

Chapter 12

Biotechnological Perspectives of Legume–*Rhizobium* Symbiosis

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12.1 Introduction

Soil microbial populations are involved in fundamental activities that ensure the stability and productivity of both agricultural systems and natural ecosystems. Certain cooperative microbial activities can be exploited as a low-input biotechnology to help sustainable and eco-friendly practices. Much attention is created toward biological legume–rhizobia symbiosis. In this symbiosis, bacteria fix nitrogen from the atmosphere and the process is known as biological nitrogen fixation (BNF). BNF is an efficient source of fixed N₂ and has the greatest quantitative impact on the nitrogen cycle; values commonly fall in the range 200–300 kg N ha⁻¹ per year (Peoples et al. 1995). In addition to fix nitrogen, legume symbiosis has potential for reforestation and to control soil erosion. The root nodule bacteria can be used in several other biotechnological niches such as production of industrially important polysaccharides, polymers such as *poly*-hydroxy butyrate, organic acids, and antibiotics (Gopalakrishnan et al. 2015). It has been observed that rhizobia could express several plant growth-promoting characteristics like increase in the availability of plant

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nutrients, biocontrol, and production of phytohormones, lipochitooligosaccharides (LCOs), lumichrome, siderophores, hydrogen cyanide (HCN), exopolysaccharides (EPS), and enzymes, which are involved in plant growth promotion and yield by direct or indirect ways. A better understanding about the rhizobia spp. and their interactions with other soil microorganisms may lead to new biotechnological approaches to improve the growth and yield of plants. This chapter presents a discussion on the *Rhizobium*–legume symbiosis and their biotechnological perspectives. The mechanism of rhizobia as plant growth-promoting rhizobacteria (PGPRs) and their interactive effects with other PGPRs and arbuscular mycorrhiza (AM) to enhance crop production have also been discussed.

12.2 Legume–Rhizobia Symbiosis: General Information

The legume symbiosis process is driven by bacteria that possess the key enzyme nitrogenase, which specifically reduces atmospheric N_2 to ammonia in the symbiotic root nodules. These bacteria belong to the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Azorhizobium* and collectively termed rhizobia (Karmakar et al. 2015). These bacteria interact with legume roots leading to the formation of N_2 -fixing nodules. The symbiosis begins with the release of root exudates such as sugars, amino acids, several classes of flavonoids, and phenolic compounds (Junior et al. 2015). These compounds behave as chemo-attractant for rhizobia and act as nodulation gene inducers (Hirsch and Fujishige 2012). These exudates are continuously secreted into the rhizosphere and their concentration increases when compatible bacteria are detected by the plant (Hassan and Mathesius 2012). These compounds activate *nodABC* genes in rhizobia that are required for the synthesis of Nod factor backbone. *nodA* encodes an *N*-acyltransferase, *nodB* encodes a chitooligosaccharide deacetylase, and *nodC* encodes an *N*-acetylglucosaminyl-transferase (Mus et al. 2016). The bacterial Nod factor perception is mediated by Nod factor receptors (NFRs), which are present in plant plasma membrane. Hence, NFRs act as host determinants for symbiotic specificity. NFRs are serine/threonine kinases like receptors and contain LysM motifs in their extracellular domains (Arrighi et al. 2006). Nod factors induce curling of root hairs and allow entry of rhizobia into infection threads.

The success of the symbiotic process depends on the compatibility and effectiveness of rhizobia with their host legume. The compatibility of this symbiosis depends on a vast range of compounds secreted by both plants and bacteria. The host produced lectins have the ability to recognize and bind to specific *Rhizobium* sp. cells due to their different carbohydrate-binding specificities. For example, soybean lectin, a galactosamine-binding protein, differs from pea lectin, a Glc-/Man-binding protein, and they both differ from their *Rhizobium* sp. also (Junior et al. 2015). In addition to Nod factors, several other bacterial compounds also affect the interaction, including EPS and nodulation outer membrane proteins (NOPs) (Frayse et al. 2003; Downie 2010). EPS is a bacterial cellular wall constituent, which is involved in both early and late stages of symbiosis. *M. loti* EPS mutants was found to fail nodule formation in

L. leucocephala (Hotter and Scott 1991). NOPs are reported to contribute in legume immune suppression during symbiosis development and modulate root cell cytoskeletal rearrangement (Gourion et al. 2015). In addition, rhizobial population also differs in the same host due to variability in soil and environmental conditions. For example, *Glycine max* is usually nodulated by *B. japonicum* globally, while in the Xinjiang region of China, *Mesorhizobium tianshanense* and *Sinorhizobium fredii* have also been recovered from this legume (Naamala et al. 2016). In another example, *Cicer arietinum* has been reported with *Mesorhizobium ciceri* and *Mesorhizobium mediterranean*, but *Sinorhizobium meliloti* was also reported from nodules of this legume under water-deficient conditions (Romdhane et al. 2009).

12.3 Biotechnological Perspectives

12.3.1 Genetic Engineering

Biotechnology embraces a wide range of genetic engineering techniques offering opportunities for researchers to increase the efficiency of BNF and reduce overall dependency on man-made forms of N fertilizer. In genetic engineering, both microsymbiont and host plant can be manipulated for increased nitrogen fixation capacity. Identification and analysis of host plant genes involved in nodule morphogenesis and functioning will be useful for genetic manipulation. On the other hand, rhizobial Nod genes are required for gene engineering (Mabrouk and Belhadj 2012).

Wild legumes in arid climate have specific traits, which may be transferred to crop legumes to improve their tolerance to the stress conditions. In addition, rhizobial strains from these wild legumes are good candidates for establishing functional symbiosis in the presence of unfavorable conditions. Mabrouk and Belhadj (2012) paid attention toward the demand of rhizobial species that could also work under stressed soil environment so that the productivity of the inoculated legumes does not suffer under derelict soils. A chromosomal DNA from the salt-tolerant *Bacillus* species was successfully transferred into *R. leguminosarum*. This rhizobial strain became salt tolerant and when inoculated with lentil, it improved plant yield and nitrogen content of soil and plant in desert soil (Talaat El-Saidi and Ali 1993). Defez et al. (2000) have successfully transferred a 10 kb DNA fragment from a wild-type strain of *Sinorhizobium* into *Rhizobium etli*, which showed multiple resistance to several antibiotics, 4% NaCl, low and high pHs, heavy metals, and high temperature. Two strains of *M. ciceri*, namely, EE-7 (salt sensitive) and G-55 (salt tolerant), were transformed with an exogenous 1-aminocyclopropane-1-carboxylate deaminase gene (*acdS*) and found to enhance shoot dry weight of plants as compared with the plants inoculated with the native strain in the presence of salt (Brígido et al. 2013).

12.4 Microbial Cooperation in the Rhizosphere

The application of appropriate rhizobia together with plant growth-promoting microorganisms is considered an effective and environment-friendly approach to increase the efficiency of the symbiotic processes and the crop yield by different mechanisms of actions under variable conditions. Multi-microbial interactions, including arbuscular mycorrhizae and PGPR, have been tested with *Rhizobium* spp. In such interactions, local isolates are recommended because of their physiological and genetic adaptation to the environment. These combinations are effective in improving plant development, nutrient uptake, N₂ fixation (¹⁵N), or root system quality. Two types of cooperations are discussed here: (1) the cooperation between rhizobia and arbuscular mycorrhizal fungi and (2) interaction between PGPR and rhizobium.

12.5 Rhizobia and Arbuscular Mycorrhizal Fungi (AMF) Cooperation

The occurrence of AMF in nodulated legumes is universally recognized that explored the research on the tripartite symbiosis of legume–AM fungi–rhizobia from the last two decades (Barea et al. 2005). The findings of tripartite symbiosis suggested the similar patterns of evolution and interaction of both the N₂-fixing and mycorrhizal symbioses (Parniske 2000). Gianinazzi-Pearson and Dénarié (1997) hypothesized that legume–rhizobia symbiosis events have evolved from already established AM symbiosis. Most of the studies have used mycorrhiza-defective mutants and isotope ¹⁵N, which allowed dissecting the common cellular and genetic programs responsible for the legume symbioses and quantify the amount of N that is fixed in that particular situation (Gollotte et al. 2002). Some AM fungi have established a specific type of symbiosis with a particular rhizobium such as *Burkholderia* genus. These bacteria have specific metabolic genes that influence AM functions (Bianciotto and Bonfante 2002). This tripartite symbiosis not only enhances N content but also improves soil fertility and quality (Requena et al. 2001). AM inoculation also improved legume–rhizobia symbiosis under stress conditions such as low water potential and salinity (Augé et al. 2001; Ruiz-Lozano 2003). For example, inoculation of AM fungi was found to protect soybean plants against the negative effects of drought stress and reduced premature nodule senescence (Porcel and Ruiz-Lozano 2004). Soliman et al. (2012) reported that the co-inoculation of *S. teranga* and AMF mitigated the salinity effect in *Acacia saligna*. They found that the co-inoculation improved nodulation, chlorophyll, carbohydrate, and proline; increased N, P, K, and Ca contents; and reduced the Na accumulation.

12.6 PGPR–*Rhizobium* Cooperation to Improve N₂ Fixation

PGPRs share common habitats with rhizobia in the root–soil interface. These PGPRs enhance the performance of rhizobia by suppressing/eliminating the effect of other rhizosphere strains and adverse environmental factors. *Azospirillum* is a widely recognized PGPR for its ability to improve nodulation and biomass of legumes as a co-inoculant of rhizobia (Remans et al. 2008). Many species of *Azospirillum* like *A. lipoferum*, *A. brasilense*, *A. amazonense*, *A. halopraeferens*, and *A. irakense* have been identified as co-inoculant of rhizobia (Gopalakrishnan et al. 2015). These bacteria produce plant hormones and siderophores and enhance total nutrient availability, which have been claimed as a mechanism for growth and biomass enhancement of various legumes (Wani et al. 2007; Dardanelli et al. 2008; Cassan et al. 2009). Other bacterial genera like *Bacillus*, *Pseudomonas*, *Azotobacter*, *Serratia*, and *Enterobacter* have also been reported as a co-inoculant of rhizobia (Naveed et al. 2015). Bano and Fatima (2009) studied the co-inoculation effect of *Rhizobium* and *Pseudomonas* on alleviation of salinity stress in maize. They observed increase uptake of Ca, P, and K and decreased leakage of electrolytes in maize. Such type of bacterial cooperations promote nodulation and biomass of various legumes via improving rhizobial colonization, number of infection sites, biocontrol of diseases, and efficient uptake of nutrients (Naveed et al. 2015).

12.7 *Rhizobia* as a Plant Growth Promoter

Besides nitrogen fixation, rhizobia have also been reported for plant growth promotion in legumes and non-legumes. Their associations modify the physiology and biochemistry of crop plants which enhanced plant growth under normal as well as stress conditions. Rhizobia can affect the plant growth in two different ways: directly or indirectly.

The direct growth promotion of the plant is regulated by producing plant hormones, regulating endogenous ethylene level, enhancing total available nutrient contents, and releasing other useful compounds like EPS, lumichrome, riboflavin, etc. (Gopalakrishnan et al. 2015). During rhizobial infection, ethylene was produced in infected roots and several times caused inhibition of nodulation in various legumes (Glick 2014). Elsheikh and Ibrahim (1999) have suggested the isolation and introduction of efficient rhizobial cultures with ACC deaminase to improve the nodulation status, seed quality, and legume productivity. There are several rhizobial strains including *R. japonicum*, *R. leguminosarum*, *R. hedsari*, *R. gallicum*, *B. japonicum*, *B. elkanii*, *M. loti*, and *S. meliloti* having an enzyme ACC deaminase which could reduce this stress by decreasing the level of ethylene in the host plant (Gopalakrishnan et al. 2015). The EPS producing rhizobial strains can relieve the effect of water deficit stress by altering soil properties. Sandhya et al. (2009) have

reported the capability of EPS producing rhizobial strain for improving water-holding capacity of the rhizosphere by developing microaggregates and biofilm around the plant roots. In another study, rhizobia alleviated water stress by altering root morphology, carbon assimilation rate, transpiration rate, and leaf stomatal conductance in non-nodulating crops (Chi et al. 2005). Various *Rhizobium* spp. are also studied for plant growth promotion via producing multiple phytohormones such as auxins, abscisic acid, cytokinins, gibberellins, ethylene, and nitric oxide (Khalid et al. 2006; Perrine-Walker et al. 2007). In addition, *Rhizobium* spp. have been found to produce lumichrome compounds, which act as an enhancer of plant growth prior to the onset of nitrogen fixation (Cooper 2007). Gouws et al. (2012) recovered lumichrome from *Rhizobium* spp. which was found to enhance nodule number when applied to *Lotus japonicus*. *Rhizobium* spp. have the ability to sequester and transport iron into plant cell via siderophores and solubilize/mineralize phosphates by releasing phosphatases or organic acids in the rhizosphere (Naveed et al. 2015).

Rhizobia can benefit plant growth indirectly by several mechanisms such as antibiosis, parasitism, competition for nutrients, and induction of systemic resistance (ISR). *Rhizobium* spp., namely, *R. leguminosarum*, *S. meliloti*, and *B. japonicum*, have been found to show parasitism against fungal pathogens belonging to genera *Macrophomina*, *Rhizoctonia*, and *Fusarium* (Siddiqui et al. 2000; Özkoç and Deliveli 2001). Several studies have demonstrated that *Rhizobium* spp. enhanced defense mechanisms of plant via ISR in non-leguminous crops (Reitz et al. 2002; Mishra et al. 2006). Siderophores are also reported to inhibit a widely occurring plant pathogen, *Macrophomina phaseolina*, by chelating irons in deficient environments (Arora et al. 2001).

12.8 Bioremediation

Legumes have attracted attention for their use in metal phytostabilization. Legume plants accumulate most of the metals in their roots and show low level of metal translocation to the shoot. The activity of phytostabilization is mainly due to their attached rhizobia spp. Different rhizobial strains were reported as an efficient biosorbent for Cd^{2+} , Ba^{2+} , Zn^{2+} , Al^{3+} , Ni^{2+} , and Co^{2+} (Pajuelo et al. 2011). Several studies have reported the alleviation of different metal stress in legume plants by inoculating *Rhizobium* spp. (Ausili et al. 2002; Panigrahi and Randhawa 2010; Dary et al. 2010). Rhizobia also produced some specific compounds such as EPS and lipopolysaccharide (LPS) that chelate different metal ions in the rhizosphere. These compounds are biopolymers that possess negatively charged ligands which instantly form complexes with metal ions through electrostatic interactions (Liu et al. 2001; Sutherland 2001).

Different approaches are used to improve metal bioremediation potential of legumes. Inoculation of legume plants with metal resistant microbes including *Rhizobium*, AMF, endophytes, and other PGPR has been proved to be a promising

and cost-effective technology for metal phytostabilization and allowing the re-vegetation of metal-contaminated areas (Pajuelo et al. 2011). The genetic engineering of rhizobia approach has been used to improve the legume–*Rhizobium* symbiosis for bioremediation purposes. Sriprang et al. (2002) constructed a genetically modified strain of *M. huakii* subsp. *rengei* B3 by the expression of metallothionein (MTL4) gene on the cell surface. This strain was found to accumulate 1.7-fold increase in Cd into nodules and root of *Astragalus sinicus* plants. In another study, the same strain was engineered with a phytochelatin synthase gene from *A. thaliana* (Sriprang et al. 2003).

12.9 Engineering the Legume Symbiosis into Cereals

Advances in our understanding of the rhizobium–legume infection process have given rise to different biotechnological approaches to develop symbiotic nitrogen fixation in cereals. The engineering of nitrogenase-encoding bacterial *nif* genes into plants is a direct approach for creating nitrogen fixation in cereals. A wide study on nitrogenase biosynthesis identified the common core set of genes and their products, which makes engineering strategy feasible into cereals (Rubio and Ludden 2008; Curatti and Rubio 2014). Both legumes and cereals have symbiosis signaling pathway for AMF association, but cereals are unable to form nitrogen-fixing nodules from this association. Hence, the perception of rhizobial signaling molecules provides an opportunity to engineer this pathway for recognition of rhizobia by cereal crops. In this regard, several studies performed engineering in plants to secrete specialized root exudates that specifically enhanced the compatibility of newly introduced nitrogen-fixing microbes in the rhizosphere (Oger et al. 1997; Savka and Farrand 1997; Mondy et al. 2014). In addition, Nod factor proteins (NFP) and their receptors are other targets for engineering the symbiosis in cereals. Since NFPs are required for responses to LCOs produced by AMF during associations, it suggests the importance of Nod receptor in cereal engineering (Maillet et al. 2011; den Camp et al. 2011).

12.10 Conclusions and Future Prospects

All strategies for transferring nitrogen fixation to non-legume crops have complex engineering problems. However, they have the potential to change the growing pattern of cereal crops and provide sustainable food production for the growing global population. Future developments in this field require collaborative and multidisciplinary efforts involving researchers with diverse skills and expertise. Further studies in functional genomics will be useful to identify the genes expressed in the rhizosphere that will allow the engineering of host and symbionts for beneficial purposes.

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