequence (IGS) analysis. Phylogenetic analysis based on the 16S rRNA gene sequences showed that the novel strains formed a subclade in the genus *Rhizobium* together with *Rhizobium galegae*, *Rhizobium huautlense*, and *Rhizobium alkalisoli*, with 99.8% gene sequence similarity between the strains. The improvement of molecular biology-based approaches will be fundamental for analyzing microbial diversity and community structure and to predict responses to microbial inoculation/processes in the environment ("ecological engineering").

Molecular tools for the identification of bacteria were used, and 16S rRNA gene analysis was intensively used to understand the phylogenetic relationships. Bacterial phylogenetic classification is based on sequence analysis of the SSU 16S rRNA molecule or its genes. Over 20,000 SSU RNA gene sequences have now been deposited in specialist r-RNA databases such as the rRNA Database Project (RDP). Given the conservation of 16S rRNA gene, at least 99% similarity seems to be a commonly accepted score for identification. Homology tree based on sequence alignment of 16S rDNA of bacterial isolates permitted rapid phylogenetic analysis. However, strains isolated from different geographic locations shared similar DNA homology. Phylogenetic analysis on the basis of 16S rDNA sequences provided better understanding in evaluation of genetic diversity of rhizobacteria isolated from same and different ecological niche; phylogenetic analysis of 500 bp of terminal region of 16S rDNA from cultivated strain has been found to show existence of large bacterial diversity (Hunter-Cerva 1998).

Researchers extensively applied the RFLP analysis of PCR-amplified 16S rRNA gene for identification of rhizobia, and thereby, several novel species have been reported during the last decade. Applying these techniques, *A. undicola* and all species of *Agrobacterium* have been reassigned to the genus *Rhizobium* (Young et al. 2001). Pandey et al. (2005) characterized PGPR isolates as *Burkholderia* from root nodules of *Mimosa* species using 16S rDNA gene

analysis. Selvakumar et al. (2008) identified nitrogen-fixing *Sinorhizobium meliloti* from *Medicago laciniata* on the basis of PCR-RFLP analyses of 16S rDNA and the intergenic spacer (IGS) sequence between 16S and 23S rDNA regions. Similarly based on the sequencing of the 16S rRNA gene, *B. thuringiensis* KR-1, *E. asburiae* KR-3, and *S. marcescens* KR-4 were characterized as non-rhizobial PGPR isolates from Nodules of Kudzu (*P. thunbergiana*). Jha et al. (2009) partially identified diazotrophic PGPR isolates from traditional Indian rice cultivars through amplification of *nifH* gene and sequencing of 16S rDNA gene. Binde et al. (2009) used rep-PCR fingerprinting and sequencing of 16s rDNA for taxonomic identification of 54 elite commercial rhizobial strains used as rhizobial inoculants in Brazil. Recently, Taghavi et al. (2009) used restriction analysis and sequencing of amplified 16S rRNA gene for identification and characterization of the endophytic bacteria exhibiting beneficial effects on growth and development of Poplar trees (a non-legume plant).

14.4 Role of Benign Nitrogen Fixers

Microorganisms represent a substantial portion of the standing biomass in terrestrial ecosystem that contributes to the regulation of C sequestration, N availability and losses, and P dynamics. Microbial biomass P turnover is rapid which is approximately twice as fast as C, suggesting the potential for microbial P pools to support plant P requirements (Kouno et al. 2002). Heterotrophs in soils with larger plant species diversity convert a greater proportion of metabolized C to biomass (Aoyama et al. 2000). The intentional management of the microbial community to enhance N retention in soils makes it possible to characterize abundance and activity of microbial functional groups. Denitrifiers in agricultural soils are more sensitive to O_2 levels that produce a greater proportion of N_2O compared to denitrifiers recovered from an early successional plant community. The rate of denitrification and the proportion of N_2O to N_2 produced affect the denitrifier community composition (Cavigelli and Robertson 2001). There is an increasing interest in understanding the cooperative activities among microbial populations because of current public concerns about the adverse effect of agrochemicals and how do they affect AESs when applied in agricultural soils (Lucy et al. 2004). Two types of interactions in the rhizosphere are recognized mainly wherein one is based on dead plant material (the detritus-based interactions), and other involves living plant roots. Both types of interactions are relevant to agronomy and ecology. Microbial activity in the rhizosphere affects rooting pattern and the supply of available nutrients to plants, thereby modifying the quality and quantity of root exudates. The specific structure and diversity of the rhizosphere bacterial community varies between plant species and over time, and the different root zones present on the same plant can support distinct bacterial communities that reflect on the qualitative and quantitative differences in root exudation (Gryndler 2000).

Some PGPRs can improve nodulation and N_2 fixation in legume plants (Lucas-García et al. 2004). Research on the mechanisms by which PGPR enhance nodule formation implicates their production of plant hormones among the co-inoculation benefits. For example, Chebotar et al. (2001) demonstrated that some *Pseudomonas* strains, but not all, increased nodule number and acetylene reduction in soybean plants inoculated with *B. japonicum*. The possibility that metabolites other than phytohormones, such as siderophores, phytoalexins, and flavonoids, might enhance nodule formation has also been proposed (Lucas-García et al. 2004). Inoculation of phosphate-solubilizing bacteria (PSB) enhanced nodulation and N_2 fixation (^{15}N) by alfalfa plants, in parallel with an increase in the P content of plant tissues. It is therefore thought that an improvement in P nutrition of the plant resulting from the presence of PSB was responsible for increased nodulation and N_2 fixation, as it is well known that these processes are P dependent (Barea et al. 2005). In a recent study, it was demonstrated that PGPR isolated from a Cd-contaminated soil increased the nodulation of clover plants growing in this soil (Vivas et al. 2005). One explanation for this effect may be that the PGPR accumulated Cd and therefore reduced solution Cd concentrations and Cd uptake by plants and rhizobia, thereby preventing Cd toxicity and enabling nodulation. In addition, an increase in soil enzymatic activities (phosphatase, β-glucosidase, dehydrogenase) and of auxin production around PGPR-inoculated roots could also be involved in the PGPR effect on nodulation.

New paradigms for sustainable crop improvement are currently arising. The above approaches do not consider the fact that plants in ecosystems have developed natural symbiotic associations for at least 400 million years (Krings et al. 2007) with a broad diversity of microbial symbionts. It is a well-accepted view that symbiotic legumes benefit companion and subsequent plant species in intercrop and rotation system. Rhizobia (species of *Rhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium, Sinorhizobium*, and *Mesorhizobium*) produce chemical molecules that influence plant development including phytohormones, lipochito-oligosaccharides, Nod factors, lumichrome, riboflavin, and H_2 evolved by nitrogenase. Nod factors stimulate seed emergence, promote plant growth, and increase grain yield when they reside in the soil. From an agricultural point of view, the most significant interactions are those of the Fabaceae–*Rhizobium* spp./*Bradyrhizobium* spp. root nodule symbioses (Squartini 2003). Recent work on root nodule bacteria has demonstrated that this interaction is not restricted to *Rhizobium*/*Bradyrhizobium* but includes N_2-fixing strains of *Ralstonia, Burkholderia*, and *Methylobacterium* that have been recovered from the nodules of several tropical Fabaceae. The plant–bacteria association has been commercially exploited wherein seed and soil inoculants of rhizobia are employed for many crops that include soybean, bean, peanut, and clover (Deaker et al. 2004).

Symbiosis between legumes and rhizobia is of considerable environmental and agricultural importance since it is responsible for most of the atmospheric nitrogen fixed on land (Graham and Vance 2003). Among the 19,000 species described so far, only a small proportion has been studied for their nodulation ability. The legume biodiversity is concentrated in tropical regions, while most studies are on

cultivated leguminous plants from temperate region wherein several symbionts capable of forming nodules and fixing nitrogen in legume roots have been documented and grouped under α and β subclass of Proteobacteria, which include *Methylobacterium nodulans* (Sy et al. 2001), *Blastobacter denitrificans, Devosia* sp. (Rivas et al. 2002), *Ochrobactrum lupini* (Trujillo et al. 2005), *Agrobacterium* like strains (Mhamdi et al. 2005), *Phyllobacterium trifolii* (Valverde et al. 2005), *Herbaspirillum lusitanum* (Valverde et al. 2003), *Ralstonia taiwanensis* (renamed as *Cupriavidus taiwanensis*) (Chen et al. 2001), *Burkholderia tuberum, Burkholderia phymatum* (Vandamme et al. 2002), and *B. cepacia* (Rasolomampianina et al. 2005) and a few γ-proteobacteria (Benhizia et al. 2004). The legume host preferred by these non-rhizobial proteobacteria possesses high diversity (Balachandar et al. 2007).

14.5 Conclusions

The plant-associated habitat is a dynamic environment in which many factors may affect the structure and species composition of the bacterial communities that colonize plant tissues. Some of these factors are seasonal changes, plant tissue, plant species and cultivar, soil type, and interaction with other beneficial or pathogenic microorganisms. An understanding of the structure and species composition of plant-associated bacterial populations is fundamental to understanding how plant-associated biological processes are influenced by environmental factors and, consequently, has important biotechnological implications. Plant growth and development cannot be adequately described without acknowledging microbial interactions.

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