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Soil Fertility Improvement by Symbiotic Rhizobia for Sustainable Agriculture

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Abstract

Soil is living medium and it acts as a precarious reserve in agriculture and food production. To enhance crop yields for ever-increasing human population, chemical fertilizers are being applied in the soil. But, the haphazard usage of fertilizers, predominantly nitrogenous and phosphorus, headed to considerable contamination of soil, air and water. Moreover, unwarranted consumption of these agrochemicals also cause lethal effects on soil microorganisms and disturbs the soil fertility. Due to current public apprehensions about the side effects of these agrochemicals, understanding plant and rhizospheric microbial interactions is gaining momentum. It is considered to be important to effectively manage level of nitrogen in soil through biological nitrogen fixation (BNF) to maintain agricultural sustainability. The fixed N is directly taken up in the plants and is less vulnerable to volatilization, denitrification and leaching. Thus, mutualistic symbiosis amongst legume plant and nodulating rhizobia plays a key role in ecological environments. Legume-rhizobia symbioses provide approximately 45% of N used in agriculture and contributions of BNF from the symbiotic association accounts for at least 70 million metric tons per year into terrestrial ecosystems. In agricultural systems, about 80% of BNF contributed by symbiotic association made between leguminous plants and species of *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium* and *Allorhizobium*. The populations of these root-nodule forming bacteria can be changed ecologically, agronomically, edaphically and genetically to increase legume production and soil productivity. Moreover, legume-rhizobia symbioses also provide non-polluting and economical ways to augment N_2-fixing potential under stress conditions. Scientists have identified numerous symbiotic systems

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tolerant in harsh situations of salinity, alkalinity, acidity, drought, toxic metals have been recognized and alteration in rhizobial population under stressed environments can be an indicator of soil fertility. Moreover, interactions among rhizobia, plant growth-promoting rhizobacteria (PGPR) and mycorrhiza as well show significant part in increasing soil fertility and crop yields. In this chapter, significance of biological nitrogen fixation in persistent food supply, influence of extreme environments on legume-rhizobia symbiosis as well as interaction of rhizobia with belowground microbial species are discussed. The eco-friendly approach to increase crop production and soil health by inoculation of symbiotic bacteria as biofertilizers is described for sustainable agriculture.

Keywords
Soil · Microbial population · Biological nitrogen fixation · Rhizobia · PGPR

7.1 Introduction

Legumes can be considered as key source of proteins in vegetarian diet in developing countries (Nedumaran et al. 2015). Therefore there is a need to improve the yield of legumes and to sustain soil fertility. Legumes utilized for human feed comprise of dry and green beans, broad beans, dry and green peas, chickpeas, lentils, soybeans, lupins, mung beans and peanuts. Nitrogen (N) and phosphorus (P) are major regulating nutrients for growth of leguminous plants. Replenishment of these nutrients to the legume crops is mostly done through application of inorganic nitrogenous and phosphate fertilizers to soil. Addition of nitrogenous fertilizers is the major external input for maximizing crop yield in agriculture. Inadequate usage of these chemical fertilizers has contaminated environment and causes various health hazards. Moreover, due to the low use efficiency of nitrogen fertilizers among plant nutrients and their continuous use leads to slow deterioration in soil health (Newbould 1989; Bockman 1997) and a decline in crop yield (Bohlool et al. 1992). Additional drawbacks of N-fertilizers include speeding up the depletion of non-renewable energy resources. Along with high usage of N fertilizers in developed countries, volatilization of N oxides (greenhouse gases) into environment and leaching of NO_3^- into ground water, is also a major threat for global N cycle.

Due to exponential growth of population, its demand of the day to implement new means of improving food production that are well-suited with sustainability and preservation of environmental quality (Sindhu and Dadarwal 1995b; Sharma et al. 2018a, b). Moreover, rates of nitrogenous and phosphatic fertilizers is continuously increasing in developing countries and these fertilizers are not only unaffordable or unavailable in many countries but also have other drawbacks. Therefore, it is actually critical task for farmers to add-on N and P fertilizers in soil to escape the nutrient insufficiencies. Viable agriculture consist of effective management of agricultural assets to fulfill shifting human requirements, while preserving or increasing environmental superiority and safeguarding natural assets. Thus sustainability deliberations requires substitutes to nitrogen fertilizer. Biological nitrogen fixation can be considered as substitute in farming practices as it uses capability of several nitrogen-fixing bacteria to transform atmospheric nitrogen into the plant usable, ammonia using the nitrogenase enzyme (Bohlool et al. 1992).

Legumes are grown approximately on 252 million hectares of land, leading to about 90 Tg of dinitrogen being fixed per year, with major contributors to overall N_2 fixation through legume–*Rhizobium* symbiosis (Smith and Giller 1992). The growth of grain legumes such as field pea (*Pisum sativum* L.), followed by the subsequent decomposition of N rich residues helps to replenish N removed by harvesting. This leads to savings of fertilizer N and brings about enrichment of soil N, which is available to subsequent crops (Jensen and Hauggaard-Nielsen 2003). By using nitrogen-fixing species of microorganisms in cropping systems dependency of agricultural crops on chemical nitrogenous fertilizers can be reduced. Moreover, biologically fixed nitrogen resides within soil organic matter in bounded form and hence it is considerably less vulnerable to chemical alterations as well as physical losses like volatilization and leaching. Considering adverse environmental effects of chemical fertilizers and growing prices, use of plant growth promoting rhizobacteria (PGPR) and rhizobia is valuable for sustainable agricultural system (Fernández et al. 2007; Shiri-Janagard et al. 2012; Uribe et al. 2012; Sindhu et al. 2018). A lot of information exists on the positive influence of *Rhizobium* and *Bradyrhizobium* on legumes in terms of biological nitrogen fixation (Werner 2005) and in cereal–legumes crop rotation systems. Moreover, coinoculation of symbiotic bacteria with PGPR is another approach which has been found to improve root and shoot weight, plant vigour, nitrogen fixation and grain production in legumes (Valverde et al. 2006; Yadegari et al. 2008; Verma et al. 2013; Sindhu et al. 2017).

This chapter describes diversity detected among different symbiotic bacteria and contribution of different rhizobia in increasing the growth and yield of legume crops as well as various biotechnological approaches undertaken for improving biological nitrogen fixation. The various limitations faced to improve crop productivity by inoculation with bacterial strains and opportunities of getting anticipated profits by confirming the establishment and survival of inoculated microbes in soil has also been discovered.

7.2 Role of Nitrogen Fixation by Bacteria in Cereal and Legume Crops

Majority of naturally augmented nitrogen in soils is from symbiotic or asymbiotic biological fixation carried out by microorganisms. As per an estimate annually roughly 100 Tg N, is needed for production of world's grain and oilseed crops (David and Ian 2000). Legume crops possess remarkable potential for biological nitrogen fixation in soil ecosystems (Brockwell et al. 1995). There exist roughly 700 genera and around 13,000 species of legumes and from such a large variety of legumes only a small part was studied for nodulation and nitrogen fixation efficiency (Sprent and Sprent 1990). Assessments showed that symbiotic association of

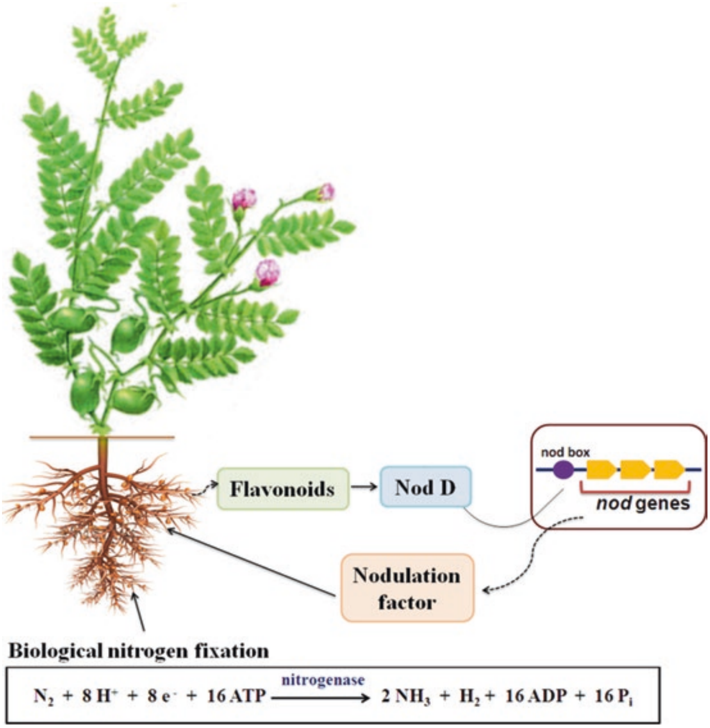

Fig. 7.1 **Nodulation and nitrogen fixation is illustrated in chickpea plant**. The coordinated and controlled expression of nodulation genes of rhizobia in response to plant-released flavonoids synthesize the nodulation factor that leads to nodule organogenesis. The differentiated bacteroids in the nodules utilize nitrogenase enzyme to convert atmospheric nitrogen into ammonia.

Rhizobium with approximately 100 agriculturally significant legumes, add roughly half the annual amount of BNF inflowing soil ecosystems (Tate 1995). Legume symbioses add approximately 70 million metric tonnes of N per yea, from which half is derived from cool and warm temperature zones and rest is resulting from tropics (Brockwell et al. 1995; Freiberg et al. 1997).

The success and sustainability of many food crops, forage and green manure legumes is mainly obligated to their symbiotic association with particular nitrogen-fixing rhizobia (Menna et al. 2006). A peculiar characteristic that distinguishes rhizobia from other nitrogen-fixing bacteria is their unique ability to elicit the development of a specialized nodule to form a symbiotic association with their legume host (Lindstrom et al. 2006) (Fig. 7.1). This association converts atmospheric inert N_2 to a renewable source of fixed N for agriculture with expected values falling in range of 57–600 kg of N ha^{-1} $year^{-1}$ (Zahran 1999; Ramankutty et al. 2018). In contrast to application of inorganic N-fertilizers. N input through the process of BNF not only maintains the soil's N reserves but can also conserve natural resources. In that way, BNF plays significant role in nourishing throughput of soils.

Table 7.1 Average biological nitrogen fixation by various plant microbe associations

Nitrogen-fixing system	Microorganisms/plants	Rate of nitrogen fixation (kg ha^{-1} $year^{-1}$)
Free-living microorganisms	Cyanobacteria (blue-green algae)	7–80
	Azotobacter	0.3–15
	Clostridium pasteurianum	0.1–0.5
Grass-bacteria associative symbioses	*Azospirillum*	15–36
	Acetobacter diazotrophicus	150–200
Plant-cyanobacterial associations	*Gunnera*	12–21
	Azolla-Anabaena	45–450
	Lichens	39–84
Rhizobium–legume symbioses	Soybeans (*Glycine max* L. Merr.)	57–94
	Cowpea (*Vigna*, *Phaseolus* and others)	84
	Clover (*Trifolium pratense* L.)	104–160
	Alfalfa (*Medicago sativa* L.)	128–600
	Lupines (*Lupinus* sp.)	150–169
Nodulated non-legumes	*Alnus* (alders, e.g. red and black alders)	40–300
	Hippophae (sea buckthorn)	2–179
	Coriaria ('tutu' in New Zealand)	60–150
	Casuarina (Australian pine)	58

Adapted and modified from Stevenson (1982)

Some bacteria and cyanobacteria have developed capacity to convert atmospheric nitrogen in to ammonia using nitrogenase enzyme and supply this important nutrient into agricultural soils. BNF take place in the free-living state, in association with or in symbiosis with plants (Table 7.1). Inoculation of various strains of diazotrophic bacteria carried out to increase amount of nitrogen as nutrients to several leguminous and non-leguminous crops. Vast areas of aerable land in Australia, India, Russia and United Kingdom inoculated with non-symbiotic N_2-fixing bacteria such as *Azotobacter*, *Azospirillum*, *Bacillus* and *Klebsiella* spp. with the goal of improving plant yield (Lynch 1983; Sloger et al. 1992; Di Benedetto et al. 2017). In symbiotic system, *Rhizobium* species have been effectively utilized globally as a bioinoculant leading to effective establishment of N_2-fixing symbiosis with leguminous crops (Eaglesham 1989; Thies et al. 1991; Dahale et al. 2016). Other N_2-fixing symbionts, such as *Frankia* spp. have also been successfully introduced into soil (Sougoufara et al. 1989; Clawson et al. 1998).

Another approach to improve nitrogen budget of crops is to inoculate symbiotic bacteria with PGPR in leguminous crops to improve root and shoot weight, plant vigour, nitrogen fixation and grain yield in several legumes (Valverde et al. 2006; Yadegari et al. 2008; Verma et al. 2013; Sindhu et al. 2017). By modulating balance of deleterious vis a vis beneficial microbial activities in rhizosphere, PGPR are known to encourage plant growth directly by producing phytohormones, by improving nutrient accessibility and acquisition or eliciting plant defense mechanisms, which in turn, leads to increased nutrient acquisition and growth (Sindhu et al. 2014, 2016) or induce systemic resistance against harmful microorganisms (Liu et al. 1995a, b). Therefore, synergistic consortia of microbes having various metabolic abilities (N_2 fixation, P mobilization, synthesis of plant growth hormones and bioactive molecules) can definitely perform better than single inoculations. However, type of inoculums, method of inoculation and agricultural practices can influence the effect of the inoculation. The effect of multiple inoculants with symbiotic N_2 fixing rhizobia, asymbiotic free-living N_2 fixing bacteria and phosphate solubilising bacteria or cyanobacteria found to stimulate plant biomass in different legumes.

Symbiotic association between leguminous plants and *Rhizobium* is the best comprehensively studied nitrogen-fixing system. This symbiotic association fixes around 70–80% of the total BNF per year (Ishizuka 1992). Nitrogen fixation capacity of symbiotic rhizobia range from 57 to 600 kg N ha^{-1} yearly (Elkan 1992). Among legumes, soybean is leading crop legume, comprising of 50% of the global crop legume area and soybean was reported to fix 16.4 million tones N annually, representing 77% of the nitrogen fixed by the crop legumes (Herridge et al. 2008). Increase in legume production are usually equal to those estimated from inoculation of 30–80 kg of fertilizer-N ha^{-1}. Inputs of fixed N for alfalfa, red clover, pea, soybean, cowpea and vetch are expected to be nearly 23–335 kg of N ha^{-1} $year^{-1}$ (Tate 1995; Wani et al. 1995). Thus, efficiency of various legume species and their microsymbionts has been found variable (Table 7.1). In general, faba bean (*Vicia faba*) and pigeon pea (*Cajanus cajan*) have been found to be very efficient; soybean (*Glycine max*), ground nut (*Arachis hypogaea*) and cowpea (*Vigna unguiculata*) to be average; and common bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) less efficient for nitrogen fixation (Hardarson 1993; Pinto et al. 2007). The *Azolla-Anabaena* symbiotic system proved to add 45–450 kg N ha^{-1} and *Frankia*-actinorhizal symbiosis deliver 2–362 kg N ha^{-1} (Elkan 1992).

Sindhu and Dadarwal (1992) carried out experiment to evaluate comparative efficiency of nitrogen fixed by *Rhizobium* strains in chickpea using non-nodulating genotype PM233 obtained from wild type nodulating genotype ICC640. Due to nitrogen fixation by *Rhizobium* strains Ca534 and Ca219 in nodulating genotype ICC640, significant increase in plant dry weight was obtained over application of 80 kg N ha^{-1} through urea in non-nodulating mutant PM233. The results reveal the fact that effective symbiosis between rhizobia and chickpea can supplement more than 80 kg N ha^{-1}. Profits of nitrogen fixation in legume crops to succeeding cereal crops are considerable and carry on for several years as a result of gradually slow mineralization. In green manuring crops greater amount of benefits of rhizobia and plant symbiosis were observed and about 532 kg N could be assimilated by 60 days with nitrogen N accumulation rate of 10.8 kg N ha^{-1} day^{-1} (Peoples and Herridge 1990).

Fixed nitrogen is also made accessible to an intercrop or succeeding crop. Generally more than 50% of the crops grown in Africa, India and Latin America are

Table 7.2 Growth promoting substances synthesized by rhizobia involved in stimulating plant growth

Rhizobia	Growth promoting substances synthesized	References
Rhizobium and *Bradyrhizobium*	Siderophores, P-solubilization, IAA, HCN	Abd-Alla (1994a, b), Antoun et al. (1998), Duhan et al. (1998), Khan et al. (2002), Deshwal et al. (2003a) and Tank and Saraf (2010)
Rhizobium sp.	Growth hormones, IAA, siderophores, HCN, ammonia, exopolysaccharides	Ahemad and Khan (2009a, 2012a), Joseph et al. (2007), Wani et al. (2007b) and Zafar-ul-Hye et al. (2013)
R. phaseoli	IAA	Arora et al. (2001)
R. ciceri	Siderophores	Berraho et al. (1997)
R. leguminosarum	Cytokinin	Zahir et al. (2010)
M. ciceri	IAA, siderophores	Wani et al. (2007c)
Mesorhizobium sp.	IAA, siderophores, HCN, ammonia, exopolysaccharides, antifungal activity	Ahemad and Khan (2009b, 2012c), Ahmad et al. (2008), Khan et al. (2002) and Wani et al. (2008a)
B. japonicum	IAA, siderophores	Wittenberg et al. (1996) and Shaharoona et al. (2006)
Bradyrhizobium sp.	IAA, HCN, ammonia, siderophores, exopolysaccharides	Khan et al. (2002), Wani et al. (2007a) and Ahemad and Khan (2011, 2012b)
R. meliloti	Siderophores	Prabha et al. (2013)

either intercropped or rotated with nitrogen-fixing crops (Fujiata et al. 1992). Hence biological nitrogen fixation assists as an efficient way to reduce reliance on chemical fertilizers by supplying nitrogen to symbiont as well as builds up soil nitrogen for subsequent crops. However, numerous soil environmental causes viz. temperature, moisture, acidity, available nitrogen, phosphorus, calcium and molybdenum content affect nitrogen fixation (Somasegaran and Bohlool 1990; Zhang et al. 1996). Application of efficient strain of rhizobia on legumes generally resulted in substantial rise in production of several legume crops (Eaglesham 1989; Thies et al. 1991) (Table 7.2). Although, numerous reports also showed unpredictability in attaining the yield increases ensuing application of rhizobial strains (Miller and May 1991).

7.3 Rhizobial Diversity

Phylogenetically rhizobia are very different, demonstrating numerous lineages. Rhizobia presently comprise of 12 genera and beyond 113 species of α- and β-proteobacteria (Sawada et al. 2003). Rhizobia are distributed in the following genera: *Aminobacter* (1), *Azorhizobium* (3), *Bradyrhizobium* (15), *Devosia* (1), *Mesorhizobium* (29), *Methylobacterium* (1), *Microvirga* (3), *Ochrobactrum* (2), *Phylobacterium* (1), *Rhizobium* (43), *Sinorhizobium/Ensifer* (13) and *Shinella* (1). Additionally, there are 9 species of β-rhizobia, namely *Burkholderia* (6), *Cupriavidus* (2) and *Herbaspirillum* (1). Many new species of rhizobia are described each year and even strains from non-typical rhizobia genera are included to list of rhizobia, as strains from the *Burkholderia* genus (Chen et al. 2003, 2008). In general, rhizobia are heterotrophic and aerobic non-sporulated rods, however, there are *Bradyrhizobium* strains having ability of anaerobic growth (Polcyn and Luciński 2003), photosynthetic bradyrhizobia (So et al. 1994) and methylotrophic *Methylobacterium* strains (Sy et al. 2001). The complete genomic sequence of photosynthetic bradyrhizobia able to induce both root and stem nodules revealed that these strains lack the canonical *nod*ABC genes required for Nod factor synthesis (Giraud et al. 2007).

Crook (2013) isolated *Rhizobium* sp. IRBG74 and *A. caulinodans* from *Sesbania aculeata* and *Sesbania rostrata* and capable of colonizing rice roots. Endophytic strain of *Rhizobium* sp. IRBG74 was also isolated from *Sesbania cannabina*, but it lacks *nif*V gene required for nitrogen fixation and hence unable to fix nitrogen. *Rhizobium* sp. IRBG74 initially grouped as Agrobacterium but as it do not possess Ti plasmid it was re-categorized as *Rhizobium*. This bacterium contain sym-plasmid having *nif*H together with *nod*A genes and it colonizes a wide range of *Sesbania* plants. Similarly, *A. caulinodans* ORS571 is capable of nitrogen fixing endophytic colonization (Chen and Zhu 2013; Venkateshwaran et al. 2013).

Plant genotype was also found to have effect on existence and dissemination of rhizobial species in soil. For example, *Phaseolus vulgaris* and *Mimosa affinis* show difference in rhizobial nodulation specificity. *P. vulgaris* is can be nodulated by six rhizobial species, viz. *R. etli*, *R. giardinii*, *R. gallicum*, *R. tropici*, *R. leguminosarum* bv. *phaseoli* and *Bradyrhizobium* spp., whereas *Mimosa affinis* showed nodulation specificity for *R. etli* alone (Wang et al. 1999). Genistoid legumes (brooms) in Canary Islands, Morocco and Spain are nodulated by four distinct rhizobial strains viz. *B. japonicum*, *B. canariense* and two unidentified species (Vineusa et al. 2005).

Abiotic factors like pH, rainfall, soil type and temperature also influence diversity of rhizobial species, whereas soil types may influence composition of rhizobial community which is ascertained from the fact that legumes grown in different geographical locations nodulated by different rhizobial species/genera. For example, *Glycine max* (soybean) generally nodulated by *B. japonicum*; but surprisingly soybean grown in Xinjiang region of China showed colonization of root by *Mesorhizobium tianshanense* and *Sinorhizobium fredii*. Sameway, *R. leguminosarum* bv. *viciae* and bv. *trifolii* geberallly nodulates beans in Leon, France, but beans grown in Andalucia region showed presence of *R. etli*, *R. gallicum* and *S. fredii* in addition to *R. leguminosarum* bv. *viciae* and bv. *trifolii* (Velázquez et al. 2001). Conventionally, *Mesorhizobium ciceri* and *Mesorhizobium mediterranean* isolated form nodules of *Cicer arietinum*, but *Cicer arietinum* grown under water deficient conditions in Tunisia showed colonization by *Ensifer meliloti* (formerly *Sinorhizobium meliloti*) (Romdhane et al. 2009). Similarly, *E. meliloti* also been isolated from *C. arietinum* plants growing in Almora and Terai region of Uttarakhand Himalayas (Rajwar et al. 2013). Type of soil also restricts distribution and diversity of rhizobia which was clearly confirmed by characterization of different rhizobial species from Caragana plant growing in three eco-regions of China differing in soil types. *Mesorhizobium* genospecies I, II, IV, VI and VII were identified from Caragana plants growing in sandy soils of Mongolia. *M. temperatum*, *M.*

tianshanense, *M. septentrionale*, *M.* genospecies III, *R. yanglingense* and *Rhizobium* sp. IV were isolated form Caragana plants grown in saline/alkaline soils and *M. plurifarium*, *M.* genospecies V and VII, and *Rhizobium* sp. IV in fertile/forest soils of Northwestern Yunnan region (Lu et al. 2009).

Delamuta et al. (2017) evaluated phylogenetic relationship between 45 *Bradyrhizobium* strains isolated from different legumes i.e., *Arachis hypogaea*, *Acacia auriculiformis*, *Glycine max*, *Lespedeza striata*, *Lupinus albus*, *Stylosanthes* sp. and *Vigna unguiculata*, based on *nodY/K* and *nifH* genes of and compared their 16S rRNA gene phylogeny and genetic diversity by rep-PCR. 16S rRNA tree revealed that strains were dispersed into two clusters – *B. japonicum* and *B. elkanii* – with numerous strains being alike within each clade. The rep-PCR examination also discovered high intra-species diversity. Grouping of strains in the *nodY/K* and *nifH* trees was undistinguishable. Thirty nine strains obtained from soybean grouped with *Bradyrhizobium* type species and five others in distinct positions. Only one strain isolated from *Stylosanthes* sp. displayed similar *nodY/K* and *nifH* sequences to soybean strains and it also nodulated soybean. *nodC* sequences comparison showed same clusters as observed in the *nodY/K* and *nifH* phylograms. The analysis of symbiotic genes showed that a large group of strains from the *B. elkanii* superclade contained new symbiovar sojae, whereas for alternative group, comprising *B. pachyrhizi*, the symbiovar *pachyrhizi* could be projected.

7.4 Nodulation of Legume Roots

Mutualistic, nitrogen-fixing relations amongst *Fabaceae* family plants and soil bacteria *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium* (as a group designated rhizobia) contribute considerably to crop yield. This symbiosis between legume plants and rhizobia also offers an interesting model to study the intricacy of various mechanisms that control plant cell partition and nodulation. In the absence of the host, free-living rhizobia are in their saprophytic phase and compete with other soil microflora for limited nutrient resources. The population densities of rhizobia are usually low when legumes are not a large component of the plant community (Woomer et al. 1988; Kucey and Hynes 1989), demonstrating that symbiotic form is crucial for formation of a considerable saprophytic inhabitants of rhizobia in the soil. Natural rhizobial population as well as inoculated rhizobia was found to be different in their tolerance to key environmental clues and thereby influence persistence and existence of distinct species in soil (Vidor and Miller 1980; Defez et al. 2017).

Nodulation (*nod*) genes of rhizobia required for infection and nodulation are classified as universal, host-specific and regulatory *nod* genes (Fig. 7.2). Nodulation genes are principally classified into three classes: (a) regulatory *nod*D and *nod*VW genes enabling activation of and host specific *nod* gene transcription, (b) the common *nod*ABC, *nod*M and *nod*IJ genes, which are functionally and physically conserved amongst different rhizobia, and (c) host specific *nod* genes, which are variable with bacterial species and strains. Alteration of host specific *nod* genes

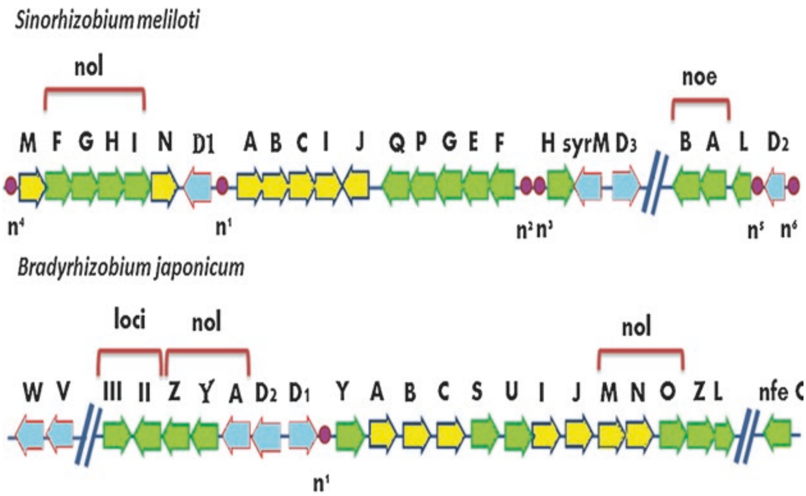

Fig. 7.2 **Nodulation genes (*nod*, *nol*, *noe*) of *Sinorhizobium meliloti* and *Bradyrhizobium japonicum*** are represented. Universal nodulation genes are depicted in yellow colour and regulatory nodulation genes are shown in light blue colour, whereas host-specific nodulation genes are shown in light green colour. Regulatory *nod*D product interact with specific flavonoids, then binds with *nod* boxes (n′) and cause activation of transcriptional operon of other nodulation genes

generally do not counterpart with cloned genes from other rhizobia. The structural arrangement and regulation of nodulation genes of *Rhizobium, Bradyrhizobium* and *Azorhizobium* has been reviewed recently (Long 1996; Spaink 1996; Hanin et al. 1999; Appelbaum 2018). Expression of structural *nod* genes was governed by flavonoid signals from plants transcription of *nod*D regulatory gene is regulated by specificity of flavanoids and hence believed to be partial determining factor of strain/host specificity. The common *nod* genes are involved in manufacturing of basic lipochitin-oligosaccharide molecule and host specific nodulation genes add various substituents at reducing or non-reducing ends of Nod factors (Perret et al. 2000; Sindhu and Dadarwal 2001a, b, c). Alteration of host specific *nod* genes may end in either a postponement in nodulation or a variation in host range (Denarie et al. 1992). Expression of structural *nod* genes results in synthesis of specific extracellular lipo-oligosaccharide compounds termed as nodulation factors (NF) which stimulate root-hair deformation, cortical cell division and other responses in prone legume root.

Legume roots release flavonoids in the root exudates and rhizobia which colonize soil in neighborhood of root hair are attracted through chemotaxis in response to the flavonoids. The flavonoids and isoflavonoids secreted by roots of legumes bind with the regulatory protein NodD, which subsequently bind to conserved *nod*-box in the promoters of bacterial nodulation genes to encourage their expression. The *nod* genes code for enzymes for synthesis of Nod factors. Strain-specific combinations of nodulation genes (nod, nol or noe) code for addition of several decorations to core structure (Sindhu et al. 1999a, b). Examples of NF substituents are

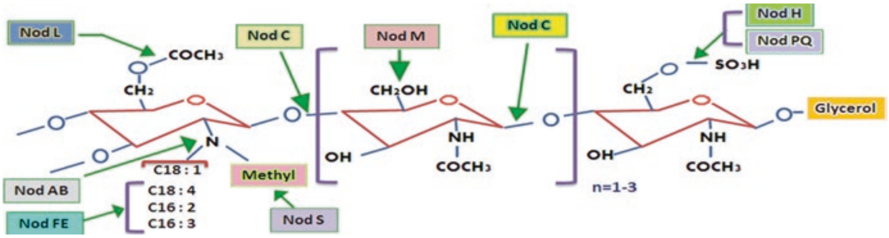

Fig. 7.3 Nodulation factor synthesized by *Rhizobium* strains. Host specific nodulation genes (*nod*, *nol* or *noe*) results in several decorations or modifications on the basic core structure

hydrogen (R1–R6), carbamoyl (R1–R3), acetyl (R1–R4), sulfate (R4), fucose (R4) and arabinose (R6) (Fig. 7.3). The perception of NFs by the plant in turn triggers several early symbiotic reactions in the plant root, for example ion fluxes, calcium spiking, root hair deformation, cortical cell division and synthesis of an infection thread that directs the bacteria to the emerging primordium. Several elements of signaling pathway leading to nodulation have been characterized: the putative NF receptors (which belong to the lysine motif receptor-like kinase family, LysM–RLK), a cation channel, a leucine-rich repeat receptor kinase (LRR–RK), a calcium/calmodulin-dependent protein kinase (CCaMK), a cytokinin receptor and various transcriptional factors. Transmembrane Nod factor receptors recognize Nod factor in a strain- and ecotype-specific manner. Alteration of Nod factor such as the length and saturation of the acyl group determine host specificity. Nod factor receptor activation stimulates root hair deformation enabling them to lodge small number of bacteria which further grow in to a colony within nodule. Modulation of host range was also done through surface polysaccharides such as EPS from *S. meliloti*. Recognition of polysaccharides by R genes present in some ecotypes or varieties of plants restricts host range which culminate in the transcriptional activation of other *nod* genes (Downie 1994; Russelle et al. 2008).

Rhizobia encourage formation of nodules on legumes by either a NF-dependent or a NF-independent process. Gully et al. (2017) reported whole genome sequence of *Bradyrhizobium* sp. strain ORS285, capable of nodulating *Aeschynomene* legumes using two different approaches that vary in requisite of Nod factors. In NF strategy, plant signals of the flavonoid family are received by bacterial NodD regulatory proteins that encourage synthesis of lipochitooligosaccharidic NFs that activate nodule organogenesis (Oldroyd and Downie 2008). Some steps of this process are subject to variation: (i) alternative plant compounds (e.g. betaines, jasmonate, xanthones, vanillin, etc.) can start *nod* gene expression but these compounds usually act at higher concentrations than (iso) flavonoids (Cooper 2007); (ii) beside NodD, additional regulators can modulate expression of *nod* genes like NolR (in some *Rhizobium* and *Sinorhizobium* species) or NolA and the two component system NodV/NodW (in *Bradyrhizobium japonicum*); and (iii) synthesis of the Nod factor support is regulated by canonical *nod*ABC genes existing in all rhizobia.

The rhizobia adhere to root hairs all over the root but root hairs that are most responsive to *Rhizobium* infection are just behind the apical meristem at the site of emergence of root hairs. In the infectible root zone, rhizobia adhere to surface of root hair either through an acidic extracellular polysaccharide or via definite calcium-dependent protein, rhicadhesin, cellulose fibrils (Mateos et al. 1995; Smit et al. 1987) and legume root lectin (Kijne et al. 1988). Lipooligosaccharides (Nod factors) produced by the infecting rhizobia cause characteristic curling and deformations of root hair and cortical cell divisions in well-suited host (Lerouge et al. 1990; Broughten et al. 2000). The deformed root hairs in various legumes may form different structures, including corkscrews, branches, twists, spirals and shepherd's crooks. Cao et al. (2017) described that a stable regulation of innate immunity is probably essential during process of nodulation starting from rhizobial infection, symbiotic establishment and maintenance. Following initial infection processes, plant immune responses can also be stimulated in nodules and expected to result in nodule senescence. Mutualism believed to be derived from a pathogenic relationship that reduced over time to a condition in which both partners can benefit. Generally rhizobia overcome host immune response by actively suppressing it to permit infection and symbiosis establishment. Whereas plants developed mechanisms to limit nutrient supply to symbiont and thereby checking number of nodules on plant so that protecting themselves form overburden.

In *Medicago sativa*, nodule development is closely linked to Nod factor (NF) synthesis by *S. meliloti* (Lerouge et al. 1990). *S. meliloti* starts two analogous, nodule-specific, procedures to develop unspecified nodules nearby root proto-xylem ends: (i) rhizobial colonization pathway, that includes infection thread development in root hairs and cortical cells, and (ii) nodule organogenesis pathway, that includes stimulation of cell divisions in root cortical, endodermal and pericycle cell layers to generate a nodule primordium and then, a nodule meristem (Timmers et al. 1999; Xiao et al. 2014; Djordjevic et al. 2015). Rhizobial NFs hurriedly trigger nuclear calcium oscillations in root hair cells (Levy et al. 2004; Miwa et al. 2006), which transcriptionally activates central symbiotic (SYM) genes e.g. nodule commencement (MtNIN), nodulation signaling pathway 1 and 2 (MtNSP1 and 2) (Kaló et al. 2005; Smit et al. 2005) and MtCLV3/ESR-related 12 and 13 (MtCLE12 and 13) (Mortier et al. 2010; Saur et al. 2011). Nodulation is also positively and negatively controlled by complex communications with numerous hormones and peptides (Mortier et al. 2010, 2012; Larrainzar et al. 2015; van Zeijl et al. 2015). Together, these signals, along with NF/SYM pathway, control nodulation process and frequency on root system (Oldroyd 2013). A large number of infection threads will not result in to nodule formation (Djordjevic et al. 1986) which shows effect of negative regulatory routes facilitated by ethylene-related and CLE-related pathways (Kassaw et al. 2015).

The rhizobia occupy root hair cell by means of host-derived infection thread, which is usually initiated from the most acutely curled region, starting as invagination of root hair cell membrane (Fig. 7.4). Rhizobia move down in root hair to cortical cell layers by interiorly budding tube-like infection thread. Rhizobia in infection thread are surrounded by mucigel composed of cell wall polysaccharides, plant-derived matrix glycoprotein and rhizobial exopolysaccharides (Callaham and Torrey 1981; Broughten et al. 2000). Growth of infection thread continues towards newly

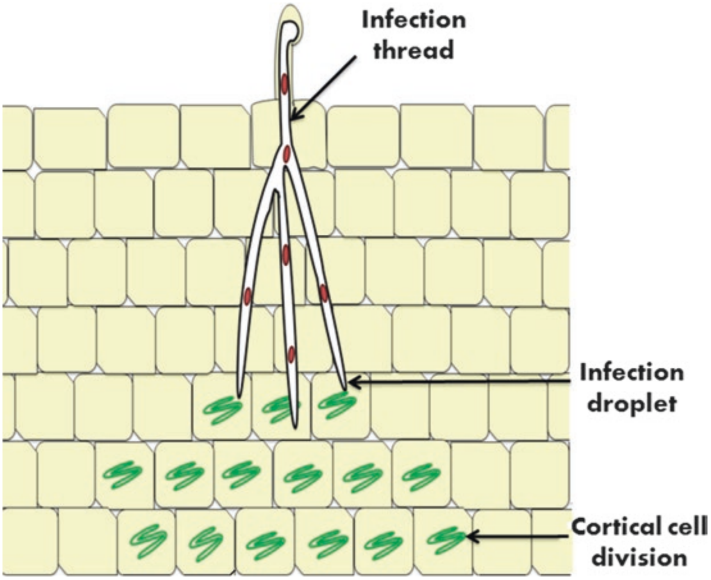

Fig. 7.4 Nodulation factor secreted by rhizobia cause root hair deformation. Rhizobia occupy root hair cell by means of a host-derived infection thread

synthesized nodule primordium which is produced by stimulation of mitotic activity in root cortex as a result of rhizobial Nod factors that afterward develops into the nodule meristem (Dudley et al. 1987). Infection thread branches and leads towards cortex and a clearly apparent nodule grow on root. Nodules may have one or more rhizobial strains and can be either determinant (lack a persistent meristem and are spherical) or indeterminate (situated at distal end of cylindrically shaped lobes) (Russelle et al. 2008). Many infections are terminated due to a failure in communication between rhizobia and the host plant leading to strict regulation of nodule number by the plant. In the root cortex, infection threads branch and enter in to individual nodule cells and a new structure, infection droplet, is formed and rhizobia get released into nodule tissue cells by a process that is similar to endocytosis (Roth and Stacey 1989a) and then occupy an organelle-like cytoplasmic compartment, designated as “symbiosome”, which is surrounded by a plant-derived peribacteroid membrane (Roth and Stacey 1989b). This process keeps microbes “outside” the plant where rhizobia are intracellular but extracytoplasmic. Peribacteroid membrane-enclosed bacteria divide until cytoplasm of every infected plant cell comprises several thousand rhizobial cells. In late symbiotic zone, infected cells are entirely occupied with bacteria that have differentiated into their pleomorphic endosymbiotic bacteroids (Brewin 1991) especially express nitrogen fixation genes. The plant uses the fixed nitrogen as nitrogen source and delivers bacteroids with photosynthates and amino acids as carbon, energy and nitrogen sources.

In the nodule primordium, rhizobia are released from infection droplet which gets differentiated into nitrogen-fixing bacteroids.

The preset senescence of nitrogen-fixing bacteroids is fundamental portion of growth sequence in indeterminate nodules (Vasse et al. 1990). At this stage, growth and division of bacteroids is ceased and lysis of N_2-fixing bacteroids as well as host cells occurs. In recent times some papain-like and legumain-like cysteine proteases, also known as vacuolar processing enzymes (VPEs), were recognized that were intensely expressed throughout the development of nodule senescence (van Wyk et al. 2014). In nodules, papain-like cysteine proteases have known functions in the regulation of bacterial symbiosis, nitrogen fixation and leghemoglobin synthesis (Vande Velde et al. 2006; Li et al. 2008). Inhibition of papain-like cysteine protease activity was found to increase soybean tolerance to drought and favoured increased nodulation (Quain et al. 2014, 2015). VPEs found to be a part in age-linked senescence and triggering of pre-proteases. With their caspase-like activity, they additionally play significant part in programmed cell death (Hara-Nishimura et al. 2005; Roberts et al. 2012). At the death of a nodule, the bounded rhizobia are exclusively positioned to obtain the plant nutrients from senescing nodule tissues to proliferate rapidly. The number of nodule-derived rhizobia entering the soil population, becomes low as numerous rhizobial cells get destroyed together with plant cells during nodule senescence (Pladys et al. 1991) and also, differentiated bacteroids could not easily shift from biotrophic to saprotrophic life in soil (Quispel 1988).

7.5 Mechanisms of Plant Growth Promotion by Rhizobia

Rhizobia acts through direct and indirect mechanisms for improvement of crop growth and yield (Fig. 7.5). Direct mechanisms for plant growth promotion includes nitrogen fixation (Machado et al. 2013), nutrient solubilization/mobilization or mineralization (Reimann et al. 2008; Yu et al. 2012; Abd-Alla 1994a; Kumar and Ram 2014; Prasad et al. 2015), production of phytohormones, vitamins etc. (Sahasrabudhe 2011; Ghosh et al. 2015; Jangu and Sindhu 2011) (Table 7.2). In addition to symbiotic N_2 fixation, rhizobia also carry out non-symbiotic N_2 fixation in association with non-legume plants. Nitrogen fixation by photosynthetic bradyrhizobia was observed in association with wild rice (Chaintreuil et al. 2000). In indirect mechanism rhizobia produces bioactive molecules which inhibits phytopathogens (Datta and Chakrabartty 2014; Sindhu et al. 2014, 2017). Functionally different plant growth promoting rhizobacteria under variable environmental situations and in crop cultivation systems may enable growth and development of plants using either one or multiple mechanisms of plant growth promotion.

7.5.1 Biological Nitrogen Fixation

Fixation of atmospheric nitrogen by microorganisms is significant constituent of sustainable agriculture systems (Sessitsch et al. 2002; Karunakaran et al. 2009). *Rhizobium* are well known for establishment of symbiotic association with leguminous crops (Patriarca et al. 2002; Gage 2004) forming nodules to transforms

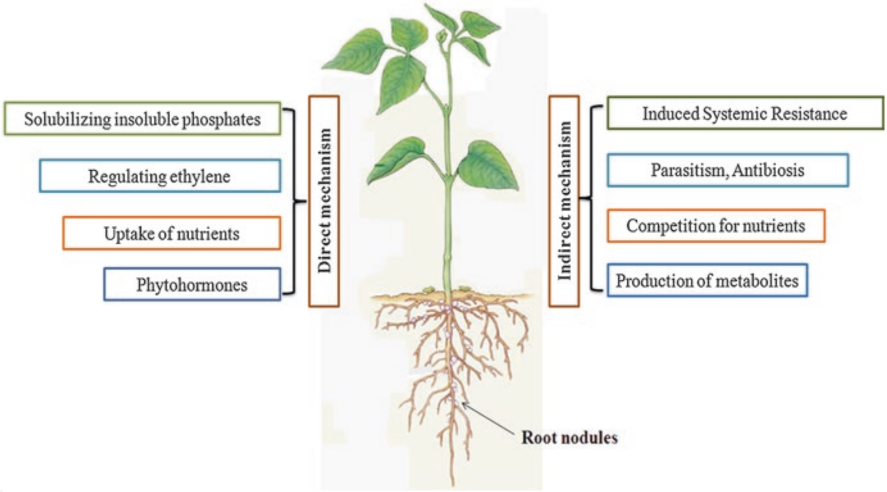

Fig. 7.5 Diagrammatic representation illustrating the direct and indirect mechanisms of plant growth promotion by rhizobia

atmospheric nitrogen in to ammonia and make it available to plants. In symbiotic relationship leguminous plants provides energy and photosynthetic materials to bacteria and bacteria in turn provide nitrogen to plants for incorporation into cellular constituents like amino acids, proteins and other essential nitrogenous compounds (Gresshoff 2003).

Nodules are generally termed as nitrogen fixation factories and millions of bacteroids inside the nodules fix the atmospheric nitrogen continually. Moreover, number of nodules formed on host plant is usually linked with amount of nitrogen fixed in particular *Rhizobium*-legume association. However, the number of nodules formed on a particular legume plant varies in different *Rhizobium*-legume systems. For example, the number of nodules formed ranges from 25–50 under sterilized chillum jar conditions in summer legumes such as green gram (*Vigna radiata* L. Wilczek), cow pea [*Vigna ungulculata* (L.) (Wilczek)], black gram [*V. mungo* (L.) (Hepper)]. Usually, 5–20 nodules are formed on cluster bean [*Cyamopsis tetragonoloba* (L.) (Taub)] and pigeon pea (*Cajanus cajan*). On the other hand, 30–60 nodules are usually formed under sterilized conditions on winter legume chick pea [*Cicer arietinum* (L.)] (Fig. 7.6). Large number of small nodules (50–120) is formed in ground nut (*Arachis hypogea*). Various environmental factors such as addition of nitrogenous fertilizers to the legume crops and level of ethylene formed due to hydrolysis of ACC in the root environment has been found to adversely affect nodulation under field conditions. Varin et al. (2009) reported that N fertilisation repressed nitrogen fixation in clover but N_2 fixation was improved by addition of sulphur (S). Sulfur fertilization improved the nodule length and number of nodules containing leghaemoglobin. Sulphur fertilization, improved photosynthesis and vegetative reproduction in white clover directly and indirectly through increase in

Fig. 7.6 Nodules formed on chick pea (*Cicer arietinum*) plant under sterilized chillum jar conditions

nitrogen fixation. Sulfur dependent response allows plants to adapt to variety of abiotic conditions but its sensitivity to S nutrition would be a shortcoming for rivalry in a state of soil sulphur poverty. Whereas, S fertilization could help sustain such plants under nitrogen limiting status.

Mathews and Carroll (2018) reported that many edaphic factors such as pH, nutrient deficiencies and toxicities, water, and temperature affect nodulation, but nitrate is unique in that it is generally not inhibitory to plant growth. Estimates of energy costs are generally greater for nitrogen fixation than for nitrate assimilation. Besides this, there are other developmental and ecological considerations which may have resulted in natural selection for nitrate inhibition of nodulation. Nitrate can be assimilated in either, or both, root and shoot tissue of plant, whereas nitrogen fixation needs development of a specific organ, root or stem nodule. In young white clover seedlings, for example, maximum activities of nitrate reductase precede the highest rates of nitrogenase activity by a matter of weeks. Indeed, plants that are dependent on nitrogen fixation as the sole nitrogen source do not grow as well as those which are supplemented with low noninhibitory or larger levels of nitrate. In the ecological context, it can be assumed that nitrate utilization by legumes decreases the amount of soil nitrate available to adjacent nonsymbiotic plants that are competing for other nutrients. Thus, preferential utilization of nitrate may be advantageous for legume species by decreasing the competitive ability of other plants that are unable to form a nitrogen-fixing symbiosis.

Sindhu and Dadarwal (2001c) evaluated efficiency of mutant *Rhizobium* strains for nodulation on chick pea (*Cicer arietinum*) grown in sterilized chillum jars. Mutants of strains Ca85 and Ca401 showed no nodulation efficiency whereas mutants of strains Ca181 and Ca534 were not able to nodulate the roots and also unable to fix nitrogen. Further mutants also displayed reduced nodulation and nitrogenase activity which in turn showed decreased shoot dry weight as compared to inoculation of wild type strains. Overall, it was concluded that acquirement of streptomycin resistance in *Rhizobium* sp. *Cicer* strains showed decreased symbiotic efficiency of the microbial strain in chick pea.

Nitrogen fixation capacity of the *Rhizobium* strains is usually deliberated as one of the key character affecting plant growth. Nitrogen fixation is detected either by growth of the organisms to grow on nitrogen free medium and can be measured via ^{15}N incorporation (^{15}N enrichment) and acetylene reduction. Urban et al. (1986) prompted *Rhizobium trifolii* strain 0403 in nitrogen free medium by treating the cells with 16.6 mM succinic acid and other nutrients and observed that organisms grew luxuriously on semisolid or liquid medium and fix nitrogen to satisfy their own requirement. Nitrogen fixation was determined through ^{15}N incorporation (18% ^{15}N enrichment in 1.5 doublings) and acetylene reduction. Nitrogen-fixing cells showed a maximum specific nitrogenase activity of 5 nmol of acetylene reduced/min/mg of protein at 0.04 atm (ca. 4.05 kPa) and 3% oxygen concentration in liquid medium. The generation time of organisms in liquid medium at 30 °C was 1–5 days, depending on oxygen concentration. Nodulation studies by *Rhizobium trifolii* strain 0403 in the white clover showed *in vitro* nitrogenase activity indicating that at least portion of population retained characteristics of wild-type strain 0403.

During nitrogen fixation, enzyme nitrogenase catalyzes reduction of nitrogen to ammonia concomitant reduction of protons to hydrogen. The energy loss in proton reduction leading to H_2 evolution varies from 40 to 60% in the absence of an active uptake hydrogenase. Some of the *Rhizobium* strains have been identified, which has the capability to oxidize the evolved H_2 leading to more nitrogen fixation. Dadarwal et al. (1985) surveyed *Rhizobium* strains nodulating summer legumes cow pea [*Vigna ungulculata* (L.) (Wilczek)], black gram [*V. mungo* (L.) (Hepper)] and cluster bean [*Cyamopsis tetragonoloba* (L.) (Taub)] and a winter legume chick pea [*Cicer arietinum* (L.)] in Northern Plains of India and screened for hydrogenase activity to determine distribution of Hup character in the native ecosystem. Around 56% of *Rhizobium* strains of summer legumes were Hup^+ winter legume, chick pea, was all Hup^-. Ex planta acetylene reduction activity was observed in most of the Hup^+ but not in the Hup^- strains of any of the host species. In summer legume, mixed inoculation of Hup^+ and Hup^- strains under sterilized as well as unsterilized soil conditions, showed that the host species were predominantly nodulated with Hup^+ strain.

Sindhu and Dadarwal (1986) reported that reduction of triphenyl tetrazolium chloride and methylene blue dyes reduction tests were ambiguous for detection of Hup character in *Rhizobium* strains isolated from green gram, black gram, cow pea, pigeon pea, cluster bean and chick pea. Hup^+ *Rhizobium* strains isolated from these legumes except Hup^- strains obtained from chick pea (*Cicer arietinum*) invariably expressed nitrogenase activity under cultural conditions. Characterization of native *Rhizobium* strains on the basis of *ex planta* nitrogenase induction showed that 94% of the *ex planta* nitrogenase positive isolate were of Hup+ phenotype, whereas all the *ex planta* nitrogenase negative isolates were of Hup^- phenotype in nodules. The expression of nitrogenase under cultural conditions was therefore, found to be a reliable method for identification of *Rhizobium* strains for Hup^+ phenotype among the rhizobia of the “cowpea miscellany”. Mutants were derived from *Rhizobium* strains of cowpea miscellany *Vigna* group i.e., S24 and GR4 having ability to express ex-planta acetylene reduction activity (ARA) after mutagenesis with nitrosoguanidine (Sindhu and Dadarwal 1992). Approximately, 70% of the mutants of strain S24 and 82% mutants of strain GR4 were found to have increased ex-planta ARA in comparison to their respective parent strains. Six mutants of strain S24 and four of GR4 strain with increased ex-planta ARA were selected to study in planta H_2 uptake and symbiotic performance in two host species: green gram (*Vigna radiata*) and black gram (*Vigna mungo*). Most of mutants showed increased H_2 uptake in nodules and symbiotic affectivity of these selected mutants was also higher than the parent strain in both the legumes. The authors suggested that it is possible to obtain symbiotically superior mutants by mutagenesis taking desirable ex-planta character for initial selection followed by plant test.

Saini et al. (1996) isolated native rhizobia from root nodules of *Sesbania bispinosa* and from root and stem nodules of *S. rostrata. Rhizobium* strains were studied for occurrence of hydrogen uptake system (Hup), nitrate respiration (NR), *ex planta* expression of nitrogenase and relative symbiotic efficiency in relation to Hup and NR activities. The rhizobia of both the host species were found to have two types of uptake hydrogenases: (i) recycling hydrogenase activity expressed *ex planta* as well as *in planta* in nodules and (ii) hydrogenase activity expressed only in nodules but not under cultural conditions. Dissimilatory nitrate reduction leading to complete denitrification was found to be common among both Hup^+ as well as Hup^- isolates. *Ex planta* nitrogenase activity was not observed in any isolates from both the *Sesbania* species. Symbiotic effectivity of Hup^+ isolates was at par with Hup isolates. There was no specificity with regard to host infectivity and the stem nodulating rhizobia from *S. rostrata* formed root nodules on *S. bispinosa* as well as on *S. rostrata*.

The amount and type of carbon sources, nitrogen level in growth medium, temperature and growth conditions have been found to affect expression of nitrogenase and hydrogenase enzymes. Sindhu and Dadarwal (1988) observed influence of temperature on nitrogenase and uptake hydrogenase activities in nodules of green gram (*Vigna radiata* L. Wilczek), black gram (*V. mungo* L. Hepper) and chick pea (*Cicer arietinum* L.), inoculated with different *Rhizobium* strains at three different temperatures. The optimum temperature for nodule nitrogenase activity was 35 °C in green gram and black gram (summer legumes), while it was below 25 °C, in the case of chick pea (winter legume). A majority of the Hup^+ *Rhizobium* strains of the summer legumes had H_2 recycling ability that recycled the evolved H_2 produced in nodules by nitrogenase. With increase in temperature from 15 to 35 °C, the H_2 uptake rates also increased in nodules. In nodules formed with Hup^- strains, although the H_2 evolution rates increased with increase in temperature in all the three legumes, however, green gram and black gram nodules (summer legumes) evolved significantly higher amounts of H_2 than chickpea (winter legume). Also, irrespective of temperature optima for ARA, at lower temperature, the relative efficiency was high in all the three legumes. Sindhu and Dadarwal (1995a) determined nodule nitrogenase and H_2 uptake activities in normal (undecapitated) and decapitated plants (removal of shoot 24 h before measurements) of green gram and black gram inoculated with two Hup^+ *Rhizobium* strains which had H recycling ability in excess to the rates of H_2 produced by nitrogenase in nodules. A significant decline in nodule nitrogenase activity was observed in decapitated plants as compared to uncut control plants at 40 and 50 days of plant growth. However, nodules of both, control and decapitated plants, of both host species showed hydrogen supported enhanced acetylene reduction activity (ARA). The H_2 uptake rates of the two strains varied depending on host as well as on stage of plant growth. However, based on the relative ratio of H consumed per mole of C_2H_2 reduced in case of normal photosynthate supply as well as from the interruption of photosynthate supply (decapitated plants), it appears that photosynthate supply remains a limiting factor in nitrogen fixation under normal conditions of plant growth during symbiosis.

7.5.2 Production of Plant Growth Regulators

Plant growth regulators are organic compounds like plant hormones that stimulates plant's physiological response at lower concentration and hence effect plant development. Based on chemical structures and mode of action, plant growth regulators are grouped into six different categories i.e., auxins; cytokinins; gibberellins; ethylene; inhibitors (abscisic acid, phenolics and alkaloids) (Ferguson and Lessenger 2006; Mishra et al. 2006); and brassinosteroids (Rao et al. 2002; Bajguz and Tretyn 2003). Concentration of plant growth regulators produced by PGPR may vary from organism to organism. Majority of PGPR and symbiotic rhizobia influence plant growth by production of auxins, cytokinins and gibberellins, strigolactones, abscisic acid and brassinosteroids.

7.5.2.1 Auxins

About 80 % of the PGPRs and rhizobia produces most efficient biomolecule for plant growth promotion i.e. auxins (Antoun et al. 1998; Schlindwein et al. 2008; Bhagat et al. 2014). Major classes of auxins synthesized by soil microbes includes indole acetic acid (IAA), indole butyric acid or analogous compounds resulting from tryptophan metabolism (Loper and Schroth 1986; Malik and Sindhu 2008; Solano et al. 2008). Auxins are phyto-hormones that encourage cell division and elongation. Vargas et al. (2009) reported a considerable difference in auxin production amongst rhizobial isolates from arrow leaf clover (*Trifolium vesiculosum*) white clover (*T. repens*) nodules. Arrow leaf clover isolates showed IAA production frequency in more than 90% isolates whereas only 15% isolates showed IAA production. IAA producing rhizobia showed more intense nodule formation as auxins was reported to influence nodulation process (Boiero et al. 2007). IAA alters root morphology by increasing number of secondary roots and thereby increasing surface area as well as size and weight of roots. Which ultimately results in to improvement of more extensive root architecture of legume plants (Dazzo and Yanni 2006). Inoculation with auxin-producing bacteria may also result in the formation of adventitious roots (Solano et al. 2008). Modification in root architecture by rhizobacterial IAA enhance nutrient absorption by plants which ultimately results in to enhancement of plant growth (Probanza et al. 1996). Similarly, Biswas et al. (2000) reported that inoculation of rice with *R. trifolii* improved dry matter and grain production, in addition to augmentation in N, P, K and Fe content in plant tissue.

7.5.2.2 Cytokinins and Gibberellins

Cytokinins influence cell division and cell enlargement, in addition to influencing seed dormancy, flowering, fruiting and plant senescence (Ferguson and Lessenger 2006). Certain strains of *Rhizobium* synthesize cytokinins in culture but is quantification and characterization was not possible (Sturtevant and Taller 1989; Wang et al. 1982) but it is found to be involved in nodule formation by rhizobia (Frugier et al. 2008) via an unknown mechanism. Gamas et al. (2017) reviewed that cytokinins were involved in the precise identification of symbiotic associates, beginning of bacterial infection in root hair cells and commencement of nodule in root cortex. Progressively multifaceted regulatory networks was found in which cytokinin (CK) play critical functions in various phases of primary symbiotic stages. Interestingly, these parts can be positive or negative, cell independent or non-cell independent, and differ, relying on time, root tissues and probably legume species. Current progresses showed interconnected role of cytokinines in establishment of symbiotic relationship with other signaling pathways during nodule initiation. Gibberellins improve seed germination (Miransari and Smith 2009), encourage general growth of plants and postpones aging (Ferguson and Lessenger 2006). Production of lower concentration of gibberellins documented from *Rhizobium* (Solano et al. 2008). Several reports showed free-living rhizobial strains can produce small quantity of gibberellin like substances. Gibberellin was also believed to play a key role in *Rhizobium*-legume symbiosis that may be significant suggestions to endophytic colonization of non-legumes by rhizobia. For example, infection of *A. caulinodans* in *S. rostrata* was through intercellular crack entrance facilitated by gibberellins which is key process of endophytic colonization of non-legumes by rhizobia and gibberellins produced by bacteria may simplify this process (Lievens et al. 2005).

7.5.2.3 Strigolactones

Strigolactones (SLs) play a key part in governing root growth, shoot branching and plant-symbionts interaction (Rehman et al. 2018). Strigolactones produced by rhizobia and PGPRs have been found to affect nodule development. The presumed constituents of SL synthesis enzymes *GmMAX1a* and *GmMAX4a* with tissue expression patterns were identified and governed by rhizobia infection and modified throughout nodule formation. Knockdown transgenic hairy root soybean lines of *GmMAX1a* and *GmMAX4a* displayed reduced nodule number due to less expression of numerous nodulation genes necessary for nodule formation. Hormone analysis showed that *GmMAX1a* and *GmMAX4a* knockdown hairy roots showed increased level of ABA and JA but considerably reduced auxin content. This study showed close interactions between SL and other hormone signaling in controlling plant development and legume-rhizobia interaction.

7.5.2.4 Abscisic Acid and Brassinosteroids

ABA produced by certain strains of rhizobia like *B. japonicum* USDA110 (Boiero et al. 2007) can provide drought tolerance to plants to some extent. However, increase of ABA concentration also showed negatively effect on nodule development in *Trifolium repens* and *Lotus japonicas*. Suzuki et al. (2004) showed inhibition of nodulation in plants inoculated with *R. trifolii* and latter supplemented with ABA. Inoculation of *L. japonicus* mutant that has lesser sensitivity to ABA, (Tominaga et al. 2010) caused improved nodulation in *M. loti* inoculated plants.

Brassinosteroids are the new group of hormones and having steroidal substances which enables plants to resist abiotic stresses. Brassinosteroids generally found to affect processes such as seed germination, rhizogenesis, flowering, senescence, abscission and maturation (Rao et al. 2002). Vardhini and Ram Rao (1999) showed that treatment of brassinosteroids in groundnut gave better nodule formation and nitrogen fixation (*Arachis hypogaea*) developed in natural soil (without inoculation of *Rhizobium*).

7.5.3 Amelioration of Abiotic and Biotic Stress by Rhizobia

Climate change is the greatest threat to world's agricultural sustainability in the twenty-first century. Drastic changes in various climatic conditions increase the incidence of abiotic and biotic stresses, which can tremendously influence the global decrease in productivity of agricultural and horticultural crops (Grover et al. 2011; Papworth et al. 2015). Global warming and alteration in precipitation patterns, lead to several abiotic stresses like extremes temperatures, drought, flooding, salinity, metal stress and nutrient stress that creates harmful effects on food production (Barrios et al. 2008; Selvakumar et al. 2012). The probability of occurrence of extreme climatic events has increased in the last couple of decades and farmers lack the management options to sustain the agricultural productivity (Kalra et al. 2013). Abiotic stress hamper growth and production of crop, causing land degradation by making soil nutrient deficient and more stress prone. The abiotic stresses are usually interconnected with one another and function as a chain due to climatic variations (Grover et al. 2011).

The improvement in crop yields under unfavourable conditions by classical breeding or gene transfer techniques pose certain limitations in terms of ethical issues and time requirements (Ashraf and Akram 2009; Fleury et al. 2010). Again, drought stress tolerance is often a complicated phenomenon involving clusters of gene networks. Apart from classical breeding and transgenic approaches, application of plant growth promoting rhizosphere (PGPR) bacteria is an alternative eco-friendly strategy for improving plant fitness under understressed environments (Kim et al. 2012) (Table 7.3). Application of beneficial rhizosphere bacteria has recently been found to alleviate the abiotic stresses. Some bacterial species such as *P. polymyxa*, *Achromobacter piechaudii* and *R. tropici* provide tolerance to drought stress in *Arabidopsis*, tomato (*Solanum lycopersicum*) and common bean (*Phaseolus vulgaris*), respectively through accumulation of abscisic acid and due to degradation of reactive oxygen species and ACC (1-aminocyclopropane-1-carboxylate) (Mayak et al. 2004b; Yang et al. 2008). Salinity tolerance in plants is conferred by application of *A. piechaudii* and *B. subtilis* (Mayak et al. 2004a; Zhang et al. 2006).

Figueiredo et al. (2008) identified enhanced antioxidant enzymatic activity in common bean plants (*Phaseolus vulgaris* L.) coinoculated with *R. tropici* and *P.*

Table 7.3 Inoculation effect of rhizobia on ameliorating the influence of various stresses in different crops

Rhizobium spp.	Host plant	Proposed mechanism(s)/ plant response	References
Drought stress			
Rhizobium spp.	*Zea mays*, *Triticum aestivum*	Encouraged drought tolerance by catalase enzyme, exopolysaccharide and IAA production	Hussain et al. (2014a, b)
Rhizobium spp., *Glomus mosseae*, *Glomus intraradices*	*Phaseolus. vulgaris*, *Zea. mays*	Enhanced growth, yield and relieved moderate drought stress	Franzini et al. (2013)
Rhizobium gallicum 8a3	*Phaseolus vulgaris*	Controlled water relations in plant	Sassi-Aydi et al. (2012)
Mesorhizobium tianshanense, *G. intraradices*	*Lotus tenuis*	Variation of proline and polyamine	Echeverria et al. (2013)
Rhizobium galegae HAMBI 1141, *Pseudomonas trivialis* 3Re27	*Galega officinalis*	Enhanced root tip colonization	Egamberdieva et al. (2013)
Rhizobium strains RhOF4 and RhOF6	*Vicia. faba*	Regulated enzymes of ascorbate-glutathione cycle and decreased glutathione	Oufdou et al. (2014)
Temperature stress			
Rhizobium sp.	*Prosopis juliflora*	Enhanced symbiosis and nitrogen fixation	Kulkarni and Nautiyal (2000)
Bradyrhizobium strains	*Glycine max*	Effective nitrogen fixation at high temperatures	Rahmani et al. (2009)
Mesorhizobium sp.	–	Improved transcriptional induction of chaperone genes	Alexandre and Oliveira (2011)
Heavy metal stress			
B. japonicum E109	*Glycine max*	Decreased symbiosis due to arsenic toxicity	Talano et al. (2013)
Rhizobium, *Sinorhizobium* spp.	*Lathyrus sativus*, *Lens culinaris*, *Medicago truncatula*, *M, minima*	Phytoremediation of Cd-contaminated soil	Guefrachi et al. (2013)
Cupriavidus necator	*Leucaena leucocephala*, *Mimosa pudica*, *Mimosa caesalpiniaefolia*	Bioremediation of Zn-, Cu-, Pb-, and Cd-contaminated soils	Ferreira et al. (2013)
Rhizobium spp.	*Lens culinaris*	Reduced uptake of Ni in contaminated soil and enhanced plant growth	Wani and Khan (2013)
Rhizobium MuJs10A	*Vigna radiata*	Improved nodulation efficiency	Mondal et al. (2017)

(continued)

Table 7.3 (continued)

Rhizobium spp.	Host plant	Proposed mechanism(s)/ plant response	References
Biotic stress			
Mesorhizobium loti	*Brassica campestris*	Suppression of white rot disease/*Sclerotinia sclerotiorum*	Chandra et al. (2007)
Rhizobia	*Cicer arietinum*	Suppression of *Rhizoctonia solani*	Hemissi et al. (2013)
R. leguminosarum strain RhOF4	*Vicia faba*	Reduction in influence of cyanotoxin biohazard	Lahrouni et al. (2013)
Rhizobium sp. *cicer* strain Ca181	*Cicer arietinum*	Improved nodulation and growth of chickpea along with decrease in wilt frequency	Khot et al. (1996)
Mesorhizobium ciceri	*Cicer arietinum*	Suppression of *F. oxysporum*, synthesis of IAA and siderophores	Yadav et al. (2015)

polymyxa under drought stress conditions. Treatment of pea plants with *Pseudomonas* spp. containing ACC deaminase somewhat removed effects of drought stress (Arshad et al. 2008). Similarly, treatment of tomato (*Solanum lycopersicum* L.) and pepper (*Capsicum annuum* L.) seedlings with *A. piechaudii* ARV8 decreased the production of ethylene (ET) that may have contributed to the observed drought tolerance (Mayak et al. 2004b). Lim and Kim (2013) showed that pepper plants treated with *B. licheniformis* K11 withstand drought stress and had better survival compared to non-treated plants. The observed drought tolerance was attributed to ACC deaminase production by PGPR that reduced ET concentrations by cleaving ACC.

Efficiency of *Bradyrhizobium* strains for alleviating effect of water stress was assessed in peanut genotypes through determination of antioxidant enzymes activities, leaf gas exchanges and vegetative growth in greenhouse with three peanut genotypes (BRS Havana, CNPA76 AM and 2012-4) (Barbosa et al. 2018). In experiment two *Bradyrhizobium* strains (SEMIA6144 and ESA123) under two levels of irrigations i.e. with and without irrigation were used. Plants grown under water deficiency showed alteration in leaf gas exchange as well as antioxidant activities and reduction of vegetative growth parameters. The plants inoculated with *Bradyrhizobium* strains SEMIA6144 and ESA123 showed increase in vegetative growth parameters, especially those inoculated with *Bradyrhizobium* sp. ESA123 strain obtained from the semi-arid region of Northeast Brazil. At in silico analyzes, ESA123 presented 98.97% similarity with the type strain of *B. kavangense*. The results uncovered beneficial effects of the peanut-*Bradyrhizobium* interaction under water stress condition.

Similarly, the consortium effect of three ACC-deaminase producing rhizobacteria - *Ochrobactrum pseudogrignonense* RJ12, *Pseudomonas* sp. RJ15 and *Bacillus subtilis* RJ46 was evaluated on drought stress alleviation in *Vigna mungo* L. and *Pisum sativum* L (Saikia et al. 2018). Consortium treatment significantly increased seed germination percentage, root length, shoot length and dry weight of treated plants. An elevated synthesis of reactive oxygen species scavenging enzymes and cellular osmolytes, higher leaf chlorophyll content, increase in relative water content and root recovery intension were observed after consortium treatment in comparison with the uninoculated plants under drought conditions. The consortium treatment decreased the ACC accumulation and down-regulated ACC-oxidase gene expression, suggesting that the consortium could be an efficient bio-formulator for crop health improvement in drought affected acidic agricultural fields.

Rhizobial species has also been found to differ in their intrinsic osmotolerance measured by capacity to tolerate and grow under variable concentration of NaCl. *B. japonicum*, *R. etli* and *R. leguminosarum* showed sensitivity to salt by complete growth inhibition at 100 mM NaCl (Boncompagni et al. 1999); whereas growth of *Mesorhizobium huakuii*, *R. tropici* IIB and *S. fredii* inhibited at 200 mM NaCl showing moderate sensitivity, but *S. meliloti* and *A. tumefaciens* found to be highly salt tolerant and grow at 300 mM NaCl (Bernard et al. 1986). *Rhizobium* spp. from nodules of *Acacia*, *Hedysarum*, *Leucaena* and *Prosopis* plants can withstand salt concentration up to 500 mM NaCl (Zhang et al. 1991). Rhizobia tolerate stress because of accumulation osmoprotectants, improved production of exopolysaccharides, ROS-scavenging enzymes and heat shock proteins and chaperons through expression of NaCl-responsive loci (Vriezen et al. 2007). Choudhary and Sindhu (2017) found fifty five rhizobacterial strains from the chickpea rhizosphere soil and selected for their salt tolerance. At 3% NaCl concentration, 41.8% rhizobacterial isolates formed colonies and only 10.9% isolates showed growth at 4% NaCl concentration.

Capacity of rhizobia to tolerate abiotic stresses like heavy metals and pesticides, aids rhizobia to accomplish their advantageous PGP activities in stress environments. Plant responses to various environmental stress is equally dependent on host plant reaction and symbiosis procedure of rhizobial symbiosis (Yang et al. 2010). Grover et al. (2011) revise importance of microorganisms in adaptation of crops to different abiotic stresses. There are widespread reports on tolerance and nodule forming efficiency of *Rhizobium* and *Bradyrhizobium* to soil acidity, salinity, alkalinity, temperature and osmotic stress conditions (Graham 1992; Kulkarni and Nautiyal 2000; Defez et al. 2017). Osmoprotectants, compatible solutes/osmolytes, similarly perform a multiple functions as showed in *S. meliloti* by proline-betaine that helps as both osmoprotectant (under high osmotic stress) and energy source (under low osmotic stress) (Miller-Williams et al. 2006).

Plants being sessile, their growth and yield are strongly influenced by biotic stress. Biotic stress is caused by various pathogens, such as bacteria, viruses, fungi, nematodes, protists and insects. Common impacts of these biotic factors include imbalanced hormonal regulation, nutrient imbalance and physiological disorder results in a substantial decrease in agricultural production (Haggag et al. 2015). Microbial diseases cause malfunction in plants which result in decrease in ability of plant to live and preserve its ecological niche. Plant diseases result either in death or may greatly impair growth and yield of the plant. Pathogenic microorganisms generally deteriorate or extinguish plant tissues and decrease crop production ranging from 25 to 100% (Choudhary and Sindhu 2015). Among the different kind of diseases, root diseases are projected to give 10–15% yield losses globally. Biotic stress also has adverse impacts on plants co-evolution, population dynamics, ecosystem nutrient cycling, natural habitat ecology and horticultural plant health (Gusain et al. 2015). Global crop yields are reduced by 20–40% annually due to pests and diseases (Strange and Scott 2005).

Yadav et al. (2015) obtained 207 strains of *M. ciceri*, from root nodules of chickpea plants and selected for antagonistic influence against *F. oxysporum* f. sp. *ciceri*. Seven strains (MC69, MC84, MC96, MC99, MC180, MC183 and MC190) showed antagonistic effects against *F. oxysporum* f. sp. *ciceri*, but none of them was observed to produce antibiotic or solubilized tricalcium phosphate. Three isolates i.e., MC84, MC96 and MC99 showed siderophore production. MC99 was found to be best antagonistic strain as it manufactured maximum quantity of siderophore.

7.5.4 Bioremediation of Organic Pollutants by Rhizobia

Many free-living rhizobial strains in the genera *Rhizobium*, *Sinorhizobium* and *Bradyrhizobium* showed resistance to polycyclic aromatic hydrocarbon (PAHs), polychlorinated biphenyl (PCBs), aromatic heterocycles (i.e., pyridine) or other toxic organic compounds (Keum et al. 2006; Poonthrigpun et al. 2006; Tu et al. 2011). Ahmad et al. (1997) isolated and characterized a different *R. meliloti* strains from soils polluted with aromatic/chloroaromatic hydrocarbons. Moreover, acenaphthylene and phenanthrene are omnipresent polycyclic hydrocarbons in the environment. *Rhizobium* sp. strain CU-A1 can completely degrade acenaphthylene (600 mg l^{-1}) within three days of inoculation via naphthalene- 1, 8-dicarboxylic acid metabolism pathway (Poonthrigpun et al. 2006). *Sinorhizobium* sp. C4 can consume phenanthrene as a only carbon source and 16 intermediary metabolites engaged in degradation pathway have been recognized (Keum et al. 2006).

Polychlorinated biphenyls are classified as persistent organic pollutants (POPs) varying in the number of chlorine atoms (1–10) bound to their biphenyl rings (Passatore et al. 2014). Tu et al. (2011) reported degradation of more than 70% of 2, 4, 4-TCB (PCB28) by *S. meliloti* ACCC17519. In trials under aerobic conditions, 2- hydroxy-6-oxo-6-phenylhex-2, 4-dienoic acid (HOPDA), the meta cleavage product in typical PCBs-degradative pathway, was recognized as principal intermediate using GC-MS during the biotransformation of 2, 4, 4-TCB by *S. meliloti*. Certain toxic aromatic acids and their hydroaromatic biosynthetic intermediates (i.e., quinate and shikimate) usually disseminated in plants and rhizosphere found to encourage growth of rhizobia (Parke et al. 1985). Mimosine [β-*N*-(3-hydroxy-4-pyrid-one)-amino propionic acid], an aromatic toxin manufactured by the roots of *Leucaena* sp., is toxic to both bacteria and eukaryotic cells (Awaya et al. 2005). Several *Rhizobium* strains forming nodules on *Leucaena* reported to have ability to use mimosine as a source of carbon and nitrogen (Soedarjo et al. 1995; Soedarjo and Borthakur 1998), emphasizing catabolic efficiency of aromatic compounds in rhizobia.

7.5.5 Impacts of Grain Legumes and Rhizobia on Atmosphere and Soil Quality

Inclusion of legumes into agricultural cycles helps in decreasing usage of fertilizers and energy in arable systems and subsequently reducing GHG (greenhouse gases) productions (Reckling et al. 2016). N fertilizer savings across Europe (Reckling et al. 2016), in cycles comprising leguminous crops, range around 277 kg ha^{-1} of CO_2 annually (Jensen et al. 2012). In view of an effectiveness of 2.6–3.7 kg CO_2 produced per kilogram of N synthesized, annual global fertilizer results in emission of 300 Tg of CO_2 into the atmosphere annually (Jensen et al. 2012). Moreover, the CO_2 exhaled from nodule containing roots of leguminous plants derives from atmosphere by photosynthetic activities. On the other hand, all CO_2 released in process of N-fertilizer production comes from fossil energy, thus defining a net influence on atmospheric concentration of CO_2 (Jensen et al. 2012). N_2O accounts for 5–6% of the total atmospheric GHG (Crutzen et al. 2007) and agriculture represents chief source about 60% of mane-made N_2O emissions; (Reay et al. 2012), because of both animal and crop production. A major quantity of these productions derives from use of nitrogenous fertilizers. Application of 100 kg of N fertilizer emits 1.0 kg of N_2O (Jensen et al. 2012) though various quantities of emission rely on number of factors viz. nitrogen application rate, soil organic carbon content, soil pH and texture (Peoples et al. 2009; Rochester 2007). Denitrification reaction is prime source of N_2O in majority of cropping and pasture systems (Peoples et al. 2004; Soussana et al. 2010).

In latest years, numerous experiments focussed on role of legumes to decrease GHG productions. Jeuffroy et al. (2013) proved that legumes produce about 5–7 times less GHG per unit area in comparison to other crops. Measurement of nitrous oxide fluxes showed that peas released 69 kg N_2O ha^{-1} which was far less than winter wheat emitting 368 kg N_2O ha^{-1} and rape producing 534 kg N_2O ha^{-1}. Clune et al. (2017) studied various life cycle assessment (LCA) experiments on GHG production for the period of 2000 to 2015 world over showing Global Warming Potential (GWP) values of pulses was very low (0.50–0.51 kg CO_2 eq kg^{-1} produce or bone-free meat). Schwenke et al. (2015) taken us two field trials in black Vertosol in sub-tropical Australia, showed 385 g N_2O-N ha^{-1} which was significantly higher as compared to emission from chickpea (166 g N_2O-N ha^{-1}), faba bean (166 g N_2O-N ha^{-1}) and field pea (135 g N_2O-N ha^{-1}). Similarly they have also reported that grain legumes showed significantly lower emission factor proving that nitrogen fixed by legumes is less emissive form of nitrogen input as compared to fertilizer nitrogen. However, the key factor determining effect of legumes for reducing greenhouse gas emission governed by management of agro-ecosystems in which they are incorporated. Senbayram et al. (2016) reported that mono cropping of faba bean showed threefold higher collective N_2O release (441 g N_2O ha^{-1}) as compared to unfertilized wheat (152 g N_2O ha^{-1}). On the other hand, intercropping of faba bean with wheat gave 31% less N_2O emissions fluxes as compared to nitrogen fertilized wheat. Nevertheless, benefits obtained by addition of legumes in crop rotations turn out to be noteworthy when market charges of Nitrogenous fertilizer are considered (Jensen et al. 2012).

7.6 Influence of *Rhizobium* Application on Yield of Leguminous Crops

Rhizobium was extensively studied because of its importance in agriculture and environment (Karaman et al. 2013; Nyoki and Ndakidemi 2014). Application of efficient strains of *Rhizobium* showed significant increase in nodulation, nitrogen absorption and crop yield (Thies et al. 1991; Wani et al. 2007a; Franche et al. 2009) (Table 7.4). Elsheikh (1998) inoculation of five guar (*Cyamopsis tetragonoloba*) cultivars, namely, HFG-75, HFG-182, HFG-363, HFG-408 and WB-195 with *Bradyrhizobium* strains TAL 169 and TAL 1371 (introduced) and strains ENRRI 16A and ENRRI 16C (local) significantly enhanced yield, protein, crude fibre and mineral content in guar under field conditions. Indigenous isolates showed higher influence on nodulation and plant growth parameters as compared to exogenous strains. Karasu and Dogan (2009) reported that seed treatment of chick pea (*Cicer arietinum*) seeds with *R. cicero* showed significantly higher seed yield, plant height, first pod height, number of pods per plant, number of seeds per plant, harvest index and 1000 seed weight as compared to treatments receiving various doses of nitrogen through ammonium nitrate (0, 30, 60, 90 and 120 kg ha^{-1}). Native genotype as crop material provided maximum yield (2149.1 kg ha^{-1}) among three chick pea genotypes utilized. Various mixtures of microorganisms utilized looking to the further research needs in this area (Gopalakrishnan et al. 2015). Utilization of appropriate species of microbes as an inoculant in N exhausted environments might be a superior method to increase legume growth and development.

Table 7.4 Growth improvement of various legumes by inoculation of selected *Rhizobium* strains

Rhizobium species	Contributions in growth improvement	References
Bradyrhizobium spp.	Increased nodulation, shoot and root growth in legumes. Enahancing plants' tolerance to drought and synthesis of indole-3-acetic acid	Shaharoona et al. (2007), Uma et al. (2013) and Gopalakrishnan et al. (2015)
Rhizobium strain MRPI	Stimulated nodulation, leghaemoglobin concentration, seed protein and seed harvest in pea plant	Ahemad and Khan (2011)
Rhizobium spp.	Significant increase in height, pod number, length and seed weight of *Vigna mungo* and *Vigna radiate*	Ravikumar (2012)
Rhizobium sp. RL9	Improved development, nitrogen content, seed protein content and seed yield of lentil plant under heavy metal stressed conditions	Wani and Khan (2013)
Sinorhizobium meliloti	Enhanced biomass diversity in black medic plant exposed to copper stress	Gopalakrishnan et al. (2015)
Bradyrhizobium strain S24	Improved nodulation, nitrogen fixation and plant biomass	Sindhu and Dadarwal (1986, 1992, 1995a)
Mesorhizobium strain Ca181	Improved nodulation, nitrogen fixation and shoot dry weight	Sindhu and Dadarwal (2001a) and Goel et al. (2002)

Table 7.5 Inoculation effect of rhizobia exerted against abiotic stress on host plants

Rhizobia	Crop species	Growth condition	Remarks	References
Drought stress				
R. tropici coinoculated with *Paenibacillus polymyxa*	Kidney bean	Greenhouse	Increased plant height, shoot dry weight and nodulation	Figueiredo et al. (2008)
M. mediterraneum LILM10	Chick pea	Field study	Higher nodulation, shoot dry weight and grain yield	Romdhane et al. (2009)
R. elti (engineered for enhanced trehalose-6-phosphate synthase)	Kidney bean	Pot studies	Superior nodulation, nitrogenase activity and biomass yield	Suárez et al. (2008)
Bradyrhizobium sp.	–	*In vitro* and pot culture	Improved drought resistance, IAA and EPS production, nodule numbers, nitrogenase activity in nodules and nitrogen content of nodules	Uma et al. (2013)
Temperature stress				
Acacia rhizobia (40 strains)	–	*In vitro*	Occurrence of small and large plasmids, buildup of free glutamate, three rhizobia strains tolerated 1.4M NaCl	Gal and Choi (2003)
M. ciceri, *M. mediterraneum* and *S. medicae*	Chick pea	Glass house	*M. ciceri* improved nodulation and CAT activity, reduction in nodule protein and SOD activity	Mhadhbi et al. (2004)
M. ciceri ch-191	Salt resistant and sensitive chick pea cultivars	*In vitro*	Reduced plant dry weight and nitrogenase activity in sensitive cultivars, normal nodule weight and shoot K/Na ratio and decreased foliar increase of Na in tolerant cultivars	Tejera et al. (2006)
Rhizobial strains	Lentil	Field study	Increased plant biomass, nodule number and nodule dry weight	Islam et al. (2013)

The response of treatment of various rhizobial species on legumes under various stress conditions depends on the host plant response (Table 7.5), but this response can also be affected by rhizobia and progression of symbiosis (Yang et al. 2010). Grover et al. (2011) has reviewed the job of microbes in adaptation of crops to different abiotic stresses. Moreover, soil acidity, salinity, alkalinity, temperature and osmotic stress conditions have been found to affect the resistance and nodulation capacity of *Rhizobium* and *Bradyrhizobium* in the soil (Graham 1992; Kulkarni and Nautiyal 2000; Defez et al. 2017).

Mfilinge et al. (2014) reported that inoculation of soybean (*Glycine max* L.) with *Rhizobium* showed significant increase in crop growth and yield components viz. number of branches bearing pod per plant, total number of pods per plant and seed number per pods. Seed treatment of *R. leguminosarum* in pea and lentil showed increased pea nodulation, shoot/root diversity and pea seed yield (Bourion et al. 2017). Likewise, seedling height, nodule and shoot biomass of lentil were increased. Bourion et al. (2017) reported increase in nodulation of chickpea by inoculation of *Rhizobium* species with significantly higher plant growth, root dry weight and number of nodules in greenhouse and field. Ravikumar (2012) reported significantly higher plant height, fresh weight, roots, nodules, leaves, shoots and pods number, pod length and seed weight of *Vigna mungo* and *Vigna radiata* inoculated with *Rhizobium* as compared to uninoculated control. Height of soybean plants treated with *Rhizobium* under field conditions was significantly higher and stem girth was also improved in greenhouse and field experiments (Tairo and Ndakidemi 2013). Likewise, Nyoki and Ndakidemi (2014) showed inoculation of cowpea with rhizobial isolates gave significantly higher plant height as compared to control treatment.

7.7 Coinoculation Effect of PGPR with *Rhizobium* Strains

The plant growth promoting effects of *Rhizobium* species are boosted when coinoculated with other microbes (Table 7.6). In coinoculation, some microbes function as assistant to improve the efficiency of the other microorganisms. Therefore, coinoculation of certain bacteria with *Rhizobium* spp. Improve efficiency of the rhizobial spp. Which ultimately results in increased crop productivity. Recently, coinoculation of *Pseudomonas, Enterobacter, Serratia* and *Bacillus* spp. with *Rhizobium/Bradyrhizobium* showed increase in number of nodules, nitrogen fixation and plant biomass of green gram, chickpea and other legumes (Sindhu et al. 1999a, b, 2002a, b; Goel et al. 2000). Therefore, combined inoculation of nitrogen fixing bacteria and PGPR could be explored for enhancing nitrogen fixation in rhizosphere of cereal and legume crops.

Coinoculation of N_2-fixing *A. vinelandii* with *Rhizobium* spp showed increased number of nodules in soybean, pea (*Pisum sativum*) and clover (*Trifolium pratense*) (Burns et al. 1981). Sameway, combined inoculation of *A. brasilense* with *Rhizobium* resulted in higher efficiency in soybean and groundnut (Iruthayathas et al. 1983; Raverkar and Konde 1988). Coinoculation of *Rhizobium* spp. and *Azospirillum* spp. showed increased root hair formation, number of root nodules and flavonoid content in root exudates in comparison to individual application of *Rhizobium* spp. (Itzigsohn et al. 1993, Burdman et al. 1997; Remans et al. 2007, 2008). Efficiency of *Azospirillum* on the legume-*Rhizobium* symbiosis was also observed to be genotype dependent. *Azospirillum* – *Rhizobium* combined inoculation in common bean (*Phaseolus vulgaris* L.) cv. DOR364 showed increase in rate of nitrogen fixation and yield at all sites in field trials (Remans et al. 2008). Coinoculation of *A. lipoferum* and *R. leguminosarum* bv. *trifolli* showed enhanced

Table 7.6 Effect of combined inoculation of rhizobia with rhizobacteria or arbuscular mycorrhizal fungi on legumes

Rhizobia	Coinoculants	Host plant	Proposed mechanism(s)/plant response	References
Rhizobium sp.	*Pseudomonas* sp. LG, *Bacillus* sp. Bx	*Paikiniana vulgaris*	P solubilization, IAA, ammonia and siderophore production	Stajkovic et al. (2011)
R. leguminosarum strain PR1	*Pseudomonas* sp. strain NARs1	*Lens culinaris*	Better growth and nutrient uptake	Mishra et al. (2011)
B. japonicum strains MN-S and TAL-102	AM fungi, *Glomus intraradices*	*Vigna radiata*	Significant increase in plant biomass and N contents	Yasmeen et al. (2012)
R. leguminosarum	PGPR, enriched compost	*Lens culinaris*	ACC deaminase activity of PGPR and symbiotic proficiency of rhizobia	Iqbal et al. (2012)
Rhizobium spp. strain Mg6	PGPR strains A1 and A2	*Phaseolus vulgaris*	ACC deaminase activity	Aamir et al. (2013)
Rhizobium sp. strain PK20	*Pseudomonas* sp. strain M9	*Vigna radiata*	ACC deaminase activity of *Pseudomonas* sp. M9	Ahmad et al. (2011, 2013)
R. leguminosarum	*Pseudomonas* spp.	*Vicia faba*	P solubilization, phytohormone and siderophore production	Saidi et al. (2013)
Mesorhizobium sp. BHURC03	*Pseudomonas aeruginosa* BHUPSB02	*Cicer arietinum*	Increased P and Fe uptake, nodulation as well as IAA synthesis	Verma et al. (2013)
Rhizobium sp.	PGPR, Phosphorus-enriched compost	*Cicer arietinum*	Increased growth and nodulation by ACC deaminase activity	Shahzad et al. (2014)
Rhizobium leguminosarum bv. *viciae*	Arbuscular mycorrhizal fungi	*Vicia faba*	Mobilization of P, Fe, K and other minerals	Abd-Alla et al. (2014)

nodule formation in white clovers (Tchebotar et al. 1998). The mechanisms behind enhanced efficacy was believed to be increase in infection sites for *Rhizobium* spp. by *Azospirillum* which in turn leads to improved nodule formation while application of *Rhizobium* and *Azospirillum* was found to increase siderophore, vitamins and phytohormones biosynthesis (Cassan et al. 2009; Dardanelli et al. 2008). *Azotobacter* came out as a potential coinoculant for *Rhizobium* as it increases vitamins and phytohormones synthesis which ultimately results in increase in nodule formation (Akhtar et al. 2012).

Coinoculation of *R. phaseoli* with *P. putida* showed increased nodulation of beans (*Phaseolus vulgaris*) under greenhouse and field trial conditions but no significant increase in bean yield was observed indicating increase in nodule numbers and *Rhizobium* infection has not direct correlation with crop yield (Grimes and Mount 1984). Bolton et al. (1990) also observed that coinoculation of *Rhizobium* and *Pseudomonas* spp. increases nodulation of pea but no significant difference were observed in shoot dry matter. On the contrary, combined inoculation of rhizobia with other rhizobacteria showed increase in nodule number, root length, plant biomass and yield in various legume crops. For example, Chanway et al. (1989) reported that individual inoculation of nine PGPR strains showed no significant impact on pea growth in field, whereas it gave against significant increase in emergence, vigour, nodule development, nitrogen fixation and root weight in lentil under field conditions. Combined inoculation of these nine strains of PGPR along with *Rhizobium* sp. *cicer* strain Ca181 showed increase in nodule numbers and growth of chickpea with simultaneous reduction of wilt disease (Khot et al. 1996).

Coinoculation of the five strains of fluorescent pseudomonad and *R. leguminosarum* biovar *viciae* enhances shoot and root length as well as dry weight of *Pisum sativum* L. cv. Capella (Dileep Kumar et al. 2001). Goel et al. (2002) reported that coinoculation of chickpea with *Pseudomonas* strains MRS23 and CRP55b, and *Mesorhizobium* sp. *Cicer* strain Ca181 showed 68.2–115.4% increase in nodule numbers at 80 and 100 days after planting, respectively as compared to inoculation of *Mesorhizobium* sp. *Cicer* strain Ca181 alone under sterile conditions. Treatments receiving combined inoculation showed 1.18–1.35 times higher shoot ratio as compared to that of *Mesorhizobium* inoculation and 3.25–4.06 times higher shoot ratio as compared to uninoculated control. Sameway combined inoculation of *B. japonicum* and *P. fluorescens* showed increase in nodule numbers and growth of 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-Unkefer 1988), *R. leguminosarum* with *P. fluorescens* strain F113 in pea (Andrade et al. 1998) and *Mesorhizobium/Bradyrhizobium* strains with *Pseudomonas* sp. in green gram [*Vigna radiata* (L.) wilczek] and chickpea (Sindhu et al. 1999a, b; Goel et al. 2000, 2002). Fox et al. (2011) reported that coinoculation of *Medicago truncatula* with *Pseudomonas fluorescens* WSM3457 and *Sinorhizobium* showed increase in number of infection sites number of root hairs. Moreover, coinoculation of *P. aeruginosa* and *Mesorhizobium* sp. showed significantly higher shoot and root dry weight, nodule numbers, grain and straw yield as well as phosphorus uptake in chickpea (Verma et al. 2013). Besides growth promotion chickpea plants receiving inoculation of consortium comprising of *A. chroococcum*, *Trichoderma harzianum*, *Mesorhizobium* and *P. aeruginosa* showed antagonistic activities against *Rhizoctonia solani* and *Fusarium oxysporum* (Verma et al. 2014).

Holl et al. (1988) stated that inoculation of *Bacillus* species to seeds or roots changed configuration of rhizosphere which ultimately increase growth and yield of various legumes. For example, Halverson and Handelsman (1991) concluded that under field conditions seed treatment with *B. cereus* UW85 gave 31 to 133% higher nodules than untreated soybeans after 28 and 35 days of planting. In soybean plant grown in sterilized soil-vermiculite mixtures, application of UW85 through seed treatment showed 34 to 61% increase in nodulation at 28 days after planting. It was suggested that UW85 influenced nodule formation afterward planting by encouraging bradyrhizobial colonization or by defeating the termination of colonization process. In another experiment, Turner and Backman (1991) showed that seed treatment of peanut seeds with *B. subtilis* enhance germination and seedling emergence, improved nodulation by *Rhizobium* spp., enriched plant nutrition, decreased incidence of root cankers caused by *Rhizoctonia solani* AG-4 and increased root growth. Srinivasan et al. (1997) reported increase in nodule numbers in *Phaseolus vulgaris* by combined inoculation of *R. etli* strain TAL182 and *B. megaterium* S49 as it increased root hair propagation and lateral root development. Podile (1995) reported increase in nodule numbers, plant dry matter and grain yield of pigeon pea by combined inoculation of *Bacillus* sp. and rhizobia. Similar effects were observed in white clover (Holl et al. 1988). Sindhu et al. (2002a) reported that combined inoculation of *Bacillus* strains with efficient *Bradyrhizobium* strain S24 gave 1.28–3.55 times increase in dry mass at 40 days after sowing. Reports suggest that *Bacillus* strains can increase nodulation and nitrogen fixation at 40 days of plant growth (Mishra et al. 2009; Singh et al. 2011; Stajkovic et al. 2011). Elkoca et al. (2007) also showed increase in root weight and yield of chickpea by coinoculation of *Rhizobium* and *Bacillus* spp. Increase in nitrogen fixation and nodule formation was observed in the pigeon pea plants receiving combined inoculation of *Azospirillum, Bacillus* spp. and *Rhizobium* (Remans et al. 2008; Rajendran et al. 2008) (Table 7.6).

Mishra et al. (2009) showed that coinocualtion of *R. leguminosarum*-PR1 and PGPR *B. thuringiensis*-KR1, obtained from the nodules of *Kudzu* vine (*Pueraria thunbergiana*), promoted plant growth of field pea and lentil (*Lens culinaris* L.) under Jensen's tube, growth pouch and non-sterile soil, respectively. Combined inoculation of *R. leguminosarum*-PR1 and *B. thuringiensis*-KR1 (10^6 cfu. ml^{-1}) showed 85 and 73% increase in nodulation in pea and lentil, respectively as compared to individual treatment of *R. leguminosarum*-PR1. Similarly there was also higher shoot weight, root weight and total biomass was observed in combined inoculation treatments as compared to rhizobial application alone. There was 1.04 to 1.15 times and 1.03 to 1.06 times increase in shoot dry weight of pea and lentil, respectively by combined inoculation of different cell density of *B. thuringiensis*-KR1 as compared to inoculation of *R. leguminosarum*-PR1 alone at 42 days of sowing. Cell population of 10^6 cfu. ml^{-1} was found to be critical as higher cell density displayed inhibitory effects on plant growth and nodulation whereas lower one showed reduced cell retrieval and plant growth. Sameway increased nodule number and biomass yield were obtained upon combined inoculation of *B. japonicum* SB1 and *B. thuringiensis*-KR1 in soybean (Mishra et al. 2009).

Coinoculation of *Rhizobium* and P-solubilizing bacteria improved more plant growth as compared to individual applications (Morel et al. 2012; Walpola and Yoon 2013). Bai et al. (2003) stated that coinoculation of *Bacillus* strains with *B. japonicum* in soybean showed significant increases in nodulation, nodule weight, shoot weight, root weight, total biomass, total nitrogen and grain yield. Tariq et al. (2012) reported improvement in nodulation efficiency and grain yield by combined inoculation of plant growth promoting bacteria with crop specific rhizobia in legumes. Remans et al. (2007) showed *Rhizobium* isolates can effectively nodulate bean plants when coinoculated with phosphate solubilizing bacteria. Barbosa et al. (2007) showed that coinoculation of *Bradyrhizobium* sp. and *Paenibacillus polymyxa* Loutit (L) and *Bacillus* sp. (LBF410) can induce nodulation and increased root dry matter in *Vigna unguiculata*. Sameway, synergistic promotion of nitrogen fixation was observed upon coinoculation of endophytic PGPB and *Rhizobium* species in lentils (Khanna and Sharma 2011; Saini and Khanna 2012). In certain cases, PGPR strain which showed ability to increase efficiency of the *Rhizobium* strains in one legume does not showed same impact with another legume. For instance, *Bacillus* sp. strain CECT450 showed ability to improve nodule formation on common bean upon coinoculation with *R. tropici* CIAT 899, whereas it decreased nodule formation in soybean upon coinoculaion with *B. japonicum* USDA 110 (Camacho et al. 2001). Sameway, Elkoca et al. (2007) showed dual and triple mixtures of PGPR with *Rhizobium* OSU-142 and M-3 displayed no substantial result on common bean yield as compared to single inoculations of these bacteria except for *B. subtilis* strain OSU-142 + *B. megaterium* strain M-3, inoculation. Difference in response of coinoculation displayed necessity to develop suitable blends of rhizobia strain and PGPR for specific sites to improve growth of common bean.

Choudhary and Sindhu (2017) reported that coinoculation of chickpea with ACC deaminase producing *Mesorhizobium* strain MBD26 and rhizobacterial isolate RHD18 produced 59 nodules per plant and showed 112.9% increase in plant dry weight in comparison to untreated plants at 50 days of sowing. In presence of salt, bacterial inoculation displayed 31.2% increase in plant dry weight in comparison to untreated plants under in vitro conditions. At 80 days of sowing, combined inoculation of *Mesorhizobium* isolate MBD26 with rhizobacterial isolate RHD18 showed significant increase in nodule number (78 nodules/plant) and 141.9% increase in shoot dry weight in comparison to uninoculated controls.

7.8 Strategies for Improving N_2 fixation

Research efforts for improving nitrogen fixation ability of various strain of nitrogen fixing free living or symbiotic microorganisms were intensified recently as they could provide an alternative source of chemical fertilizers and thereby reduce our reliance on chemical nitrogenous fertilizers. Selection of appropriate approach for improving nitrogen fixation ability of microbial strains depends largely on state in which microbial strain carry out nitrogen fixation either free-living or symbiotically and genes to be targeted for strain improvement i.e. either nitrogen fixation (*nif, fix*) or nodulation (*nod*, *nol*, *noe*) genes. Till date, efforts to improve nitrogen fixation capacity of symbiotic nitrogen fixers of genus *Rhizobium* and *Bradyrhizobium* were intensively done as they form symbiotic relations with agronomically significant legumes (Shantharam and Mattoo 1997; Schmidt et al. 2017).

Numerous approaches were projected to enhance nitrogen fixation in legume crops either by (i) improvement of nodulation and extending host range by transfer of symbiotic plasmid or cloning of nodulation genes; (ii) enrichment of nitrogen fixation; (iii) breeding of legume cultivars for improved nodulation with efficient strains and (iv) nodulation and nitrogen fixation in non-legume crops. Another methodology used comprises the genetic management of non-legumes to integrate *nif* genes from bacteria (Dixon et al. 1997; Gough et al. 1997) or expansion of host range for symbiosis amongst rhizobia and non-legumes (Trinick and Hadobas 1995; Sindhu and Dadarwal 2001b).

7.8.1 Intensification of Nodulation and Expansion of Host Range

Rhizobium-legume relations are frequently host specific and there exist concept of cross inoculation groups wherein specific *Rhizobium* strain can efficiently colonize specific host plant which make them restricted to colonize narrow range of crops (Brewin 1991). Some particular rhizobia linked with the families *Cicereae*, *Trifolieae* and *Vicieae* have limited host ranges (Broughten and Perret 1999). In other symbiotic relationships, host specificity differs significantly between the symbiontic partners. *A. caulinodans* nodulates only *Sesbania rostrata* and *R. meliloti* nodules found on *Medicago, Melilotus* and *Trigonella* plants, while *Rhizobium* sp. NGR234, nodulates above 137 genera of legumes and non-legumes like *Parasponia andersonii* (Young and Johnston 1989).

In actual field situations, legumes encounter large number of rhizobial strains and there are chances of legume facilitated genetic interchange amongst rhizobia or genetic altercation between rhizobia and other types of rhizosphere bacteria (Osborn 2006). Kinkle et al. (1993) showed that exchange of plasmid among populations of *R. leguminosarum* bv. *viciae* and *B. japonicum* respectively, in non-sterile soil. Souza et al. (1994) provided indication that gene transfer was regular between native soil populations of *R. etli*. Because of genetic recombinations occurring in nature, rearrangements of genetic material between bacteria in soil occurs which ultimately results in evolution of new rhizobial populations dissimilar from that of inoculated one (Sullivan et al. 1995; Vlassak et al. 1996). Many times such genetic manipulations could develop greatly adaptable rhizobial population that will govern nodule development in succeeding years.

7.8.1.1 Transfer of Symbiotic Plasmid

Large number of *Rhizobium* strains own plasmids containing genes influencing nodulation (*nod, nol* and *noe* genes), nitrogen fixation (*nif* and *fix* genes) as well as additional cellular functions (Denarie et al. 1992; Fischer 1994). Symbiotic (*sym*) plasmids of *R. leguminosarum* and *R. meliloti* differ in size from 140 kb to 1500 kb (Beynon et al. 1980; Long 1989). Number and size of these plasmids differs between various strains. Transfer of *sym* plasmid of *R. leguminosarum* to other closely related rhizobia belonging to either bv. *trifolii*, bv. *viciae* or bv. *phaseoli* normally induce development of normal nitrogen-fixing nodules on host plants of donor strains (Beynon et al. 1980; Brewin et al. 1980) but when the *sym* plasmid of *R. leguminosarum* was transferred to distantly related species of *R. meliloti,* the transconjugants induce non-nitrogen fixing root nodules on pea and vetch (Kondorosi et al. 1980; Young and Johnston 1989). Similarly, Kondorosi et al. (1982) also observed that transconjugants of *Lotus* rhizobia or tropical cowpea miscellany rhizobia, carrying the symbiotic megaplasmid pRme41b of *R. meliloti* strain 41, formed white non-nitrogen-fixing nodules on *Medicago sativa*. When the *sym* megaplasmid (pRme41b) of *R. meliloti* was mobilized into *Agrobacterium tumefaciens* by cloning a *mob* region into the *sym* megaplasmid (Kondorosi et al. 1982), the transconjugants were capable to induce ineffective nodule like deformations on alfalfa roots. Introduction of *R. leguminosarum* or *R. trifolii sym* plasmid into *Agrobacterium tumefaciens* conferred the ability to nodulate pea and clover, respectively but the nodules formed were ineffective without formation of bacteroids (Hooykaas et al. 1981, 1982). Djordjevic et al. (1983) showed that transfer of plasmid pBRIAN (encoding clover specific nodulation and nitrogen fixation functions) to *A. tumefaciens* strain ANU109 enabled the strain to nodulate white clovers, whereas the same strain carrying the plasmid pJB5JI (encoding pea-specific nodulation and nitrogen fixation) failed to nodulate peas.

Truchet et al. (1984) mobilized the *sym* megaplasmid of *R. meliloti* strain 2011 into *A. tumefaciens* with the help of plasmid RP4 or PGM142. The consequential transconjugants encouraged root distortions on homologous hosts *Medicago sativa* and *Melilotus alba* but not on the heterologous hosts *Trifolium repens* and *T. pratense*. Cytological interpretations showed that bacteria entered only in shallow layers of host tissue by an uncommon infection progression. Sindhu and Dadarwal (1993) constructed recombinant strains by protoplast fusion between *R.* sp. *Vigna* and *R.* sp. *Cicer* that formed effective nodules on green gram but ineffective pseudonodules on chickpea. These results indicated that infection and nodule commencement genes could be expressed in heterologous rhizobia which leads to expansion of host range but bacteroid formation and formation of efficient nitrogen fixing nodules is challenging to attain.

A cryptic plasmid, pRmeGR4b, reported to affect nodulation capacity and competitiveness in *R. meliloti* GR4 (Sanjuan and Olivares 1989). Mutations in the relevant locus, spanning 5 kb region, delayed nodule formation and also reduced nodulation competitiveness. Nucleotide sequence analysis revealed the occurrence of two neighboring genes, *nfe*1 and *nfe*2 (nodule development proficiency), preceded by a functional σ54 and a NifA-dependent promoter (Soto et al. 1993). The *nfe* genes were not present in four other strains of *R. meliloti* and transfer of *nfe* genes by conjugation in these strains was found to increase nodulation efficiency in two of strains (Sanjuan and Olivares 1991a). Expression of both *nfe*1 and *nfe*2 is perhaps triggered in infection and nodule formation by alteration to microaerobic situations that trigger NifA synthesis. Adding of several replicas of *nif*A from *Klebsiella pneumoniae* correspondingly conferred improved nodulation effectiveness of constructed *R. meliloti* strains (Sanjuan and Olivares 1991b). However, Dillewijn et al. (1998) reported that this observed increase in nodulation was not reliant on plasmid-borne *nif*A activity however it was dependent on sensitivity of non-resistant strains to streptomycin carried over from growth cultures. Rogel et al. (2001) revealed that *Ensifer adhaerens* ATCC 33499, could not form nodules on *Phaseolus vulgaris* (bean) and *Leucaena leucocephala*. Transferring symbiotic plasmid of *R. tropici* CFN299 into *E. adhaerens* enables it to form nitrogen fixing nodules on both hosts. *R. tropici* was carefully chosen as donor as its *sym* plasmids deliberated nitrogen fixing nodule formation ability to *A. tumefaciens* on *Phaseolus vulgaris* and *Leucaena leucocephala* (Martinez et al. 1987). The plasmids “a” and “b” were co-transferred from *R. tropici* CFN299 together with plasmid “c” (carrying *nod-nif* genes) into *A. tumefaciens. A. tumefaciens* recombinant strains comprising three plasmids showed better nodulation and nitrogen fixation as compared to recombinant with only plasmid “c”.

7.8.1.2 Transfer of Cloned Nodulation Genes

Rhizobia possess coordinately regulated operons containing nodulation genes either one on symbiotic plasmids (p*sym*) or one on chromosome. Till date, above 60 diverse nodulation genes were described in various rhizobia (Sindhu and Dadarwal 2001a, b, c; Loh and Stacey 2003; Delamuta et al. 2017). Spaink et al. (1989) created chimeric *nod*D gene, containing 75% of *nod*D1 gene of *R. meliloti* at the 5′ end and 27% of *nod*D gene from *R. leguminosarum* bv. *trifolii*. Its expression in *R. leguminosarum* bv. *trifolii* and *R. meliloti* lead to expansion of host range for nodulation up to tropical legumes *Macroptilium atropurpureum*, *Lablab purpureus* and *Leucaena leucocephala*. Expression of chimeric *nod*D gene in *R. leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *viciae* similarly lead to substantial escalation of nitrogen fixation rates during symbiosis with *Vicia sativa* and *Trifolium repens*. Bender et al. (1988) moved *nod*D1 gene from *Rhizobium* strain NGR234 to a limited host range *R. leguminosarum* bv. *trifolii* strain and this exchange widened nodulation ability of beneficiaries to new hosts comprising non-legume *Parasponia andersonii*. Point mutations in *nod*D of *R. leguminosarum* bv. *trifolii* showed expansion of host range even to non-legume *Parasponia* (McIver et al. 1989).

The transfer of a 14 kb HindIII fragment on recombinant plasmid pRt032 (carrying *nod*ABC and *nod*D genes from *sym* plasmid of *R. leguminosarum* bv. *trifolii* strain ANU843) to other *Rhizobium* species or to *A. tumefaciens* provide capacity to nodulate clover by recipients (Schofield et al. 1984). The conjugative transfer of 14 kb HindIII fragment on plasmids pRt032 and pRKR9032, to *R. fredii* USDA192 strain, extended the host range of *R. fredii* even to clover (Yamato et al. 1997). Transconjugant strain NA102 and YA101 produce non nitrogen fixing small and whitish nodules on clover. The Nod factors synthesized by the transconjugants in presence of apigenin and genistein flavonoids also varied from those of their receiver strains. Concurrent inoculation of *Glycine max* and *Vigna unguiculata* roots with NodNGR factors and *nod*ABC mutants of strain NGR234 or *B. japonicum* USDA110 enabled bacteria to produce nitrogen fixing nodules on corresponding hosts (Relic et al. 1994). NodNGR factors also enabled entrance of *R. fredii* USDA257 into the roots of non-host *Calopogonium caeruleum* (Relic et al. 1994) and of *nod*ABC mutant of NGR234 into *Macroptilium atropurpureum* (Relic et al. 1993).

The allocation of the host-specific *nod*FEGHPQ genes of *R. meliloti* to strains of *R. leguminosarum* bv. *trifolii* or bv. *viciae* provided capacity of nodule formation on alfalfa (Putnoky and Kondorosi 1986) but intensely repressed nodulation on usual host plants, white clover and vetch, respectively (Debelle et al. 1988; Faucher et al. 1989). Mutations in the *nod*H gene of *R. meliloti* (involved in transfer of sulfate on lipo-oligosaccharide Nod factor) intensely repressed nodulation on common host *Medicago sativa* and directed to hindered nodulation on *Melilotus alba* but provided capacity to nodulate non-host plant, vetch (Faucher et al. 1988; Roche et al. 1991). Mutation in *nod*Q gene also expanded host range of *R. meliloti* to vetch (Schwedock and Long 1992). Transfer of *R. meliloti nod*HPQ genes into *R. leguminosarum* bv. *trifolii* or *R. leguminosarum* bv. *viciae*, none of which owns these genes, indicates production of sulphated Nod signals and prolonged the host range of these strains to alfalfa (Denarie et al. 1996; Long 1996). Mutation of strain NGR234 *noe*E gene (involved in fucose-specific sulfotransferase) obstructed nodulation of *Pachyrhizus tuberosus*, while its overview into closely linked strain USDA257 prolonged host range of *R. fredii* to encompass *Calopogonium caeruleum* (Hanin et al. 1997).

*Nod*L gene is essential for accumulation of an O-acetyl residue at terminal non-reducing glucosamine remainder in *R. meliloti* Nod factors (Ardourel et al. 1994). In strain NGR234, interruption of flavonoid-inducible *nol*L gene results in synthesis of NodNGR factors that lack 3-O- or 4-O- acetate group (Berck et al. 1999). The transconjugants of *R. fredii* strain USDA257 comprising *nol*L of NGR234 formed acetylated Nod factors and nodulated non-hosts *Calopogonium caeruleum*, *L. leucocephala* and *L. halophilus*. Acetylation of Nod factors' fucose of *R. etli* similarly deliberated effective nodulation on some *P. vulgaris* cultivars and on different host *Vigna umbellata* (Corvera et al. 1999). *Nod*Z gene, encodes a fucosyltransferase, which is essential for nodulation of legume siratro by *B. japonicum*, but alteration in *nod*Z of *B. japonicum* does not affect nodulation in soybeans considerably (Nieuwkoop et al. 1987; Stacey et al. 1994). NodZ$^-$ mutants of NGR234 vanished the ability to nodulate *Pachyrhizus tuberosus* (Quesada-Vincens et al. 1997). Allocation of *nod*Z gene to *R. leguminosarum* bv. *viciae* lead to in synthesis of fucosylated Nod signals and widen host range to comprise *Macroptillium* (Lopez-Lara et al. 1996). Inactivation of gene *nod*S, involved in methylation of Nod factors of *A. caulinodans*, NGR234 and *R. tropici* eliminated nodulation of *Leucaena leucocephala* and *Phaseolus vulgaris* (Lewin et al. 1990; Waelkens et al. 1995). Transfer of either *nod*S or *nod*U gene into *R. fredii* USDA257 expanded host range to include *Leucaena* spp. (Krishnan et al. 1992; Jabbouri et al. 1995). These outcomes shown that numerous replacements or alterations at reducing or non-reducing terminus of Nod factors could broaden the host range.

Castillo et al. (1999) utilized precise DNA amplification (SDA) approach to create *S. meliloti* strains CFNM101 and CFNM103, that demarcated 2.5 to 3 copies of symbiotic region (containing *nod*D1, *nod*ABC and *nif*N of p*sym* plasmid). Application of these strains to alfalfa created escalation in nodulation, nitrogen fixation and growth of alfalfa plants in environmentally controlled situations. Likewise, Mavingui et al. (1997) employed random DNA amplification (RDA) in symbiotic plasmid of *R. tropici* to get strains with improved competency for nodulation.

7.8.2 Improvement of Nitrogen Fixation

Structural or regulatory *nif* genes of the nitrogenase enzyme complex can be altered to enhance efficiency of nitrogen fixation. It was proposed that increasing *Nif*A construction, which is the transcriptional activator of other *nif* genes, could improve expression of entire N_2-fixing system (Szeto et al. 1990). Initially greenhouse experiments showed that certain *R. meliloti* strains with higher *nif*A gene expression exhibited a 7–15% rise in alfalfa plant biomass in comparison to parents (Williams et al. 1990). Meanwhile regulatory stage in nitrogen fixation appears to be process of attaching reduced dinitrogenase reductase (Fe-protein, the *nif*H gene product) to dinitrogenase (MoFe-protein) followed by one electron transfer. It was observed that increase in copy numbers of *nif*H gene and its products result in increase in throughput rate of nitrogenase which seems to be reason for occurrence of more than one copy of the *nif*H gene in certain diazotrophs such as *A. vinelandii* (Jacobson et al. 1986), *Rhizobium phaseoli* (Quinto et al. 1985) and *A. sesbaniae* (Norel and Elmerich 1987).

Alteration in expression of the C4- dicarboxylate transport (*dct*) genes could increase substrate transport which in turn increase nitrogen fixation efficiency (Ronson et al. 1990). Root nodules contains photosynthetic energy and utilize roughly 10% of the plant's net photosynthates for nitrogen fixation. Therefore, nitrogen fixation in the *Rhizobium*-legume symbiosis is supposed to be partial by amount of plant-derived photosynthetic outputs accessible to bacteroids (Hardy and Havelka 1975; Sindhu et al. 2003). Birkenhead et al. (1988) proposed that increasing efficiency of endosymbiont to use photosynthate in nodule may results in improved nitrogen fixation rates. Recombinant strains of *R. meliloti* and *B. japonicum* with better expression of *dct*A (structural gene for dicarboxylate transport) and *nif*A genes exhibited 15% escalation in nitrogen fixation rates (Ronson et al. 1990).

Certain nitrogen fixing bacteria like *Rhizobium*, *Azotobacter, Azospirillum* etc. found to increase efficacy of nitrogen fixation by oxidizing hydrogen by means of hydrogenase enzyme, that concurrently formed and developed during nitrogen fixation (Sindhu et al. 1994; Garg et al. 1985). This oxidation of hydrogen enhance ATP biosynthesis. Improved nitrogen fixation efficiency described in nodules and bacteroids of soybean, pea and *Vigna* group of hosts designed by application of Hup$^+$ strains (Emerich et al. 1979; Dadarwal et al. 1985; Evans et al. 1987). Improved hydrogenase activity in root nodule bacteroids showed increase in soybean yield by use of near isogenic strains of *B. japonicum* (Hanus et al. 1981; Hungaria et al. 1989). Second strategy to increase yields could be to increase the activity of the hydrogenase in bacterial strains that previously own it. Mutants of Hup$^+$ *B. japonicum* strains (Merberg and Maier 1983) or *Rhizobium* sp. strains (Sindhu and Dadarwal 1992) were developed with enhanced hydrogenase activity. Inocualtion of mutants of *Rhizobium* sp. strains showed higher in dry matter yield of green gram and black gram. The *hup* genes, coding biosynthesis of uptake hydrogenase was cloned and utilized to transform Hup$^-$ strains. These Hup$^+$ recombinants exhibited increased nitrogen fixation (Pau 1991).

7.8.3 Breeding for Enhanced Nodulation

Changing the genetic make-up of plants to influence both endophytic and external populations suggest likelihood of creating favorite rhizosphere communities (O'Connell et al. 1996; Sindhu et al. 2018). Plant breeding strategy could be used to combine preference traits from several sources to generate plant genotypes capable of excluding nodulation by ineffective indigenous rhizobia. Hardarson et al. (1982) showed that the selection of alfalfa for physiological and morphological traits associated with nitrogen fixation capability altered the preference of the host plant for effective strains of *R. meliloti*. Nutman (1984) reported that red clover bred for improved nitrogen fixation maintained its superiority against a range of *R. leguminosarum* bv. *trifolii* strains. These studies illustrated the potential for developing broad-spectrum effectiveness for genetically diverse indigenous rhizobia in some legume species. Mytton et al. (1984) assessed genetic variation in nitrogen fixation in different cultivars of *M. sativa* inoculated with diverse strains of *R. meliloti*; one of these cultivars was found relatively insensitive to changes in *Rhizobium* genotype and maintained high average yield.

The specific compatibility between *nod*X of *R. leguminosarum* bv. *viciae* strain TOM and *sym*2 of *Pisum sativum* cv. Afghanistan could be utilized to avoid native rhizobia from nodulating and to permit inoculated strains to nodulate. The *sym*2 gene has already been crossed into a desirable pea cultivar (Trapper) and *nod*X was transferred in effective N_2-fixing *Rhizobium* strain. Performance of these manipulated host cultivar and rhizobial strains appeared promising enough under field studies (Fobert et al. 1991). A similar combined approach involving alteration of both soybean host and *Bradyrhizobium* strains has also been carried out to improve symbiotic N_2 fixation in soybean-*B. japonicum* symbiosis (Cregan et al. 1989; Sadowsky et al. 1991). This strategy involves use of soybean genotypes that restrict the nodulation of indigenous competitive strains and allow nodulation only with desired added strains (Sadowsky et al. 1995). In this way, improved strains produced by genetic engineering or other techniques can be targeted to specifically improve soybean varieties. Thus development of legume cultivars with broad-spectrum effectiveness for genetically diverse indigenous rhizobia could be an alternative beneficial plant breeding strategy to obviate the requirement for legume inoculation (Brockwell and Bottomley 1995). This requires an understanding of the genetics of host and rhizobia, and offers real promise for genetically well-defined systems such as alfalfa and soybean.

Alternative approach of improving number of nodules by alteration of host genome is also utilized to increase nitrogen fixation ability in symbiotic microbes. Proposed strategy is based on hypothesis that nodule formation in legume is suboptimal and obviously increase in nodule numbers results into increased rate of nitrogen fixation. Hypernodulating mutants of soybean developed 100 times more nodules as compared to parent plant (Carroll et al. 1985; Betts and Herridge 1987). Scientists have isolated number of soybean mutants with the higher nodulation efficiency even in presence of nitrate (Carroll et al. 1985) which can produce 3–40 times higher number of nodules as compared to parent crop and demonstrated improved nitrogen fixation capacity (Hansen et al. 1989). Unluckily, these mutants were found to be poor agronomic performers (Pracht et al. 1994) due to fact that plant used up large extent of energy in hosting root nodules and thereby restricting energy required for the nitrogen fixation (Kennedy et al. 1997). Sato et al. (1999) altered source-sink association in hypernodulating soybeans by reducing infection dose so that nodulation is optimized to standard level and resolved that autoregulatory control may play crucial role in improving the number of nodules in soybeans and total nitrogen fixation activity.

7.8.4 Nodulation and Nitrogen Fixation in Nonlegume Hosts

Certain non-legume plants are able to establish nitrogen-fixing symbiosis. The *Frankia* are of great importance which nodulates woody angiosperms like *Alnus* or *Casuarina*. These nodules have simple, branched structures; indicative of solidified lateral roots, however their capability to fix nitrogen is comparable to that in legumes (Clawson et al. 1998). Likewise, non-legume nodulation and nitrogen fixation was seen with *Bradyrhizobium* application in *Parasponia* (Trinick and Hadobas 1995; Webster et al. 1995) with high capacity of nitrogen fixation and structurally related to actinorhizal nodules.

*Nod*D gene of rhizobia proved to regulate the initial level of host specificity (Denarie et al. 1992). Transfer of *nod*D1 gene from NGR234 into *R. leguminosarum* bv. *trifolii* expanded host range to nodulate non-legume *Parasponia andersonii* (Bender et al. 1988). Plasmids containing *nod*DABC genes of *R. leguminosarum* bv. *trifolii* were reassigned to *A. tumefaciens, P. aeruginosa, Lignobacter* sp., *A. brasilense, E. coli* and different non-nodulating mutant rhizobia (owning *sym* plasmid deletions) which enabled them to perform root hair curling and alterations on clover and large number of other non-host legumes (Plazinski et al. 1994), proposing manifestation of *nod*DABC genes in varied array of soil bacteria may spread or effect normal growth of plant root hairs of a varied kind of host and non-host legumes. Attempts to enhance nitrogen fixation by modification of macrosymbiont host plant have been done with leguminous crops such as soybean and alfalfa. In recent times, two model legumes *Medicago truncatula* and *Lotus japonicus* are identified for genetic examination of nodule formation and operational facts of root nodules, by which transgenics can usually be produced. These legumes will perform as tools for the identification and genetic characterization of plant genes engaged in nodule development as well as provides idea about mechanisms controlling root nodule formation.

Present studies to transfer the nitrogen fixation capability to nonleguminous plants showed nodule like structures could be formed on rice and wheat roots with *Rhizobium* strains in artificial conditions by means of hormones or cell wall degrading enzymes (Al-Mallah et al. 1989; Cocking et al. 1994). A precise investigation of these nodule-like structures shown accumulation of bacteria at the spot of lateral root formation and get enter through cracks. *Rhizobium* strains obtained from *Aeschynomene indica* (strain ORS310) and *Sesbania rostrata* (strain ORS571) were observed to produce nodule like structures on developing secondary roots of rice, wheat and maize (Cocking et al. 1994) and displayed considerable nitrogen fixation activity. Wheat plants inoculated with *A. caulinodans* showed higher nitrogen fixation activity whereas uninoculated plants as well as those inoculated with *nif*$^-$ strain of *A. caulinodans* showed absence of nitrogen fixation (Sabry et al. 1997). *A. caulinodans* strain ORS571 having *lac*Z reporter gene was found to be present in cracks of developing lateral roots in rice and wheat (Webster et al. 1997). Sameway, *lac*Z containing *A. caulinodans* strain ORS571 could enter *Arabidopsis thaliana* roots through cracks developed during lateral root formation. The flavonoids, naringenin and diadzein at low concentration considerably roused incidence of lateral root cracks and intercellular colonization of *A. thaliana* roots by *A. caulinodans.*

Tchan and Kennedy (1989) reveled induction of ‘para nodules’ on wheat by application of 2, 4-dichlorophenoxyacetic acid (2, 4-D) or with auxins IAA and NAA (Naphthyl-acetic acid), along with inoculation of rhizobia or *Azospirillum.* Rolfe and Bender (1991) demonstrated formation of paranodules on rice roots by inoculation of *Rhizobium* having *nod*D allele whose gene product interacts with rice root exudate but could not display nitrogenase activity. It was discussed that transformation of rice may induce new genes and thereby provides great chance to examine the probability for nitrogen fixation in rice. In addition, certain early nodulin homologous genes in legumes were detected in rice genome which is yet to be studied in depth.

In depth research understanding about different physiological and genetic processes in legume plants and bacteria as well as detection of key characters for nodulation in legumes might provide opportunity to enable nonlegumes like rice and wheat to be engaged in symbiosis with nitrogen fixing bacteria (Kennedy and Tchan 1992). Consequently, widespread fundamental studies are required to realize relations between *Rhizobium* and cereal plants with specific weightage on signal exchange mechanisms. Additionally, these altered nodule like structures on lateral roots of cereals must develop microaerobic environment for protection of oxygen sensitive nitrogenase. To develop oxygen protection mechanism, plant could be engineered to accumulate polysaccharides or other O_2 eliminating material within intercellular space upon infection. Plentiful efforts and management are needed in genetics, molecular biology and developmental biology to attain a comprehensive understanding of the *Rhizobium* legume symbiosis and to discover future opportunities for attaining final objective of expressing active nitrogenase in cereal crops (Dixon et al. 1997; Shantharam and Mattoo 1997).

7.9 *Rhizobium* Based Commercially Available Inoculants

Strategies to improve crop production by inoculating plant growth promoting bacteria is accelerated as developing technology because of their environment friendly potentials. Bioinoculants like biofertilizers has been popularized since many years to get advantage positive effects of various soil microbes to boost plant growth and yields. Biofertilizers are microbial inoculants comprising of microbial strains having capacity of nitrogen fixation, phosphate solubilization/mineralization, phytohormone production and biocontrol activities. Rhizobial strains generally utilized as biofertilizers (singly or in mixture) contain a number of genera: *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* (Table 7.7). Prime focus points for development of rhizobial biofertilizer technology are development of appropriate formulation with suitable carrier and adoption of appropriate application methods. Rhizobia-based inoculants generally used for improvement of growth and yield of leguminous crops, whereas *Azotobacter* and *Azospirillum* for enhancement of cereal growth. On contrary *Bacillus* and *Pseudomonas* are utilized as biocontrol agents (as biopesticides) against plant diseases (Fravel 2005; Bravo et al. 2011). Table 7.7 displays certain selected

Table 7.7 Marketed *Rhizobium*-based biofertilizers

Bacteria	Product	Company
Rhizobia	VAULT® HP plus INTEGRAL®	Becker Underwood Corporate, USA
Delftia acidovorans and *Bradyrhizobium*	BioBoost	Brett Young Seeds Ltd., Canada
Rhizobium sp.	SeedQuest®	Soygro (Pty) Ltd., South Africa
Rhizobium sp.	Legumefix	Legume Technology Ltd., UK
Bacillus subtilis and *Bradyrhizobium japonicum*	HiStick N/T, Turbo-N	Becker Underwood Corporate, USA
B. subtilis and *B. japonicum*	Patrol N/T	United Agri Products (UAP) Inc., Canada
Burkholderia cepacia type Wisconsin	Deny	Market VI LLC, Vern Illum 6613 Naskins Shawnee KS 66216, USA
Rhizobium spp.	*Fasloon Ka Jarasimi Teeka*	AARI, Faisalabad, Pakistan
Rhizobium spp.	BioPower	NIBGE, Faisalabad, Pakistan
Rhizobium spp.	Biozote	NARC, Islamabad, Pakistan
Rhizobium spp. and PGPR	Rhizogold	ISES, UAF, Faisalabad, Pakistan
Bradyrhizobium spp. *Mesorhizobium* sp. *ciceri* and PGPR	Rhizoteeka, Azoteeka and phosphoteeka	CCS Haryana Agricultural University, Hisar, India

commercially existing rhizobial inoculants with their producers/trade name. The development of mass production technology for commercial manufacturing of microbial inoculants like biofertilizers is the key point to be considered for spreading wide use of biofertilizers.

7.10 Performance and Limitations of Inoculant Strains

Rhizobial inoculation in soil have showed colonization of soil as well as plant roots to a extent adequately high for proposed aim. In majority of the cases expected effect of biofertilizers inoculation is not witnessed under field conditions in legume or cereal plants and frequently fails to increase crop yield (van Elsas and Heijnen 1990; Akkermans 1994). Regulating factors for performance of microbial strains under field conditions includes abiotic soil factors such as texture, pH, temperature, moisture content and substrate accessibility which should be determined crucially as they showed great influence on survival and activity of inoculated microorganisms (Hegazi et al. 1979; Sindhu and Lakshminarayana 1982; van Veen et al. 1997; Hansena et al. 2018). Efficiency of inoculated nitrogen fixing bacterial strain is determined by genetic and physiological efficiency of bacterial strain (Brockwell et al. 1995). Insertion of genetic markers viz. antibiotic resistance genes or other metabolic markers could assist to mark out introduced strains, whether it is rhizobia, cyanobacteria, azotobacter or azospirilla (Wilson et al. 1995).

A main factor limiting feat of rhizobial inoculants is its inability to survive under competitive stress with the native strains for nodulation (Sindhu and Dadarwal 2000; Sindhu et al. 2003). Rhizobia produces bacteriocins which can inhibit growth of and nodulation by the native ineffective strains (Goel et al. 1999; Sindhu and Dadarwal 2000). Transfer and expression of genes involved in trifolitoxin synthesis i.e. *tfx* genes in rhizobia resulted in to constant synthesis of trifolitoxin and controlled nodulation by indigenous trifolitoxin-sensitive strains on many leguminous crops (Triplett 1988, 1990). Though, efforts to manipulate some rhizobial genes in particular legume rhizosphere places for improving competence failed to show notable results (Nambiar et al. 1990; Sitrit et al. 1993; Krishnan et al. 1999).

Biotechnological methods for improving nitrogen fixation and crop production having narrow utility in field conditions. For example, recombinant strain of *R. meliloti* and *B. japonicum* showed higher expression of *nif*A and *dct*A genes indicating intensification in rate of N_2 fixation but in field conditions, recombinant strains didn’t performed well for nitrogen fixation or yield enhancement (Ronson et al. 1990). Alteration of nodulation genes to increase bacterial competence generally resulted in either no nodulation, delayed nodulation or inefficient nodulation (Devine and Kuykendall 1996). Mendoza et al. (1995) improved NH_4^+ assimilating enzymes in *R. etli* by adding an extra copy of glutamate dehydrogenase (GDH), ultimately showed retardation of nodulation on bean plants. Such inhibitory effect was minimized by *Nif*A and thereby postponing the inception of GDH activity after nodule formation (Mendoza et al. 1998). In the same way, efforts to manipulate hydrogen uptake (Hup^+) ability by cloning hydrogenase genes into Hup^- strains of *Rhizobium* showed success only in parts where soybeans are cultivated under restricted photosynthetic energy (Evans et al. 1987). Efforts to construct self-fertilizing crops for nitrogen was also disappointment due to complex nature of nitrogenase enzyme system under unavailability of oxygen safeguard system in eukaryotes (Dixon et al. 1997). Stimulation of nodule formation (pseudonodules) in wheat and rice crops by lytic enzyme of hormonal treatment displayed nitrogenase activity and nitrogen integration in plants. However, the activity expressed is >1% of the significance seen in legumes (Cocking et al. 1994).

7.11 Conclusion

Biological nitrogen fixation provides nitrogen to leguminous crops and hence considered to be significant process for improving yield. Symbiotic nitrogen fixing systems like rhizobia and legumes can fix significant quantity of nitrogen by acclimatizing with varied ecological conditions. So that, influence of rhizobia on legumes cannot be ascertained exactly under harsh environment and there is need to isolate stress tolerant rhizobial strains to act under stress in soil ecosystem which in turn ensures survival and growth of inoculated legumes in challenging soil. We are enriched with the research about molecular mechanism of nitrogen fixation but it is yet to be involved in applied aspects under field studies. As a way out of issue regarding establishment of microbes after inoculation, diazotrophic inoculants should be chosen from native ecological boundaries and re-inoculated in similar environment for ensuring anticipated benefits. Forthcoming research should concentrate on unveiling in situ physiology of inoculant and means to manipulate the same. On applied side, idea development of mixed inoculum with ecologically different strains having same roles should be tested as an alternative of monoculture. The coinoculation of diazotrophic bacteria with rhizosphere bacteria or the inoculation of microbial consortia is preferable because these microorganisms might express beneficial functions more frequently in a soil or rhizosphere system, even under ecologically diverse and/or variable circumstances. Hence, both customary and biotechnological methodologies can be used to improve nitrogen fixation efficiency and crop production in sustainable agriculture.

In general, inoculative application of *Rhizobium* provide 10–15% yield increase in leguminous crops. On the other hand, anticipated effect of biofertilizer application on legumes is generally not attained in field conditions. Commercial inoculants generally fails under field condition because of incompetence to strive with the native, ineffective microbes, which offers a competitive obstruction to inoculated strains. Efforts to operate some rhizobial genes in particular legume rhizosphere environment to improve survival under competitive stress were not successful up to mark. This chapter emphasize potential of plant growth promoting rhizobia for sustainable agriculture as well as highlighted exceptional characteristics to cope up with various biotic and abiotic stresses on a various agricultural crops. Thus, development of broad knowledge on screening approaches and concentrated selection of superlative *Rhizobium* strains for rhizosphere competence and survival is required to improve field efficiency of applied strains. Characterization of such prospective rhizobial strains and evolving a strong technology for farmers is still in developing phase. Current developments of 'omics' technologies provided prospects to exploit genomic, transcriptomic, proteomic and metabolomic means to alter the characters of 'biological designers' to maximize their plant growth promotion proficiency. Bioengineering could possibly be used to operate the tolerance, accumulation and degradation potentials of plants and microbes against pollutants.

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