Chapter 8
Recent Trends in Identification and Molecular Characterization of Rhizobia Species

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8.1 Introduction

Agricultural sustainability may represent the greatest encumbrance to increasing food production. On the other hand, as a component of sustainability, replacement of chemical fertilizers by bio-fertilizers has the potential to lower costs for farmers, to increase yields, and to mitigate greenhouse-gas emissions and pollution of water and soil. Rhizobia and plant growth-promoting rhizobacteria (PGPR) have been broadly used in agriculture. Sustainability probably represents the greatest challenge to increase food production. The agricultural sector is forced to adopt new technologies dynamically to maintain high yields—without clearing new land for agriculture—and to minimize degradation of land that is occurring worldwide. Since the Green Revolution, the use of chemical fertilizers has played a key role in increasing yields; however, costs are often a major limitation to farmers in developing and poor countries, whereas, for developed countries, pollution of water and soil by fertilizers and greenhouse-gas emissions are sources of concern. Rhizobial inoculants have been applied to legume crops as bio-fertilizers for over half a century (Okon and Labandera-Gonzalez 1994; Bashan and Bashan 2005; Hungria et al. 2005).

Rhizobia have been widely used in agricultural systems for enhancing the ability of legumes to fix atmospheric nitrogen (Teaumroong and Boonkerd 1998). Nitrogen was known to be an essential nutrient for plant growth and development. Intensive

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A.P. Hansen et al. (eds.), *Rhizobium Biology and Biotechnology*, Soil Biology 50,
DOI 10.1007/978-3-319-64982-5_8

farming practices that accomplish high yields need chemical fertilizers, which are not only cost-effective but also may create environmental problems. Nitrogen is essential in plant cells for synthesis of enzymes, proteins, chlorophyll, DNA, and RNA, thus essential for plant growth and production of food and feed (Matiru and Dakora 2004). Nitrogen is the most limiting nutrient for the growth of leguminous plants like common beans, soya beans, cow peas, and garden peas because that present in the soil cannot support growth (Howieson and Committee 2007).

The extensive use of chemical fertilizers in agriculture is currently under debate due to environmental concern and fear for consumers' health. Consequently, there has recently been a growing level of interest in environmentally friendly sustainable agricultural practices and organic farming systems (Rigby and Caceres 2001; Lee and Song 2007). Increasing and extending the role of bio-fertilizers such as *Rhizobium* would decrease the need for chemical fertilizers and reduce adverse environmental effects (Ogutcu et al. 2008).

The rhizobia, which are widely used in agricultural systems, are represented by 7 genera containing about 40 species as *Alphaproteobacteria: Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Sinorhizobium* (Wei et al. 2002), and a species in the genus *Methylobacterium* (Sy et al. 2001). Recently, symbiotic nitrogen-fixing species have also been defined among the genera *Burkholderia* and *Cupriavidus* within the beta subclass of proteobacteria (Moulin et al. 2001). The diversity of the rhizobia is however not clear, particularly considering the large number of leguminous species and their wide geographical distribution (Wei et al. 2002). Since rhizobia are taxonomically very diverse (Wolde-Meskel et al. 2004), efficient strain classification methods are needed to identify genotypes displaying such as superior nitrogen-fixation capacity (Sikora et al. 2002). Molecular techniques have helped to develop easy and quick methods to microbial characterization including works distinguish genera, species, and even strains (Schneider and De Brujin 1996; Giongo et al. 2008). The polymerase chain reaction (PCR) and the use of primers corresponding to consensus repetitive sequences scattered in the eubacteria genome, such as enterobacterial repetitive intergenic consensus (ERIC) and enterobacterial repetitive sequences (BOX) can create highly characteristic patterns when distinguished in agarose gels, providing well separation on strain level (Adiguzel 2006). ERIC sequences are highly protected among rhizobia genomes, and they were used to select and classify different rhizobia strains in population works and to evaluate the environmental effect in defined populations (Giongo et al. 2008). Recently, wild legumes and their symbionts have drawn the attention of ecologist because of their tolerance to extreme environmental conditions such as severe drought, salinity, and elevated temperatures. For rhizobial inoculants, a molecular dialogue between the host plant and the bacterium results in root nodulation and nitrogen fixation, involving plant flavonoids and bacterial nodulation (Nod) factors, identified as lipochitooligosaccharides (LCOs) (Ferguson et al. 2010); however, the roles of other molecules, such as those related to type-III secretion systems and exopolysaccharides (EPSs) (Downie 2010) have also been emphasized.

Many researchers have focused on the genetic diversity of *Rhizobium* isolated from several countries around the world (Mutch et al. 2003; Moschetti et al. 2005; Shamseldin et al. 2009). However the taxonomy of *rhizobia* is very diverse (Wolde-Meskel et al. 2004), molecular techniques based on the PCR provided easy and quick methods to microbial characterization (Shoukry et al. 2013). The 16S rRNA gene sequencing is an excellent tool for molecular characterization of the different isolates of *Rhizobium* (Ismail et al. 2013).

Due to their considerable agricultural and environmental significance, *Rhizobium* species have been extensively studied. During the last decades, the assessment of diversity within rhizobial natural population in various regions of the world has received increased attention. The development of numerous molecular genetic methods and other serological techniques has been greatly contributed to these investigations. The availability of several sensitive and accurate PCR-based genotyping method (Jensen et al. 1993) has enabled the differentiation among closely related bacterial strains and detection of a higher rhizobial diversity than previously considered. Consequently, the taxonomy of root- and stem-nodulating bacteria has been deeply changed in recent years.

8.2 Approaches for Identification of Rhizobia

Rhizobia are Gram-negative soil bacteria capable of inducing formation of nodules in the leguminous plants in which atmospheric nitrogen is reduced to ammonia. This mutualistic relationship between rhizobia and legumes is the most important biological mechanism for providing nitrogen to the plants as an alternative to the energy expensive ammonia fertilizer (Freiberg et al. 1997). Inoculation of leguminous seeds with the selected rhizobial strains is being widely practiced to ameliorate the plant yield by enhanced root nodulation and nitrogen uptake of the plant (Babic et al. 2008). Due to their paramount environmental and agricultural significance, these legume symbionts are being extensively characterized. Recently, the assessment of diversity within rhizobial natural populations in various regions of the world has received increased attention.

8.2.1 Culture Characteristics

In a combined approach of phenotypic and genotypic characterization, Rai et al. (2012) obtained 28 indigenous rhizobial isolates from different chickpea growing regions in peninsular and northern India and analyzed their diversity. The field isolates were compared to two reference strains TAL 620 and UPM-Ca142 representing *Mesorhizobium ciceri* and *M. mediterraneum,* respectively. Phenotypic markers such as resistance to antibiotics, tolerance to salinity, temperature, pH, phosphate solubilization ability, growth rate, and also symbiotic efficiency showed considerable diversity

among rhizobial isolates. Their phenotypic patterns showed adaptations of rhizobial isolates to abiotic stresses such as heat and salinity. Two salt-tolerant strains (1.5% NaCl by T1 and T4) with relatively high symbiotic efficiency and two P-solubilizing strains (66.7 and 71 µg/ml by T2 and T5) were identified as potential bioinoculants. Molecular profiling by 16S ribosomal DNA restriction fragment length polymorphism (RFLP) revealed three clusters at 67% similarity level. Further, the isolates were differentiated at intraspecific level by 16S rRNA gene phylogeny. Results by these workers assigned all the chickpea rhizobial field isolates to belong to three different species of *Mesorhizobium* genus. 46% of the isolates grouped with *Mesorhizobium loti* and the rest were identified as *M. ciceri* and *M. mediterraneum*, the two species which have been formerly described as specific chickpea symbiont. This was the first report on characterization of chickpea-nodulating rhizobia covering soils of both peninsular and northern India.

Naz et al. (2009) aimed to isolate and characterize PGPR (Rhizobia) from rhizosphere (EC: 2300 µS/cm; pH: 8.6) of four halophytes: *Sonchus arvensis* L., (sow thistle), *Solanum surratense* Burm. F., (yellow berried night shade), *Lactuca dissecta* D. Don., (wild lettuce), and *Chrysopogon aucheri* (Boiss.) Stapf (golden beard grass) collected from Khewra Salt Range and compared with *Rhizobium* isolate from *S. surratense* Burm. F. of arid soil (EC: 210 µS/cm; pH: 7.9) of Attock (treated as control). The isolates were identified and characterized on the basis of colony morphology and biochemical traits viz. Gram staining, catalase and oxidase tests, and carbon and nitrogen source utilization pattern. The survival efficiency of isolates was measured in culture (colony forming unit/g soil). The genetic diversity among the isolates assessed by RAPD-DNA fingerprinting and PCR was done for the presence of 16S rRNA gene. On the basis of carbon/nitrogen source utilization patterns, *Rhizobium* isolates placed in five different groups and were designated as Rkh1, Rkh2, Rkh3, Rkh4, and Rak5 but random amplified polymorphic DNA (RAPD) tests categorized the isolates into two clusters. The RAPD results were further analyzed by MVSP software; similarity matrix was measured and converted into dendrogram using UPGMA clustering method.

A study was undertaken by Wagh et al. (2015) to investigate the occurrence of nitrogen-fixing bacteria (NFB) from soil and root nodule of Nashik area. Four soil samples and two root nodule samples were collected randomly to estimate microbial population which used plate count method. The study characterized NFB strains isolated from leguminous plant species. The characterization of isolated cultures was done through colony morphology analysis, cellular morphology and biochemical properties which included Gram staining, catalase test, methyl red test, Voges Proskauer test and citrate utilization and nitrate reduction pattern. Isolation of DNA was done with the modified CTAB method. The isolated DNA was subjected to Agarose gel electrophoresis and observed under UV light. The presence of nifH check by using PCR which gave 700 bps amplicon in 03 isolates. These isolates could be useful to produce efficient bio-fertilizers for agriculture.

Zhang et al. (1991) studied on the diversity of *Rhizobium* bacteria isolated from the root nodules of leguminous trees. 60 rhizobial strains isolated from the root nodules of *Acacia senegal* and *Prosopis chilensis* in Sudan were compared with

37 rhizobia isolated from woody legumes in other regions and with 25 representatives of recognized *Rhizobium* species by performing a numerical analysis of 115 phenotypic characteristics. **C**ultures were grown to log phase in YEM agar or broth before inoculation. The strains were then streaked onto YEM agar plates and several tests like hydrolysis of urea, precipitation of calcium glycerophosphate, reduction of nitrate, production of melanin, utilization of carbon sources were performed as well as resistance to intrinsic heavy metals and antibiotics and tolerance of NaCl were also determined.

Suliasih and Widawati (2005) investigated the occurrence of NFB from soil samples of Wamena Biological Garden (WbiG). Eleven soil samples were collected randomly to estimate microbial population which used plate count method. During their work, yeast extract mannitol agar (YEMA) was used for growing *Rhizobium* and Mannitol Ashby agar medium for isolating *Azotobacter* and Okon medium for *Azospirillum*. The number of bacterial colony was estimated after 7 days of incubation at room temperature and then were identified following the methods of *Bergey's Manual of Systematic Bacteriology*.

Khan et al. (2008) reported the isolation and identification of nitrogen-fixing microorganisms during the seedling (30 days after seed sowing) stage of rice (BR 10) rhizosphere soil grown in Non-calcareous grey flood plain soil of Bangladesh. Four individual strains were microbiologically identified based on the selection criteria, and it was found out that their biochemical tests were strictly similar to *Enterobacter spp*., for strain-1, *Klebsiella spp*. for strain-2, *Bacillus spp*. for strain-3, and *Azospirillum spp*. for strain-4. They were anaerobic in nature.

Messaoud et al. (2014) isolated and characterized phenotypically the rhizobial strains from the soils belonging to the Meknes-Tafilalet region in order to select strains that were able to nodulate *Bituminaria bituminosa*. Samples from 23 different sites belonging to the Meknes-Tafilalet region were collected in order to select rhizobial strains that are able to nodulate *B. bituminosa*. The morphological, cultural, and phenotypic parameters of isolated strains were studied. The phenotypic characteristics included colony morphology, growth speed, tolerances to temperature, salt, and pH. The results highlighted the important biodiversity of the isolated rhizobial strains and opened opportunities for the development of new bio-fertilizer.

8.2.2 Physiological and Biochemical Approaches

Shoukry et al. (2013) obtained eight locally isolates of *Rhizobium* sp. from different soil locations were characterized and determined of their nodulation efficiency. The obtained results exhibited that all the isolated strains were *R. leguminosarium* bio *viciae* and the eight isolates produced the highest mean of nodules per plants which reached (104 nodules) for isolate RL8. The obtained results after salt tolerance and antibiotic response of the *R. leguminosarium* strains showed that the isolate No. RL7 was the superior strain for salt tolerance. The following strain for salt

tolerance was the isolate (RL2). The high growth rate of the *R. leguminosarium* strains could be considered as an indicator of salt tolerance. Moreover, the isolated strains (RL3, RL4, and RL7) showed the highest level of antibiotic resistance since they were resistant to five antibiotics.

In a study carried out by L'taief et al. (2007), several phenotypic markers were used to determine the biodiversity of rhizobial strains nodulating *Cicer arietinum* L. in various areas of Tunisia. They included symbiotic traits, the use of 21 biochemical substrates, and tolerance to salinity and pH. Numeric analysis of the phenotypic characteristics showed that the 48 strains studied fell into three distinct groups.

The investigation was carried out by Mishra et al. (2013) who isolated bacteria-nodulating cluster bean from the areas of arid and semiarid regions of Rajasthan to identify effective and competitive strains tolerant to various abiotic stresses such as temperature, pH, and salinity. A total of 15 rhizobium isolates from cluster bean nodules were characterized on the basis of cultural analysis and screened for physiological traits. Most isolates were tolerant to pH between 6.0 and 8.0, NaCl concentration between 0.5 and 3% and temperature between 37 and 42 °C. These rhizobial isolates were characterized by RAPD to estimate their relationship on molecular basis and compared with their tolerance to various abiotic stresses.

Park et al. (2005) isolated and characterized diazotrophic growth-promoting bacteria from rhizosphere of agricultural crops of Korea. Burk's nitrogen-free semisolid and solid medium was used throughout the study and the pH of the medium was adjusted to 7 ± 0.1. Physiological and biochemical characters of the bacterial isolates were examined according to methods described in *Bergey's Manual of Systematic Bacteriology*. Gram reaction was performed as per standard procedures and tests like plate assays and fatty acid analysis were also done.

In a study by Singh et al. (2013), root-nodulating bacteria were isolated and characterized from the root nodules of *Sesbania sesban* leguminous plants growing in regions of Mumbai and its suburban areas. A total of 17 isolates isolated on YEMA medium. These isolates were further studied for their morphological characters and biochemical characterization along with one reference culture (NCBI-TUR1). Out of 17, morphologically six Rhizobium strains were rod-shaped, Gram-negative, and mucous producing. These isolates were unable to grow in the presence of 0.1% methylene blue and lactose. With the help of biochemical characterization, it was confirmed that those all six isolates were *Rhizobium* species. These isolates were studied for different salt concentration range from 5 to 9.0%, and culture shows less growth as the concentration of salt increased. All isolates grow at pH 6.0–11.0 but none of the isolates grow at pH 12.0. The optimum physical parameters for the growth of fast-growing rhizobia were found in pH between 7.0 and 8.0 and 28 °C temperature. BIOLOG test was performed in order to understand the nutrient requirement and utilization pattern of *Rhizobium* species. The goal of isolation of *Rhizobium* species associated with *Sesbania sesban* sample would be the assessment of rhizobial genetic diversity. The tolerance to high salinity, pH, and their survival in such harsh environmental conditions make these rhizobial isolates valuable to improve the productivity of the leguminous plants cultivated under extreme environments.

Mirza et al. (2001) isolated nitrogen fixing, phytohormone-producing bacteria from sugarcane and their beneficial effects on the growth of micropropagate sugarcane plantlets. For isolation, serial dilutions of the bacterial growth in the semisolid medium in ARA-positive vials were spread on LB agar plates and incubated at 30 °C for 24–48 h. Colonies appearing on plates were picked and streaked on fresh LB agar plates. All the different types of colonies were again inoculated in N-free semisolid media and were assayed for confirmation of acetylene reduction activity. Physiological and biochemical tests were performed using the QTS-20 miniaturized identification system (DESTO Laboratories, Karachi, Pakistan). Oxidation fermentation test and catalase test were also performed for the identification of the isolates.

Holguin et al. (1992) worked on the isolation of new diazotrophic bacteria, *Listonella anguillarum* and *Vibrio campbellii,* and one non-nitrogen-fixing bacterium, *Staphylococcus sp.,* from the rhizosphere of mangrove trees. The cellular morphology of the pure isolates was determined with light microscopy (Zeiss). Species identification was done by FAME analysis through gas chromatography of cell fatty acid methylesters that have a chain length between 9 and 18 carbons long. During their trials, it was found that *Staphylococcus sp.,* indicates interaction with the above two diazotrophic bacteria and results in increase or decrease in the nitrogen-fixing capacity of the two diazotrophs.

A total of 251 bacteria were isolated from the surface-sterilized roots of 11 legumes except *Lathyrus odoratus* (Ozawa et al. 2003). Thirty one isolates of these bacteria showed ARA of 2.6–450 nmol/h/culture when grown in the JNFb media. 16S rRNA gene sequence analysis and physiological characteristics of the 31 isolates showed that the isolates were *Agrobacterium radiobacter, A. tumefaciens, Azospirillum lipoferum, Bradyrhizobium elkanii, Burkholderia cepacia, Frateuria aurantia, Klebsiella oxytoca, K. pneumonia, Rhizobium gallicum, R. sp., Starkeya novella*, and *Xantobacter flavus*.

Prabudoss and Stella (2009) isolated *Gluconacetobacter diazotrophicus* from sugar-rich crops like sugarcane (root, stem bud, and leaves), sweet potato, pine apple, and wild cane. Nitrogen-fixing efficiency of the isolates was evaluated by using acetylene reduction activity following the standard procedure. It was found out that the nitrogen-fixing ability of the isolated strains were comparatively high from that of the reference strains, indicating the superiority of the isolates.

Thirty nodule isolates from bean (*Phaseolus vulgaris* L.) grown in Eskißehir, Turkey were studied by Kucuk et al. (2006) for their physiological and biochemical characteristics. Most isolates produced abundant extracellular polysaccharides, tolerated high salt concentration (5% NaCl), grew at a temperature of 42 °C, and synthesized melanin. They were able to grow at pHs ranging from 3.5 to 9.0. The majority of the isolates showed an intrinsic resistance to the antibiotics chloramphenicol (20 and 50 μg/ml), erythromycin (30 μg/ml), kanamycin (10 μg/ml), and streptomycin (40, 80, and 100 μg/ml).

Hassen et al. (2014) evaluated nodulation of bacterial strains isolated from the root nodules of indigenous and exotic forage legumes. The nodulation authentication trial was conducted following the Koch's postulate experiment under glasshouse

condition and revealed that all bacterial strains were root nodule microsymbionts of the forage legumes from which they were initially isolated. Nodulation and improved growth of the legumes was achieved by the tested strains with statistically significant ($p = 0.05$) increase in plant biomass and nodule number in comparison with the un-inoculated controls. To elucidate their identity and phylogenetic relatedness, the effective strains were selected and characterized by means of the 16S ribosomal RNA sequence analysis. The analysis confirmed that the isolates nodulating *Macrotyloma axillare*, *Desmodium uncinatum*, *Indigofera spicata* var. *spicata,* and *Stylosanthes gracilis* predominantly belong to the genus *Bradyrhizobium. Vigna unguiculata* and *Vigna* sp. were nodulated by strains belonging to members of the genus *Bradyrhizobium*, *Sinorhizobium,* and *Rhizobium.*

An attempt was made by Ahmed and Abdelmageed (2015) to evaluate the diversity of 16 *Rhizobium leguminosarum* bv. *viceae* strains isolated from different schemes in Shendi area, Sudan. Morphological and cultural characteristics were performed viz. colony morphology, Gram staining, and motility. Biochemical and physiological tests include acid–base production, oxidase and catalase tests were carried out. The effects of antibiotics on the growth of *Rhizobium* strains on YEMA media were tested using measurement of diameters of the growth inhibition zones. Growth of pure rhizobial isolates on (YEM) medium having variable range of pH (5.5–8.5) and different concentrations of NaCl (0.5–6.6%) were recorded. The isolates studied were motile, Gram-negative, and rod-shaped and catalase- and oxidase-positive bacteria. Regardless the location of isolation, *Rhizobium* strains tested showed significant differences ($p < 0.05$) in their sensitivity to the antibiotics. Strain SHUOS1F34, SHUMAF6, and SHUGF37 were the most sensitive strains whereas strain SHUJ15F2, SHUSSF4, and SHUR2F36 were the most tolerant ones to almost all antibiotics tested. With the exception of strain SHUNOF35, which tolerate high pH values up to 8.5, the growth rate of all strains increased steadily with increasing pH from 5.5 reaching the maximum at pH 6.5–7.5 and then declined to 8.5. In all the tested strains, the growth rate decreased with increasing salt concentration from 0.5 up to 6%.

The diversity of rhizobia associated with introduced and native *Acacia* species in Algeria was investigated from soil samples collected across seven districts distributed in arid and semiarid zones by Boukhatem et al. (2012). The in vitro tolerances of rhizobial strains to NaCl and high temperature in pure culture varied greatly regardless of their geographical and host plant origins but were not correlated with the corresponding edaphoclimatic characteristics of the sampling sites, as clearly demonstrated by principal component analysis. Based on 16S rRNA gene sequence comparisons, the 48 new strains isolated were ranked into 10 phylogenetic groups representing five bacterial genera, namely, *Ensifer, Mesorhizobium, Rhizobium, Bradyrhizobium*, and *Ochrobactrum. Acacia saligna*, an introduced species, appeared as the most promiscuous host because it was efficiently nodulated with the widest diversity of rhizobia taxa including both fast-growing ones, *Rhizobium, Ensifer*, and *Mesorhizobium*, and slow-growing *Bradyrhizobium*. The five other *Acacia* species studied were associated with fast-growing bacterial taxa exclusively.

8.2.3 Nucleic Acid Hybridization and Sequencing

The variability in the effectiveness of native *Rhizobium* isolates even on a single cultivar of a legume crop and gives the impression that the nitrogen-fixing ability of *Rhizobium* could be improved either by strain selection or by genetic manipulation. Since the nitrogen-fixing ability is expressed only in symbiotic association, it is not possible to find out whether the restriction on the bacterial gene expression is due to the bacterial genome or the plant. Among fast-growing rhizobia, on the basis of physiological properties and nucleic acid hybridization studies, a cluster could be identified grouping strains of *R. leguminosarum, R. phaseoli,* and *R. trifolii,* which is called the *R. legurninosarum* cluster, whereas strains of *R. meliloti* form a separate cluster (Gibbins and Gregory 1972). Recent genetic evidence has confirmed this separation in two clusters (Kondorosi et al. 1980).

The genetic diversity of ten rhizobial isolates (*R. leguminosarum*) isolated from root nodules of broad beans (*Vicia faba* L.) growing in ten locations in Egypt were investigated by Ismail et al. (2013) using 16S rRNA gene partial sequence. The average genetic distance among the studied isolates was low (0.193) with the lowest genetic distance between isolates collected from South Sinai and Zefta and the highest genetic distance between North Sinai and Quesna isolates. The studied isolates formed two main groups based on cluster analyses and principal coordinate analysis. The grouping pattern of the isolates in both analyses was independent of their geographic location. Further, the isolation-by-distance analysis showed no correlation between genetic and geographic distance of the studied isolates ($r = 0.118$, $p = 0.71$). The results suggested that the genetic diversity of Egyptian rhizobia across the studied locations was very low, probably due to the narrow genetic background of Egyptian rhizobia.

A Gram-negative, nonmotile, fast-growing, rod-shaped, bacterial strain VKLR-01 T was isolated by Kesari et al. (2013) from root nodules of *Pongamia* that grew optimal at 28 °C, pH 7.0 in the presence of 2% NaCl. Isolate VKLR-01 exhibited higher tolerance to the prevailing adverse conditions such as those of salt stress, elevated temperatures, and alkalinity. Strain VKLR-01 T consisted of the major cellular fatty acid as $C_{18:1}\omega7c$ (65.92%). Strain VKLR-01 T was found to be a nitrogen fixer using the acetylene reduction assay and PCR detection of a *nif*H gene. On the basis of phenotypic, phylogenetic distinctiveness and molecular data (16S rRNA, *rec*A, and *atp*D gene sequences, G + C content, DNA–DNA hybridization, etc.), strain VKLR-01 T = (MTCC 10513 T = MSCL 1015 T) was considered to represent a novel species of the genus *Rhizobium* for which the name *Rhizobium pongamiae* sp. nov. was proposed.

de Freitas et al. (2014) determined the characteristics of native rhizobia isolates from nodules of *Mimosa tenuiflora* and *Mimosa paraibana* grown in pots with soils collected under Caatinga vegetation and compared the restriction ribosomal DNA profiles of the isolates with those of 16 reference strains. All plants formed abundant indeterminate nodules and all nodule isolates formed fast-growing colonies. No colony altered the medium to an alkaline reaction and most of them

produced low or medium amounts of extracellular polysaccharides. White and creamy colonies predominated among the isolates but orange and green colonies were present. Differences among the isolates from the *Mimosa* species tested were indicated by the greater phenotypic diversity of those obtained from *M. tenuiflora*. The analysis of the 16S rDNA gene suggested that the isolates from *M. tenuiflora* and *M. paraibana* were closely related and closer to β-rhizobia than to α-rhizobia. However, the similarity with all the tested β-rhizobia reference strains was relatively low which suggested that the isolates might belong to different bacteria species.

8.2.4 PCR Fingerprinting Technologies

For molecular characterization of the rhizobial strains, various types of PCR technologies could be utilized. Suitable technique should be used out of the several PCR techniques such as RAPD analysis, RFLP analysis, amplified ribosomal DNA restriction analysis (ARDRA) analysis, or 16S rDNA sequencing. PCR-based fingerprinting techniques, such as terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP), have been widely used in the study of rhizobial communities. In general, fingerprinting techniques rely on the amplification of 16S rRNA gene fragments by PCR, but 16S rRNA gene copy number per genome vary from 1 up to 15 or more copies depending on the bacterial species. The numbers of rRNA gene copies are related to the life strategy of bacteria; taxa with low copy numbers and inhabit low nutrient environment (Kang et al. 2010).

The quantitative PCR or qPCR is a molecular technique widely used for detection and quantification of specific genes and their expression from DNA and RNA samples from various environments. It is a sensitive technique allowing detection of the signal produced by DNA fragment amplification in real time during each cycle of the PCR reaction. This technique could provide important insight into the distribution of specific rhizobial genes in the rhizosphere and bulk soils (Sørensen et al. 2009) because it is not only used to detect and quantify 16S rRNA genes but also functional genes involved in relevant processes in the rhizosphere, such as nutrient cycling and phytopathogen biocontrol.

A study was done by Al-Judy and Majeed (2013) where ten of rhizobial isolates and strain studied were either local isolate from chickpea root nodules or non-local (Syrian and Turkish) obtained from ICARDA. These isolates were identified and characterized on the basis of colonies morphology and biochemical tests including Gram staining, catalase and oxidase tests. The genetic diversity among the isolates was assessed by RAPD-PCR fingerprinting by using five primers. The RAPD result showed high ability to detect genetic polymorphism in Rhizobia and have the ability to generate unique bands (marker) especially in Shiekhan 3(10)bands Mosle(8)bands isolates that were isolated from chickpea plants.

Oraon and Singh (2013) studied diversity of *Rhizobium* spp. in agricultural lands of Madhya Pradesh. Physicochemical properties of soil were studied using standard methods while molecular methods used to study diversity within *Rhizobium* species. Further, population analysis of *Rhizobium* species in relation to genetic diversity was carried out using 16S rDNA-RFLP PCR. *Rhizobium* were identified and genetically by determining the %G + C content of the whole genome, followed by restriction enzyme (Mbo I, Hap II, Taq I, Msp I, Cfo I, Nde II, Dde I, Rsa I) treatment of PCR amplified product of 16S rDNA segment was performed. The sequences recognized by the restriction enzymes are distributed at variable intervals in the genome of an organism and also vary in number. The separation carried out by electrophoresis (1.6–2% agarose gel) resulted in specific banding pattern differing within as well as among different species.

Molecular diversity studies of 19 rhizobia isolates from chickpea were conducted by Yadav et al. (2013) using simple sequence repeats (SSR) and 16S rDNA-RFLP markers. Phenotypic characterization with special reference to salinity and pH tolerance was performed. These isolates were identified as different strains of *Mesorhizobium, Rhizobium, Bradyrhizobium*, and *Agrobacterium*. Twenty SSR loci of *M. ciceri* distributed across the other rhizobial genome clearly differentiated 19 rhizobial isolates. Analogous clustering supported the results of 16S rDNA sequence-based phylogeny. Analysis of the 16S rDNA sequences from *M. ciceri* strains revealed that nucleotide variables (signature sites) were located at 20 different positions; most of them were present in the first 820 bp region from 5′ terminal. Interestingly, 14 signature sites were located in two main regions, the variable region V1 (nt 527–584), and variable region V2 (nt 754–813). The secondary structure and minimal free energy were determined in these two regions.

In a study carried out by Suneja et al. (2016), the normal nodulating chickpea cultivar (HC5) and revertant of non-nodulating cultivar ICC 4993 NN designated as ICC 4993 (R) were used to study and characterize the rhizobia infecting both cultivars. On the basis of growth characteristic and nodulation, 43 rhizobial isolates from revertant of non-nodulating cultivar and 8 rhizobial isolates from normal nodulating cultivar were selected. Heterogeneity of the rhizobia infecting both of the cultivars was estimated by ERIC as well as RFLP analysis of 16S rDNA sequence. Based on the presence of different ERIC profiles, rhizobial isolates from cv. ICC4993 (R) formed eight different clusters and those from cv. HC5 formed three clusters at 80% similarity. A combined dendrogram of all the mesorhizobial isolates from the two cultivars showed two clusters at 70% similarity and eight subclusters at 80% similarity level. Similarly, RFLP patterns showed that rhizobial isolates from cv. ICC4993 (R) formed 11 clusters while those from cv. HC5 formed two clusters at 80% similarity. A combined dendrogram of mesorhizobial isolates from the two cultivars formed 12 clusters at 80% level of similarity. Using both methodologies, heterogeneity (if any) of mesorhizobia-nodulating cvs. ICC4993 (R) and HC5 could not be ascertained. Further sequencing of partially amplified 16S rDNA of three rhizobial isolate from cv. ICC4993 (R) and one from cv. HC5 showed more than 98% similarity with *Mesorhizobium muleiense* and *Mesorhizobium mediterraneum*. The phylogenetic analysis of 16S

rRNA partial sequence revealed 11 monophyletic clades. The isolates NN78 and HC 1065 were clustered along with *M. mediterraneum* strain PECA20 while NNs13 and NN90 formed a separate cluster.

The study carried out by Prasad (2014) established a phylogenetic relationship between four Rhizobia species isolated from different plant nodules by RAPD marker. The genomic DNA of good quality without any degradation was successfully isolated from Rhizobia. In total four Rhizobia isolates were studied for their polymorphism at molecular level. Out of four random primers three primers, namely, OPZ 8, 9, and 10 produced clear banding patterns. From the electrophoretic banding pattern, cluster analysis was carried out using frequency similarity coefficient. The results of this study indicated that RAPD provided a high degree of discrimination between the strains.

Ogutcu et al. (2009) conducted a study to determine the phenotypic and genotypic differences in *R. leguminosarum* subsp. *ciceri* strains isolated from perennial wild chickpeas (*Cicer anatolicum*) from high altitudes (2000–2500 m) in mountains of Erzurum, Eastern Anatolia, Turkey. In this study, rep-PCR (ERIC-, REP-, and BOX-PCR) fingerprinting methods were used for the genotypic characterization and phylogenetic analysis of *R. leguminosarum subsp. cicero* strains isolated from perennial wild chickpeas. The results showed a high intraspecies diversity among the strains in terms of rep-PCR (ERIC-, REP-, and BOX-PCR) profiles.

In a study, Shamseldin et al. (2005) recovered 12 rhizobial isolates from nodules of common bean (*P. vulgaris*) grown in two different locations of Egyptian soils. The most effective strains for nodule formation and nitrogen fixation were selected. Strain specificity with the bean cultivars Saxa, Canoca, and Giza 6 from Germany, Colombia, and Egypt were studied. The strains were characterized by ARDRA of 16S and 23S rDNA, plasmid DNA content, and 16S rDNA sequencing. A high degree of genetic diversity was observed among the strains used. The strains were separated into three genotype groups. Genotype A was displayed by seven isolates classified as *Rhizobium etli*, while genotype B was displayed by a single isolate, classified as *R. gallicum*. Genotype C included four isolates which were unable to renodulate *P. vulgaris*, which were related to *Agrobacterium tumefaciens*. Single strains were further characterized by specific physiological tests and measurements such as acetylene reduction activity, nodule/root biomass ratio, and shoot and root fresh weight.

Toolarood et al. (2012) studied the genetic diversity of 48 rhizobia isolated from root nodules of alfalfa, cultivated in different regions of Iran, was studied by RFLP analysis of PCR-amplified intergenic region (IGS) and 16 s rRNA gene. Analysis of the intergenic region between 16S and 23S rDNAs (IGS) showed a considerable diversity within these microsymbionts. At the similarity of 70%, these rhizobia were clustered into four groups: I, II, III, and IV. Two genera, *Sinorhizobium* and *Agrobacterium,* were identified among the isolates by PCR-RFLP of 16S rRNA gene.

Costa et al. (2014) evaluated the diversity of rhizobial isolates obtained from root nodules of pigeon pea plants grown at the eastern edge of the Brazilian Pantanal. The bacterial isolates were isolated from root nodules from field-growing

pigeon pea grown in two rural settlements of the Aquidauana municipality. The bacterial isolates were characterized phenotypically by means of cultural characterization, intrinsic antibiotic resistance (IAR), salt and high incubation temperature tolerance, and amylolytic and cellulolytic activities. The molecular characterization of the bacterial isolates was carried out using ARDRA and Box-PCR techniques. In addition, the symbiotic performance of selected rhizobial isolates was evaluated in a greenhouse experiment using sterile substrate. The molecular fingerprinting of these bacterial isolates also showed a highly diverse collection, with both techniques revealing <25% similarity among bacterial isolates. The results obtained in this study indicated the presence of a highly diversified rhizobial community nodulating the pigeon pea at the eastern edge of the Brazilian Pantanal.

In one study, Orel et al. (2016) collected crown gall-affected grapevine samples from major vineyards, located in different Turkish provinces. One hundred and three bacterial strains were obtained from 88 vineyards and 18 grapevine varieties; they were tumorigenic when inoculated in tobacco, sunflower, and *Datura stramonium* plants and were identified as *Rhizobium vitis* using biochemical and physiological tests as well as PCR and specific primers. Nineteen *R. vitis* strains presented a number of anomalous biochemical and physiological characters. PCR and opine-specific primers revealed the presence of octopine/cucumopine-type plasmid in 82 *R. vitis* strains, nopaline-type plasmids in 18 strains, and vitopine-type plasmids in three strains. Clonal relationship of strains was determined using pulsed field gel electrophoresis following digestion of genomic DNA with the restriction endonuclease *Pme*I. The greatest genetic diversity was found for the strains from Denizli, Ankara, and Nevşehir provinces. Nopaline and vitopine types of *R. vitis* were detected for the first time in Turkey.

Reinhardt et al. *(*2008) isolated and identified 14 strains of NFB from different agricultural plant species after performing the culture characteristics tests and molecular characterization. The strains were characterized by RAPD, ARDRA, and 16S rDNA sequence analysis. Out of 14 strains 13 were assigned to known groups of nitrogen-fixing bacteria, including organisms from the genera *Azospirillum*, *Herbaspirillum*, *Pseudomonas,* and *Enterobacteriaceae*. Remaining one strain was grouped with *Acidovorax avenae,* but with low similarity and phenotypic results were inconclusive.

In a study by Sankhla et al. (2015), the phenotypic as well as genetic diversity of 20 root nodule bacterial strains isolated from root nodules of *Crotalaria medicagenia* growing in Thar Desert of India were investigated. All bacterial isolates were fast growing and highly diverged in their phenotypic characteristics such as salt and pH tolerance. All isolates distributed into three groups based on ARDRA pattern while formed 11 groups on the basis RAPD patterns, which indicates high genetic diversity among them. The 16S rDNA sequencing and BLAST result of seven isolates suggested that microsymbionts of *C. medicagenia* belong to the only *Ensifer* genus. On the basis of phylogenetic analysis of 16S rRNA gene sequences, these *Ensifer* strains closely related to old world rhizobia (*E. kostiensis, E. saheli, E. terangae*) as well as new world rhizobia (*E. mexicanus, E. americanus*), but formed new lineages, thus these are novel

strains of *Ensifer*. It was suggested that in the alkaline soil of the Thar Desert *C. medicagenia* was nodulated by diverse *Ensifer* species.

Sessistsch et al. (1997) found that *P. vulgaris* L. (common bean) was nodulated by rhizobia in the fields around the Seibersdorf laboratory despite the fact that common bean was not been grown for a long time. Using PCR analysis with repetitive primers, plasmid profiles, nifH profiles, PCR-RFLP analysis of the 16S rRNA gene and of the 16S rRNA–23S rRNA intergenic spacer and the nodulation phenotype, two well-differentiating groups could be distinguished. One group showed high similarity to *Rhizobium* sp. R602sp, isolated from common bean in France, while the other showed the same characteristics as *R. etli*. The researchers detected little variation in the symbiotic regions but found higher diversity when using approaches targeting the whole genome. Many isolates obtained in the study might have diverged from a limited number of strains; therefore, the Austrian isolates showed high saprophytic and nodulation competence in that particular soil.

Saeki et al. (2010) demonstrated various experimental approaches to characterization of soybean-nodulating rhizobial communities in relation to host genotype and geographical distribution which included using environmental DNA, including DGGE, terminal RFLP (T-RFLP) analysis, and automated ribosomal intergenic spacer analysis (ARISA), targeting 16S rDNA, the 16S–23S rDNA ITS region, and other genomic and RNA sequences. The study highlighted the diversity and geographical distribution of indigenous soybean-nodulating bradyrhizobia in Japan and suggested that bradyrhizobial communities occupy different niches from north to south determined by environmental factors such as temperature and host-plant diversity. These results were, however, based on limited numbers of isolates from nodules. For further advances in rhizobial ecology, a direct method should be developed to characterize the rhizobial ecology of indigenous rhizobia in relation to interactions with host soybean cultivars. In particular, rhizobial communities in the soil should be compared with soybean-nodulating rhizobial communities to clarify ecological interactions among host plant, rhizobia, and environmental factors, such as temperature.

Silva et al. (2012) characterized rhizobia isolated from the root nodules of cowpea (*V. unguiculata*) plants cultivated in Amazon soils samples by means of ARDRA and sequencing analysis to know their phylogenetic relationships. The 16S rRNA gene of rhizobia was amplified by PCR using universal primers Y1 and Y3. The amplification products were analyzed by the restriction enzymes *Hinf*I, *Msp*I, and *DdeI* and also sequenced with Y1, Y3, and six intermediate primers. The clustering analysis based on ARDRA profiles separated the Amazon isolates in three subgroups, which formed a group apart from the reference isolates of *Bradyrhizobium japonicum* and *B. elkanii*. The clustering analysis of 16S rRNA gene sequences showed that the fast-growing isolates had similarity with *Enterobacter*, *Rhizobium*, *Klebsiella,* and *Bradyrhizobium* and all the slow-growing clustered close to *Bradyrhizobium*.

Genetic diversity in rhizobial strains was studied by Sajjad et al. (2008) using RAPD markers. The strains isolated, using dilution plate method, from nodules of lentil plant grown at different sites of Punjab, Pakistan were used in the study.

Slow- and fast-growing colonies of rhizobial isolates were selected, isolated, and purified by streaking. About 10 ml of broth culture of each isolate was used for DNA extraction. PCR was performed using decamer oligonucleotide primers to study DNA polymorphism among strains. Cluster analysis divided the strains into two distinct groups A and B. The data showed that only the strains L-5, L-10, L-22, and S-26 were able to solubilize soil phosphates.

A survey of native legume *Rhynchosia minima* was conducted by Tak et al. (2014) at various sites/villages of district Jodhpur, Nagaur, Barmer, Bikaner, and Jaisalmer of arid region of western Rajasthan in the months of September to November during post-monsoon periods. Rhizobia trapping experiments were also performed with rhizospheric soil collected from various sites in arid regions of Thar Desert. The nodules in *R. minima* were found to be indeterminate with bark and lenticels on the surface. More than 80 bacterial strains were isolated from *R. minima* and around 57 bacterial isolates were purified and characterized at phenotypic, biochemical, and molecular level. Bacteria isolates were screened for salt (NaCl) and pH tolerance. Genetically diverse bacteria were grouped on the basis of DNA fingerprinting like ARDRA and RAPD using RPO1 primer. On the basis of ARDRA and RAPD pattern, selected root nodule bacteria were characterized by partial 16S rRNA gene sequences and identified by closest match in BLAST. Phylogenetic analysis reveals that *Ensifer*sp is the most competent nodulating bacteria in this native legume growing in alkaline soil of Thar Desert. 16S rRNA gene NJ tree showed a separate clade of novel *Ensifer* sp. diversifying from old world rhizobia *Ensifer arboris* TTR 38 T (Z78204) and *Ensifer saheli* LMG 7837 T (X68390).

The diversity of a collection of 21 bradyrhizobial isolates from Lima bean (*Phaseolus lunatus* L.) was assayed by Ormeno-Orrillo et al. (2006) using molecular methods. Moderately high to high genetic diversity was revealed by multilocus enzyme electrophoresis (MLEE) analysis of seven enzyme loci and genomic fingerprints with ERIC and BOX primers. Two groups with differences in growth rate were found among the isolates and their differentiation as two divergent bradyrhizobial lineages was supported by PCR-RFLP of the rpoB gene and sequence analysis of the 16S rDNA and dnaK genes. Isolates with slow growth (SG) were identified as Bradyrhizobium yuanmingense, while extra-slow growing isolates (ESG) constitute a new lineage different from all described Bradyrhizobium species. Three distinct symbiotic genotypes were detected among Lima bean bradyrhizobia by PCR-RFLP and sequence analysis of the nifH and nodB genes. One genotype was found in the ESG lineage and two in *B. yuanmingense*. Another symbiotic genotype was detected in *B. yuanmingense* isolated from Lespedeza plants. The identified bradyrhizobial lineages constituted sympatric species effectively nodulating Lima bean on the coast of Peru.

El-Fiki (2006) used RAPD fingerprinting for strain identification and the assessment of genetic diversity within a field population of *Rhizobium* (*Bradyrhizobium archus*, *B. japonicum,* and *R. leguminosarum* bv. *Trifolii*). Total genomic DNAs from different field isolates were amplified using two different arbitrary primers. Different band patterns were obtained for all strains. Cluster analysis showed the relationship of *R. leguminosarum* bv. *Trifolii* with *B. archus* (69%) and *B. japonicum*

(63%). The results indicated that RAPD is a very discriminative and efficient method for differentiating and studying genetic diversity of *Rhizobium* strains.

The study was conducted by Boakye et al. (2016) to assess the characteristics and diversity of the rhizobia that nodulate some prominent tree legumes in three soils of Ghana. Five introduced and/or indigenous tree legumes were initially assessed for nodulation in three Ghanaian soils. After 12 weeks of growth in nursery pots the 200 rhizobial strains isolated from their nodules were characterized culturally, metabolically, and phenotypically. Sixty of these isolates were selected randomly and their genotypic characteristics determined using PCR-RFLP of 16S rRNA and intergenic spacer (IGS) genes. Each tree legume was nodulated by isolates classified as fast or very fastgrowers or by isolates classified as slow- or very slowgrowers with 54% of all the 200 isolates belonging to fast or very fastgrowers. Morphologically, 85% of the colonies formed on YEMA were wet and gummy while 70% were acid tolerant, i.e., they were able to grow at a pH of 3.5. Combined restriction of the 16S rRNA genes of the 60 rhizobial isolates with five restriction enzymes clearly distinguished seven different clusters at 80% similarity level. The majority of *A. lebbeck* isolates were distinct from those of the Acacias and *L. leucocephala*. The *M. thonningii* isolates were related to *L. leucocephala* isolates. Simple PCR of the ITS DNA provided several distinct band sizes indicating great variation among the isolates and restriction of the ITS with three different enzymes did not yield many further differences. Molecular techniques revealed a great diversity among the rhizobia that nodulate tree legumes in the tropics, and this may explain why many introduced and/or indigenous trees are able to form nodules with indigenous rhizobia in this region.

A total of 40 bacterial isolates from the root nodules of *Caragana microphylla* growing in desert soil in Ningxia, China, were analyzed for genetic diversity and phylogenetic position by Dai et al. (2012). These isolates were classified into seven types of 16S ribosomal DNA (rDNA) using PCR-RFLP analysis. They were grouped into four clades, *Rhizobium-Agrobacterium*, *Sinorhizobium*, *Phyllobacterium*, and *Bradyrhizobium*, when the phylogenies of 16S rDNA, *recA*, and *atpD* genes were applied. Phylogenetic analysis showed that the tree generated from the 16S rDNA sequencing agreed with that produced from the *recA* and *atpD* genes. By analyzing phylogenetic relationship using the three loci, the isolates in the branches of *Phyllobacterium* and *Sinorhizobium* could be identified as *P. brassicacearum* and *S. meliloti*. The isolates in the branch of *Rhizobium–Agrobacterium* were the most abundant microsymbiont of *C. microphylla* and were designated *R. leguminosarum*, *R. galegae*, *R. alamii*, and *A. tumefaciens*. Two isolates with low sequence similarity to the known species of *Bradyrhizobium* might be novel species in this genus.

Paffetti et al. (1996) investigated the genetic diversity of 96 *Rhizobium meliloti* strains isolated from nodules of four *Medicago sativa* varieties from distinct geographic areas and planted in two different northern Italian soils. The 96 isolates, which were phenotypically indistinguishable, were analyzed for DNA polymorphism with the following three methods: (i) an RAPD method, (ii) an RFLP analysis of the 16S–23S ribosomal operon spacer region, and (iii) an RFLP analysis of a 25-kb region of the p*Sym* plasmid containing *nod* genes. Although the bacteria

which were studied constituted a unique genetic population, a considerable level of genetic diversity was found. The analysis of molecular variance (AMOVA) method was used to estimate the variance among the RAPD patterns. The results indicated that there was significant genetic diversity among strains nodulating different varieties.

Abi-Ghanem et al. (2013) collected isolates of *R. leguminosarum* from several pea fields in Washington, examine genetic diversity among these isolates and several commercial isolates of *R. leguminosarum*, and compare genetically distinct isolates for their ability to fix N in a range of pea hosts. Seventy-nine isolates were collected from pea root from four un-inoculated pea fields. Sequence-related amplified polymorphism (SRAP) markers generated by PCR were used to discriminate among isolates. Isolates fell into 17 clusters with robust bootstrap support values. Nearly half of the isolates fell into a single large cluster, but smaller clusters were also detected for isolates from all four-field locations. The majority of commercial isolates fell into a distinct cluster. Four genetically distinct isolates were compared for their efficiency in fixing N in a greenhouse experiment. Host plant variety effects were significant for plant biomass due to N fixation and also for the quantity of N fixed per variety. Significant effects of *R. leguminosarum* isolates were observed for the quantity of N fixed per isolate, plant biomass, and the quantity of N per plant.

In a study carried out by Loureiro et al. (2007), soybean nodules were collected from 12 sites in the State of Mato Grosso, in the Brazilian Cerrados, where both exotic soybean [*Glycine max* (L.) Merrill] and bradyrhizobial strains have been introduced from 1 to 18 years before. All soils were originally devoid of rhizobia capable of effectively nodulating soybean and varied in terms of chemical and physical properties, inoculation procedures, and cropping systems. Rhizobial genetic diversity was assessed on 240 isolates by rep-PCR fingerprinting with BOX primer, and indices of diversity (abundance-based coverage estimator and traditional and modified Shannon indices) were applied to the profiles obtained. The genetic diversity was much greater than expected, as after the introduction of a maximum of four strains, up to 13 profiles were identified, some sharing many similar bands with the inoculant strains, but others quite distinct from the putative parental genotypes. The increase in the number of rep-PCR profiles could be attributed to genetic variability due to the stressful tropical environmental conditions, but also indicated that indigenous rhizobia became capable of nodulating the host legume. After the third year of cropping with the host legume, inoculation did not affect rhizobial diversity. A high content of clay decreased diversity in comparison with that seen in a sandy soil, probably due to reduced aeration. Diversity was higher under the no-tillage system when compared to the conventional tillage management, highlighting the importance of maintaining crop residues in tropical soils.

Wei et al. (2008) characterized 29 rhizobial isolates from root nodules of the wild legumes *Astragalus, Lespedeza,* and *Hedysarum* growing in the north-western region of China, by numerical taxonomy, RFLP, and sequencing of PCR-amplified 16S rDNA genes, and cross-nodulation with selected legume species. Based on the results from numerical taxonomy, the isolates could be divided into two main groups

(Clusters 1 and 2) and some single isolates at 82% similarity. Cluster 1 contained six isolates from *Astragalus, Lespedeza,* and *Hedysarum* spp. Cluster 2 consisted of nine isolates from *Astragalus* and *Hedysarum* species. The phylogenetic analysis based on 16S rRNA gene sequences showed that SH199, representing cluster 1, belonged to the *Rhizobium–Agrobacterium* group, and SH290B, representing cluster 2, was closely related to *R. galegae* and *R. huautlense*.

Distribution and diversity of rhizobial strains associated with *A. senegal* (L.) Willd. in relation.

to seed provenances in soils from arid (Dahra) and semiarid (Goudiry) zones of Senegal were investigated by Bakhoum et al. (2014). PCR-RFLP performed on 16S–23S rDNA intergenic spacer (IGS) of nodule crude extracts revealed a high genetic diversity of rhizobial strains, which was higher in the semiarid region than in the arid region. The distribution of rhizobial populations was influenced by soil physical and chemical characteristics and by *A. senegal* provenances as shown by the analysis of correspondence. In contrast, the phenotypic diversity of rhizobial strains was not correlated with the soil origin. The phylogenetic tree (performed by the maximum likelihood algorithm) of IGS 16S–23S sequences showed that most of the rhizobial strains nodulating *A. senegal* were closely related to *Mesorhizobium plurifarium*. Our results showed that rhizobial taxa associated with *A. senegal* were mainly distributed according to soil physical and chemical characteristics and *A. senegal* provenances. A large subset of *A. senegal* root-nodulating bacteria had high diversity that correlated with the most favorable environmental conditions.

In a study carried out by Pandey et al. (2004), diversity of rhizobia recovered from five medicinal legumes, *Trigonella foenum graecum*, *Abrus precatorius*, *Mucuna pruriens*, *Melilotus officinalis,* and *Vicia angustifolia* was investigated. Nine bacterial strains isolated on YEMA and showing tolerance to 2% NaCl were analyzed along with reference strains, *B. japonicum* SB102, *Sinorhizobium meliloti* 102F 34, and *R. gallicum* R602spT using restriction patterns produced by amplified DNA coding for 16S rDNA (ARDRA) with two enzymes *Hae*III and *Msp*I and were placed in six genotypes. Four isolates from *Trigonella*, i.e., PP1, PP2, PP3, and PP4 were placed in two genotypes: one genotype was closely related to *S. meliloti* 102F 34 and the other to *B. japonicum* SB 102. Genetic diversity was also assessed by repetitive PCR using BOX primers, wherein all the isolates were placed in five genotypes. Based on repetitive PCR, isolates from nodules of *Trigonella* were placed in single genotype. Nitrogen-fixing ability of the isolates was confirmed by amplification of 781 bp *nif*H fragment in five isolates, PP1, PP2, PP3, PP4, and PP9.

8.2.5 Serological Techniques

Serological techniques have been used in the study of rhizobia for strain identification, ecological investigation of serological relatedness of strains, and their antigenic composition (Graham 1976). The techniques have also been used to identify some organisms depending on their cells immune response to foreign

organism that enters their body (Sadowsky 1983). The technique applies agglutination, immuno-diffusion, and immuno-fluorescence techniques in investigation of rhizobia cells (Kapoor and Dudeja 1995). Agglutination reaction has been used to assess the serological relatedness of strains and species of rhizobia (Ahmad et al. 1981). The studies conducted in Hawaii to examine serological relatedness of 25 strains of slow-growing *Rhizobia japonicum* by agglutination identified six somatic serogroups (Koontz and Faber 1961). Raposeiras et al. (2006) reported that SLA 2.2 native rhizobia strain and CIAT 899 commercial strains are competitive strain for bean inoculation in soils with low fertility and reduced rhizobia population. Gao et al. (2004) reported that strain 042B could form nodules and fix nitrogen to both alfalfa and soybeans with nodule occupancy ranging from 82 to 90% while that of strain USDA110 ranging from 78 to 46%.

Bizarro et al. (2011) reported that 27/75 isolates from soybeans were similar to original strain with strong correlation obtained in their genetic variability. Immunodiffusion is another technique used extensively to investigate the serological relationships between various strains and species of *Rhizobium* (Amarger 2001). It has the resolving power in distinguishing between antigenically identical and closely related but not identical strains (Dudman 1971). The studies for immuno-diffusion of 62 fast-growing strains of lotus rhizobia indicated that while fast and slowgrowers shared no common somatic antigen, internal antigen were shared by fast-growing strains (Sadowsky 1983). Fluorescent Antibody (FA) is among the most used technique for direct examination and identification of rhizobia strains in the culture media, nodules, and direct enumeration of specific strains from the soil (Crozat et al. 1987). The technique is essential because it needs only small amount of antigen and antibody and is the only technique capable for the study of rhizobia in situ (Assmus et al. 1997). Enzyme-linked immunosorbent assay (ELISA) is another technique mostly used for identification of bacteria in the soil or in plants. It uses antibodies and color change to identify a substance. Moawad et al. (2004) assessed the competition for nodulation using FA technique and reported that Phaseolus 163 inoculant strain occupied 30–40% in both soils and 38–50% of nodules on Bronco cultivar and at least 50% of the nodules on the Bronco were occupied by native rhizobia.

8.2.6 Microarrays

DNA microarrays are widely used for transcriptome analysis, single-nucleotide polymorphism and mutation detection, resequencing, comparative genomics, and identification of bacterial species (Lockhart and Winzeler 2000). However, although DNA microarray technology holds promise for microbial ecology and diagnosis, few microarray studies have been conducted to detect the presence of particular genes in biological or environmental samples. The analysis of transcriptome profiles of rhizobial strains by microarrays could give information

on gene expression involved in the synthesis of several signals to control the bacterial activity (Wu et al. 2011).

Bontemps et al. (2005) used a particularly well-suited microarray that consisted of the nodulation gene *nodC*, which is shared by phylogenetically distant rhizobia. 41mer and 50mer oligonucleotides featuring the nucleotide diversity of two highly conserved regions of the NodC protein were spotted on glass slides and cross hybridized with the radioactive-labeled target genomic DNA under low-stringency conditions. Statistical analysis of the hybridization patterns allowed the detection of known, as well as new, *nodC* sequences and classified the rhizobial strains accordingly. The microarray was successfully used to type the *nodC* gene directly from legume nodules, thus eliminating the need of cultivation of the endosymbiont. This approach could be extended to a panel of diagnostic genes and constitute a powerful tool for studying the distribution of genes of interest in the environment, as well as for bacteria identification.

vanPuyvelde et al. (2011) studied the transcriptome of *Azospirillum brasilense* demonstrating that auxin indole-3-acetic acid is a signal molecule affecting its arsenal of transport proteins and cell surface proteins. However, microarray analysis relies on known genes from bacterial species; however, unknown genes are not detected.

Peng et al. (2014) investigated the differential transcriptomes of *Mesorhizobium huakuii* 7653R 7653R bacteroids and free-living cells using RNA-Seq and microarrays. The two approaches identified several thousand differentially expressed genes. The most prominent up-regulation occurred in the symbiosis plasmids, meanwhile gene expression is concentrated to a set of genes (clusters) in bacteroids to fulfill corresponding functional requirements. The results suggested that the main energy metabolism is active while fatty acid metabolism is inactive in bacteroid and that most of genes relevant to cell cycle are down-regulated accordingly. For a global analysis, the researchers reconstructed a protein–protein interaction (PPI) network for 7653R and integrated gene expression data into the network using cytoscape. A highly interconnected subnetwork, with function enrichment for nitrogen fixation, was found, and a set of hubs and previously uncharacterized genes participating in nitrogen fixation were identified.

8.2.7 Biosensors

Biosensors are defined as bacterial cells harboring a reporter gene, which is usually a fluorescence marker such as a green fluorescent protein (GFP) cassette expression (Sørensen et al. 2009). This system allows detection of activity and colonization of rhizobia at the single cell level in rhizosphere microsites or inside the plant roots by epifluorescent and confocal microscopy.

Bacteria of the genus *Bradyrhizobium* are able to establish a symbiotic relationship with peanut (*Arachis hypogaea*) root cells and to fix atmospheric nitrogen by converting it to nitrogenous compounds. Quorum sensing (QS) is a cell–cell communication mechanism employed by a variety of bacterial species to

coordinate behavior at a community level through regulation of gene expression. The QS process depends on bacterial production of various signaling molecules, among which the *N*-acylhomoserine lactones (AHLs) are most commonly used by Gram-negative bacteria. Some previous reports have shown the production of QS signaling molecules by various rhizobia, but little is known regarding mechanisms of communication among peanut-nodulating strains. Nievas et al. (2012) identified and characterized QS signals produced by peanut-nodulating bradyrhizobial strains and to evaluate their effects on processes related to cell interaction. Detection of AHLs in 53 rhizobial strains was performed using the biosensor strains *A. tumefaciens* NTL4 (pZLR4) and *Chromobacterium violaceum* CV026 for AHLs with long and short acyl chains, respectively. None of the strains screened were found to produce AHLs with short acyl chains, but 14 strains produced AHLs with long acyl chains. These 14 AHL-producing strains were further studied by quantification of β-galactosidase activity levels (AHL-like inducer activity) in NTL4 (pZLR4). Strains displaying moderate to high levels of AHL-like inducer activity were subjected to chemical identification of signaling molecules by high-performance liquid chromatography coupled to mass spectrometry (LC-MS/MS). For each AHL-producing strain, we found at least four different AHLs, corresponding to *N*-hexanoyl-DL-homoserine lactone (C6), *N*-(3-oxodecanoyl)-L-homoserine lactone (3OC10), *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12), and *N*-(3-oxotetradecanoyl)-L-homoserine lactone (3OC14). Biological roles of 3OC10, 3OC12, and 3OC14 AHLs were evaluated in both AHL-producing and -non-producing peanut-nodulating strains. Bacterial processes related to survival and nodulation, including motility, biofilm formation, and cell aggregation, were affected or modified by the exogenous addition of increasing concentrations of synthetic AHLs. Their results clearly demonstrate the existence of cell communication mechanisms among bradyrhizobial strains symbiotic of peanut. AHLs with long acyl chains appear to be signaling molecules regulating important QS physiological processes in these bacteria.

Salome et al. (2009) described an electrochemical method for the determination of the nitrate and nitrite reductase activities of *Rhizobium japonicum*. The advantage of the method lied in the use of whole cells for the analysis and the team developed this protocol for the assay of NO. The results obtained were comparable to the spectrophotometric Griess assay. As the method is based on electrochemical reduction, the commonly interfering biological components like ascorbic acid, uric acid, dopamine, etc. could not interfere with the analysis. This method could be extended to the fabrication of biosensors for nitrate and nitrite using the same principle.

8.2.8 Proteomics

Proteomics is a high-throughput technology that has been used to investigate a wide range of biological aspects including phylogenetic, molecular divergence studies,

plant responses to different stresses, detailed studies on the structural components, and biochemical pathways involved in symbiotic nitrogen fixation. Approaches, such as transcriptome, proteome, and metabolome analysis in both symbionts, promise to reveal much more detail about the metabolic flows in the nitrogen-fixing nodule or even to description the novel unknown aspects (Khatoon et al. 2012).

In a study, Natera et al. (2000) identified root nodule proteins in *Melilotus alba* during 12 days after inoculation by *S. meliloti*, including *S. meliloti* and bacteroid proteins. Proteins involved in nodule formation and regulated by auxin have also been identified in *Medicago truncatula* infected by *S. meliloti* (van Noorden et al. 2007).

Extracellular proteome of *R. etli* strain during different growth stages was described by Meneses et al. (2010). Their results revealed that secretome of *R. etli* consists of actively secreted proteins, which mostly are extracellular enzymes (mostly degradation enzymes) and proteins that bind nutrients and extracellular appendages, and proteins that have functions in the cytosol and are not actively secreted but may be released into the culture medium. Function of many identified proteins in extracellular proteome is still unknown.

To analyze the nodule organogenesis in legumes at the protein level in a time-course study with soybean over the first 48 h, Salavati et al. (2012) coupled 2-D gel electrophoresis with quantitative RT-PCR to analyze isolated proteins at different time points from infected soybean root hairs at both transcriptional and translational levels. Analysis of 56 proteins revealed the differential expression of plant proteins associated with important events, such as metabolism, cell signaling, and disease/defense response. The formation of infected legume nodules capable of fixing nitrogen requires the bacteria to activate two plant programs: one leading to nodule morphogenesis and the other leading to nodule infection.

Proteomic studies in combination with transcriptomics studies such as quantitative RT-PCR can advance symbiosis analysis to a new level (Resendis-Antonio et al. 2011). In combination with the genome sequencing, proteomics has been recently become a powerful investigation of the most detailed physiological events in plant, animal, and microorganisms (Thibivilliers et al. 2009).

While remarkable progress in proteomic study of symbiosis has been made in model plants, a quite advancement in developing proteomic approaches in other crops has been reached. The biggest obstacle to these proteomic applications is the scarcity of well-annotated protein databases and sequences of proteins. Although some techniques such as de novo sequencing and proteogenomics recompense this paucity, there is still an urgent need to expand and curate plant protein databases. Many existing databases, including Soybean, *Medicago,* and rice proteome database should be expanded and integrated in the future (Sun et al. 2009).

It is assumed that the development and improvement of techniques, such as metagenomics, metaproteomics, and metatranscriptomics, will provide more accurate evaluation of the activities and compositions of microbial communities in rhizospheres than classical molecular techniques, generating new questions about the roles and functions of these microbial communities (Sørensen et al. 2009).

8.2.9 *Metagenomics, Metaproteomics, and Metatranscriptomics*

Metagenomics (environmental genomics) is the genomic analysis of microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms. The development of metagenomics stemmed from the ineluctable evidence that as-yet-uncultured microorganisms represent the vast majority of organisms in most environments on earth. This evidence was derived from analyses of 16S rRNA gene sequences amplified directly from the environment, an approach that avoided the bias imposed by culturing and led to the discovery of vast new lineages of microbial life (Niedringhaus et al. 2011).

In order to study and evaluate the richness, distribution and activity of rhizobial communities in bulk and rhizosphere soils, it is important to understand the ecological functions of each species. Modern molecular techniques have shown that bacterial diversity of bulk and rhizosphere soil is much greater than was predicted. Soil metagenome study of 16S rRNA gene and ITS1 region using next generation sequencing or second-generation sequencing technologies have revealed that 1 g soil sample may contain 33,346 bacterial and archaeal OTUs (Mendes et al. 2011), 3320 fungal OTUs (Schmidt et al. 2013), 145–200 of fungal OTUs and 300 archaea OTUs (Pires et al. 2012). Both Roche 454 and Illumina platforms have been used to address the bulk and rhizosphere soils. Roche 454 pyrosequencing platform produces long read length (<450 bp) and high consensus accuracy more than Illumina platform (Unno 2014). Uroz et al. (2010) by 454 pyrosequencing compared the bacterial diversity of oak rhizosphere and bulk soil, finding that *Proteobacteria, Acidobacteria, Actinobacteria,* and *Bacteroidetes* were the dominant taxa.

Metraproteomics provides a direct measure of proteins present in an environmental sample such as soil, offering information about the functional roles of soil microorganism, such as biogeochemical processes, degradation, or bioremediation processes (Bastida et al. 2012). Nevertheless, numerous metaproteomics studies have revealed the diversity of proteins that are expressed by the interactions between plants and soil microbial communities.

Metatranscriptomics involves the characterization of a set of messenger RNA (mRNA) (transcripts) produced in all cells, which would provide insight into the metabolic processes of a microbial community (deMenezes et al. 2012). Consequently, metatranscriptomics analysis has the potential to discover novel genes and functions, allowing identification of active community members in both bulk and rhizosphere soils, and to correlate them with their metabolic activities (Kim et al. 2014). However, metatranscriptomic approaches have not been widely used in the rhizosphere, probably due to the instability of mRNAs and difficulties in their extraction from complex ecosystems. Among the more notable methodological challenges are their short half-lives, difficulties in the separation of mRNA from other RNA types (i.e., tRNA, rRNA, miRNA) and interference from humic compounds that co-extract with nucleic acids from soil (Simon and Daniel 2011). In addition, few studies have applied metagenomics, metaproteomics, and metatranscriptomics due

also to the difficulties and expense related to simultaneous extraction of nucleic acids and proteins from soil samples. Moreover, the majority of studies in bulk and rhizosphere soils are mainly focused on bacteria, demonstrating the need for further studies to obtain further insights into the molecular ecology of other microorganisms present in rhizosphere microbiome, such as fungi, archaea, microalgae, and protozoa.

8.2.10 Conclusions and Future Prospects

An important bacterial group in the rhizosphere is defined as PGPR, which are able to promote the growth, nutrient uptake, and pathogen biocontrol in plants (Mendes et al. 2011). The most abundant groups of bacteria which potentially can promote plant growth are *Actinobacteria, Firmicutes, Proteobacteria (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria) and Bacteroidetes* and particularly the genera *Rhizobium*, *Azospirillum*, *Burkholderia*, *Pseudomonas,* and *Enterobacter*. During the last few decades, a wide variety of molecular techniques have been developed and used as valuable tools for the study of diversity and function of bacteria in the rhizosphere. However, the biases of each molecular technique must be considered and evaluated during their application in the study of rhizobacteria.

Diversity in rhizobium has been revealed by many studies, and almost all of the data reported previously indicate that there is a high level of genetic diversity in these bacteria. An assessment of the genetic diversity and genetic relationships among strains could provide valuable information about bacterial genotypes that are well adapted to a certain environment. The advances in soil molecular and post-genomic techniques have and will continue to improve understanding of the compositions and activities of rhizobial communities, to target the rare or low abundance rhizobacterial populations in the symbiotic relationships, and to predict in situ responses, activities, and growth of rhizobial communities.

Acknowledgement The authors are grateful to Hon'ble Vice-Chancellor, Kurukshetra University, Kurukshetra for proving necessary infrastructure for carrying out research and literature survey. The authors also acknowledge their gratitude to the Director, University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra and Chairman, Ambala College of Engineering and Applied Research, Devsthali, Ambala, Haryana for providing infrastructural facility.

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