CHAPTER – 2

Review of Literature

2.1. LITERATURE REVIEW

A study on the isolation and characterization of Diazotrophic bacteria was carried by Ozawa *et al.,* (2003) from the surface-sterilized roots of eight species of legumes: *Arachis hypogeal, Astragalus sinicus, Crotalaria juncea, Glycine max, Medicago sativa, Phaseolus vulgaris, Pisum sativum, Sesbania cannabina, Trifolium incarnatum, Trifolium pratense* and *Trifolium repens*. The nitrogen fixing activity of the isolates in the semi-solid media was assessed by Acetylene Reduction Acitivity (ARA) method and 31 isolated bacteria showed ARA activity of 2.6 to 450 nmol h^{-1} $culture^{-1}$ when grown in the JNFb media. Analysis of 16S rRNA gene sequence (Devereux and Willis 1995) and examination of physiological characteristics (Smibert and Krieg 1994) of the 31 isolates showed that the isolates belong to *Agrobacteriun radiobacter, A. tumefaciens, Azospirillum lipoferum, Bradyrhizobium elkanii, Burkholderia cepacia, Frateuria aurantia, Klebsiella oxytoca, K. pneumoniae, Rhizobium gallicum, R.* sp., *Starkeya novella* and *Xantobacter flavus.*

Zhang *et al.,* (1991) did their work on the diversity of *Rhizobium* bacteria isolated from the root nodules of Leguminous Trees. In their work 60 rhizobial strains isolated from the root nodules of *Acacia senegal*and *Prosopis chilensis* in the 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. After this strains were 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.

A work regarding the Genetic diversity of rhizobia from *Leucaena leucocephala* nodules in Mexican soils was undertaken by Wang *et al.,* (1999). In the study a total of 150 isolates were obtained from the nodules of two *L. leucocephala* cultivars growing in different soils. After performing restriction fragment length polymorphism (RFLP) of amplified 16S rRNA genes, twelve rDNA types were

identified which are found to clustered into groups conforming to *Mesorhizobium, Rhizobium* and *Sinorhizobium*. Most of the isolates corresponded to *Sinorhizobium.*Based on the different combinations of electrophoretic patterns of 13 metabolic enzymes, forty-one electrophoretic types (ETs) were identified among the isolates which were clustered into groups in general agreement with the rDNA types.Among the isolates diverse plasmid patterns were obtained and symbiotic plasmids were identified among the isolates, except for the *Mesorhizobium* isolates. The impact of soil cultivation on the soil populations of rhizobia and the affinities of host cultivars for different rhizobial groups were analysed from the estimation of isolation frequencies and diversity. The results of the study showed the differences in rhizobial populations in cultivated and uncultivated soils and also differences in rhizobia confined by *L. leucocephala* cv. Cunninghamor Peruvian.

Suliasih and Widawati., (2005) undertake a study to investigate the occurrence of phosphate solubilizing bacteria (PSB) and nitrogen-fixing bacteria (NFB) from soil samples of Wamena Biological Garden (WbiG). Eleven soil samples were collected randomly to estimate microbial population which used plate count method (Ravina et al, 1992; Thompson, 1989; Vincent, 1982). 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 Systemic Bacteriology* (Kreig and Holt, 1984)**.**

Identification and characterization of *Rhizobium* associated with woody legume trees grown under Saudi Arabia condition was undertaken by Shetta *et al*., (2011). For the work six woody legume tree species, *Acacia ehrenbergiana* (Hayne.), *Acacia nilotica* (Willd.), *Acacia saligna* (Labill.), *Acacia tortilis* (Forssk.), *Acacia tortilis* var. *reddiana* (Savi.) and *Leucaenaleucocephala* (Lam.) were selected and the growth characteristics and resistance of native isolated Rhizobium strains from root zone surrounding trees to environmental stresses- antibiotic, high temperature, salinity and acidity were studied. Physiological properties of all isolated strains were found to be fast growing and had the same colony morphology and produced high, slimy

transparent to creamy coloured colonies on YEMA plates after 3 days of incubation at 28°C. Acid production was observed among the isolates after 72 hours. In the study, the strains showed resistance to antibiotics, temperature, salinity and pH fluctuation. Based on the utilization of carbon and nitrogen, the results indicates that the strains probably belongs to one of the two groups, *Rhizobium* or *Sinorhizobium*. The isolates from thestudy could be useful to increase the symbiotic nitrogen fixation in legume trees and it provides basis for further research on the phylogeny of Rhizobial strains nodulating the legume treesas well as their use as inoculants to improve growth in arid lands of the central region of Saudi Arabia.

Mirza*et al.,* (2001) reported about the isolation of nitrogen fixing, phytohormone producing bacteria from sugarcane and their beneficial effects on the growth of micropropagated sugarcane plantlets. For isolation serial dilutions of the bacterial growth in the semi solid 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 (Hugh and Leifson, 1953) and catalase test (MacFaddin 1980) were also performed for the identification of the isolates. Finally PCR-amplification and 16S rRNA sequence analysis was carried out for differentiating the isolates.

Fourteen strains of nitrogen-fixing bacteria were isolated by Reinhard *et al.,* (2008) from different agricultural plant species, including cassava, guinea grass, maize, sugarcane and tomato using the selective media NFb described by Hartmann *et al.,* (2006). It was found that the isolated strains from the above plants reduced acetylene on the chromatography analysis and hence indicating their N_2 fixing ability. All potentially nitrogen-fixing strains tested showed positive hybridization signals with *nif*H probe derived from *Azospirillum brasilense*. RAPD, ARDRA and 16S rDNA sequence analysis were also performed for the characterization of the strains. ARDRA and 16S rDNA sequence analyses allowed the assignment of 13 strains to

known groups of nitrogen-fixing bacteria, including organisms from the genera *Azospirillum, Herbaspirillum, Pseudomonas* and Enterobacteriaceae. Further their work has been analyzed by performing Dot blot Hybridization.

Prabudoss and Stella., (2009)isolated *Gluconacetobacter diazotrophicus* strains from samples of sugar rich crops like sugar cane (root, stem, bud, leaves), sweet potato, pine apple and wild cane by following the methodology of Cavalcante and Dobereiner, (1988) . The nitrogen fixing efficiency of all the isolates were found to be efficient with the reference strain indicating the superiority of the local isolates.

Khan *et al.,* (2008) reported about 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 *Enterobacterspp.*, for strain-1, *Klebsiella spp.* for strain-2, *Bacillus spp.* for strain-3 and *Azospirillum spp.* for strain-4. They were anaerobic in nature.

An investigation on the occurrence and genetic diversity of rhizobia nodulating *Sesbania sesban* in African soils was carried out by Bala *et al*., (2002). The distribution, diversity and relative abundance of Sesbania sesban rhizobia in African soils were investigated by host-trapping and counting of rhizobia during the study. Characterization of the rhizobia was performed using restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA and the internally transcribed spacer (ITS) between the 16S and 23S rRNA genes. Isolates representative of the diverse 16S rRNA groups from the various soils were selected for sequence analysis of the first 750 bp of the 16S rRNA. The study found out that compatible rhizobia were detected in only 15 out of 55 soils, and were present generally in soils with more than 10% clay, and those from low-lying areas. The rhizobia nodulating S. sesban were found out to be genetically diverse, with isolates bearing 16S rRNA sequences similar to those of rhizobia belonging to the genera *Rhizobium, Mesorhizobium, Sinorhizobium* and *Allorhizobium* and onlyabout 1% of the isolateshad sequences with close homology with*Agrobacterium tumefaciens*. The

study indicates that Mesorhizobium group was dominant in all soils examined despite the wide phylogenetic distribution of the rhizobial isolates, accounting for 90% of the isolates on average, with individual soil populations usually being comprised of two genera. There was a strikinginconsistency in the sequence and size of the ITS region among rhizobia nodulating Sesbania which specifies a broad diversity of 'strain' types both within and between soil populations, and within and between rhizobial genera.

Sharma et al., (2005) undertook a study on the diversity in a promiscuous group of rhizobia from three *Sesbania* spp. colonizing ecologically distinct habitats of the semi-arid Delhi region. Diversity in symbiotic properties, LPS profiles, *Sym*plasmid and rhizobiophage sensitivity of 28 root- and stem-nodulating bacterial isolates of three *Sesbania* species (*S. sesban*, *S. aegyptica*and *S. rostrata*) inhabiting six ecologically distinct sites of semi-arid Delhi region was analyzed. The isolates were highly promiscuous among the symbiotic partners (*Sesbania* spp.). The root nodules formed by all the isolates were morphologically similar but they differed in their symbiotic efficiency and effectiveness. 16S rDNA sequence analyses revealed that root nodule isolates of sesbanias belong to diverse rhizobial taxa (*Sinorhizobium saheli*, *S. meliloti*, *Rhizobium huautlense*) whereas stem-nodule isolates were strictly *Azorhizobiumcaulinodans*. *Sinorhizobium* spp. seem to dominate as microsymbiont partner of *Sesbania* in the Delhi region. The genetic diversity revealed by cluster analyses based on NPC-PCR reflects sorting of isolates across the ecological gradient. Parallel diversity was also observed in the grouping based on LPS profiles and *sym*plasmid (NPC- PCR). Segregation of different rhizobial taxa into distinct types/clusters based on LPS and NPC-PCR analyses suggest its significance in the circumscription of the taxa. However, subtypes and subclusters showed their sorting across the ecological gradients. *Sesbania* rhizobia showed extremely high specificity to rhizobiophages. Enormous diversity in LPS profiles and high specificity of rhizobiophages might be the result of environmental selection pressures operating in ecologically distinct habitats. The ability of sesbanias to enter into effective symbioses with different rhizobial taxa and colonize diverse habitats with various biotic and abiotic stresses appears to contribute to its wide ecological amplitude.

Holguin *et al.,*(1992)*;* worked on the isolation of two new diazotrophic bacteria, *Listonellaanguillarum* 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 methyl esters that have a chain length between 9 and 18 carbons long. During their work it was found that *Staphylococcus sp.,*indicates interaction with the above two diazotrophic bacteria and results in the increased or decreased in the nitrogen fixing capacity of the two diazotrophs.

Gaby and Buckley.,(2012); performed a comprehensive evaluation of PCR Primers to amplify the *nifH* Gene of nitrogenase. They performed an *in Silico* analysis of the specificity and coverage of 51 universal and 35 group-specific *nifH* primers by using an aligned database of 23,847 *nifH* sequences. Their work also suggest other workers that many of the primers will amplify gene that do not mediate nitrogen fixation and thus are advisable for researchers to screen their sequencing results for the presence of non-target genes before analysis. Universal primers that performed well *in sillico* were also tested empirically with soil samples and with genomic DNA from a phylogenetically diverse set of nitrogen-fixing strains. Their study will be of great utility to those engaged in molecular analysis of *nifH* genes from isolates and environmental samples.

A study regarding Rhizobium compatibility and nitrogen fixation of *Leucaena sps.* was carried out by Thoma (1983). The study was primarily for the determination of the compatibility and effectiveness of potentially nitrogen fixing associations between two Rhizobium strains and thirteen *Leucaena leucocephala,* five other species of *Leucaena* and *Albizia occidentalis.* Determinations about the appearance, dry weight and nitrogen content of plants grown with and without *Rhizobium* was done. A comparison was also made of nitrogen fixed per plant and per unit weight of plant, and the ratio of plant weight (grown with *Rhizobium*) to plant weight (grown without *Rhizobium*). The various collection of *L. leucocephala* was to made it compatible and equally effective nitrogen fixing associations with the two strains of *Rhizobium*. The study revealed the compatibility and effectiveness of nitrogen fixing

associastions with *L. pulverulenta, L. esculenta, L. diversifolia*and *L.collinsii.* Less effective nitrogen fixing associations was found to occur with *L. retusa* and the same happened with *A. occidentalis* although root nodules were formed on it.

Hongrittipun *et al.,* (2014), during their study, isolated endophytic bacteria from one-month-old seedlings of five rice (*Oryza sativa L.*) varieties growing without nitrogen fertilizer in the farmers' field. In their study one hundred and twenty-three isolates of endophytic bacteria were obtained from roots, stems and leaves of these rice varieties and their nitrogenase activity was determined by acetylene reduction assay. Seven isolates with highest nitrogenase activity were then identified through phylogenetic analysis of 16S rRNA genes, and were found belonging to *Burkholderia cepacia, Citrobacter* sp., *Bacillus amyloliquefaciens* and *B. Thuringiensis.* It was found that inoculation of*Bu. cepacia* and *Citrobacter* sp. to the seedlings of local rice variety (Muey Nong 24) significantly increased nitrogen concentration in the roots of rice.

Zahran 2001, gave a detailed report about the diversity, taxonomy, ecology, nitrogen fixation and biotechnology of rhizobia from wild legumes distributed in arid regions. The paper described that wild legumes in arid zones harbour diverse and promiscuous rhizobia in their root-nodules of which majority of rhizobia are with wide host range and specificity existed only in few rhizobia. It was also mentioned that they exhibit higher tolerance to the prevailing adverse conditions, e.g, salt stress, elevated temperatures and desiccation.Moreover, intercropping of some nitrogen-fixing tree legumes (e.g, *Lablab, Leucaena, Sesbania* etc.) to pasture grasses improved biomass yield and herb quality. Suggestion was also given that these bacteria may have specific traits that can be transferred to other rhizobia through genetic engineering tools or used to produce industrially important compounds. It is thus concluded that these bacteria are very important from economic and environmental points of view.

A work on the phenotypic and genotypic characterization of*Rhizobium* species isolated from the root nodules of *Sesbania sesban* found in Mumbai and its suburban areas was carried out by Singh et al., (2013).A total of seventeen bacterial

isolatesRoot were isolated and characterized from the root nodules of Sesbania sesban growing in regions of Mumbai and its suburban areas using Yeast Extract Mannitol Agar medium. These isolates were further studiedmorphologically and biochemically along with one reference culture (NCBI-TUR1). Out of seventeen, morphologically six Rhizobium strains were found to be rod shaped, Gram negative, mucousproducing and were unable to grow in the presence of 0.1% Methylene blue and Lactose.Biochemical characterization of all six isolates confirmed them to be of Rhizobium species. Furtherpolymerase chain reaction and 16S rRna gene sequencing were used for the genotypic analysis of Rhizobium species. After nif gene amplification, only six strains out of seventeen isolates were selected as Rhizobium. Further six isolates were selected as fast growing Rhizobium species based on Bromothymol Blue test. The study showed less growth of the isolates as the concentration of salt increased.All isolates were found to grow at pH 6.0 to 11.0 but none at pH 12.0. The optimum physical parameters for growth of fast growing rhizobia were also found in pH between 7.0-8.0 and 28ºC temperature. Lastly, BIOLOG test was done in order to know the nutrient requirement and utilization pattern of Rhizobium species.The objectiveof the study was solely for the assessment of Rhizobial genetic diversity.

A study was conducted by Satapute *et al.,* (2012) with the goal of isolation, screening and characterization of the most potential nitrogen fixers from agricultural soil. Five bacterial isolates were isolated and characterized from the rhizosphere of ground nut plant and *Bacillus subtilis* was identified as the potential isolate based on the morphological, biochemical, cultural and 16s rDNA identification. The study proves that the soil isolate *Bacillus subtilis strain* is salt tolerant, free living nitrogen fixing bacteriathat could be exploited as soil inoculants and can be used for nitrogen fixation in soil withhigh concentration of salt, which is of long run, eco-friendly and cost ineffective.

Isolation and characterization of diazotrophic growth promoting bacteriawas performed by Park *et al.,* (2005) from rhizosphere of seven different agricultural crops grown in Chungbuk Province, Korea namely sesame, maize, wheat, soybean, lettuce, pepper and rice. Physiological and biochemical characters of the bacterial isolates

were examined according to methods described in *Bergey's Manual of Systematic Bacteriology* (Holt *et al*., 1994). Nitrogen fixing activity of the isolates was determined by the acetylene reduction assay (Hardy *et al*., 1968). Five isolates with nitrogenase activity above 150 nmol h^{-1} mg^{-1} protein were identified based on phenotypic and 16S rDNA sequences analysis after which the strainwere identified as *Stenotrophomonas maltophilia, Bacillus fusiformis* and *Pseudomonas fluorescens*.Production of IAA by the cultures were also estimated.

Characterization of symbiotic root nodulating rhizobia isolated from Lentil (*Lens culinaris medic.*) was undertaken by Rashid *et al.,* (2009) using molecular techniques along with traditional techniques such as physiological, biochemical and intrinsic antibiotic resistance. Two molecular techniques based on PCR amplification such as repetitive extra genomic palindromic (REP-PCR) sequences and restriction fragment length polymorphism (RFLP) analyses were used in the study. Groupings generated by PCR DNA finger printing with either extragenomic palindromic repetitive primers or two different primers were correlated with similar levels of resolution. In the study nitrogen fixation nature of all the strains was confirmed by amplification of *nif*H gene and variations were not observed among the strains. Variations in 16S rRNA between *Rhizobium leguminosarum* and Bangladeshi strains observed by RFLP in the study revealed that Bangladeshi lentil symbiont might be different from *Rhizobium leguminosarum*.

Hung and Annapurna., (2004) carried out an investigation to analyse the phenotypic and genotypic diversity in the bacterial endophytes of two species of soybean *viz. Glycine max* and *G. Soja*. During the study a total of 65 bacterial endophytes were isolated from three tissues: stem, root and nodule. The isolates were screened for Gram reaction, secretion of hydrolytic enzymes (pectinase and cellulose), fluorescent pigment production and motility, resistance to streptomycin, capsule formation and IAA production. Preliminary characterization of the 65 endophytes showed that approximately equal percentages of gram positive (49%) and gram negative (55%) bacteria were present, 80% were motile, 33% and 70% secreted pectinase and cellulose, respectively and 17% did not produce IAA *in vitro*. Molecular characterization 35 selected endophytes was carried out by PCR

amplification of 16S rDNA gene, and its restriction analysis using three tetra cutters, *HaeIII, MboI* and *MspI*. The study revealed that the genetic variation was more among endophytes isolated from *G. max* tissues than *G.soja*.

A valuable work on the Isolation ofan Endophytic Diazotroph, *Klebsiella oxytoca,* from the stem of Sweet Potatocultivars (Beniotome [BO], Koganesengan [KS], and Shiroyutaka [SYD in Miyakonojo, Miyazaki, Japanwas undertaken by Adachi et al., (2002) by using a semi-solid nitrogen free medium. The population density ofendophytic bacteria in the stem offield-grown sweet potato was found to range from 102 to 104 cells g-l fresh weight sample. Two isolates, BO-l and BO-5, isolated from cv. Beniotome in September 1999 and 2000, respectivelyshowed a positive reaction in the acetylene reduction activity (ARA) test out of the eleven strains isolated from the stems. Isolates BO-l and BO-5 were found to exhibit a similar colony colour in potato sucrose agar slants, produced bubbles in a modified semi-solid medium, acidified the medium, and displayed similar characteristics using the API 20NE rapid diagnostic kit after Morpho-physiological characterization of the isolates. It was found out that BO-l revealed a 100% similarity (491 bp) to that of *Klebsiella oxytoca* after partial sequence analysis of 16S rRNA. The other isolates in the study were found to show a negative reaction in the ARA test, marginally acidified or did not acidify the medium. After partial sequence analysis of the 16S rRNA, it was shown that isolate SY-2 corresponded to *Methylobacterium* sp. (99.3%similarity for 1,241 bp), BO-3 to *Pantoea agglomerans* (99.1% similarity for 469 bp), and BO-8 to *Sphingomonassanguinis* (98.8% similarity for 419 bp).

A study undertaken by Sugitha and Kumar., (2009) deals on the identification of *nif* genes of heterotrophic and endophytic diazotrophs associated with rice (*Oryza sativa* L.,) by targeted DNA finger printing. The diazotrophs were isolated from rhizosphere soil, rhizoplane, roots and stems of different rice varieties. A total of thirteen isolates obtained were subjected to acetylene reduction assay (ARA) and eight isolates recorded significant amount of nitrogenase activity in a range of 31.65 to 91.95 nmoles of ethylene mg^{-1} cells h^{-1}. Targeted PCR fingerprinting using *nif* H primers generated specific DNA band of approximately 750 bp, confirming the presence of *nif* genes in these isolates.

To analyse the diversity and relationships ofrhizobia in the subtropical and tropical zones of China, Liu et al., (2007)characterized 67 bacterial strains isolated from root nodules of five legumespecies in the genera *Trifolium*, *Crotalaria*and *Mimosa***.**Incorporationof PCR-amplified 16S rDNA RFLP, numerical taxonomy, SDS-PAGE of whole cell proteins, sequencing of 16S rDNA and DNA–DNA hybridization grouped the isolates into 17 lineages belonging to *Bradyrhizobium*,*Mesorhizobium, Rhizobium*, *Sinorhizobium* and *Burkholderia*, as well as a non-symbiotic group of *Agrobacterium*. The *Rhizobium* group was found tocomprise of twenty strains isolated from *Mimosa pudica*, *Crotalaria pallida* and two species of *Trifolium* where fifteen of them were *R. leguminosarum*. Twenty-one strains that were isolated from four species of *Trifolium,Crotalaria* and *Mimosa* were categorised into five groups of *Bradyrhizobium*, including *B. japonicum*. *Agrobacterium*group composed of 20 isolates from *M. pudica*, *C.pallida* and *T. fragiferum*.Moreover, several strains of *Sinorhizobium* and *Mesorhizobium* associated with *Trifolium* and *Burkholderia* associated with *Mimosa pudica* were also identified during the study. The predominance of *Bradyrhizobium*in the nodules of *Trifolium* was a novel finding and the study concluded that the nodule microsymbionts might be selected by both the geographic factors and the legume hosts.

Wang et al., (2003) undertook a study for the characterization of diverse *Mesorhizobium pluriferium populations* native to Mexican soils. During the study,forty-six *Mesorhizobium* strains associated with the leguminous plants *Leucaena leucocephala* and*Sesbania herbacea* in an uncultivated Mexican field were characterized using a polyphasic approach. The strains were identified as *Mesorhizobium plurifarium* based upon the close relationships with the reference strains for this species by using PCR-based restriction fragment length polymorphism analyses, sequencing of 16S rRNA genes, DNA-DNA hybridization and multilocus enzyme electrophoresis. Although cross-nodulations were observed in the laboratory from the strains isolated from both plants and forming the same group in multilocus enzyme electrophoresis, different electrophoretic types were acquired from the two plants grown in natural soils, signifying the existence of a better association between the plants and the rhizobia. *M. plurifarium* strains from Mexico and the reference

strains from Africa and Brazil were found to formed different phenotypic clusters in a numerical taxonomy. Besides, the Mexican strains failed to grow at 37 °C and shows sensitivity to salty-alkaline conditions, whereas the reference strains from Africa and Brazil grew at 42 °C and showed more resistance to salty-alkaline conditions. Thus the results of the study established that both the plants and environmental factors affected the evolution of rhizobia and concludes that the Mexican strains had adapted to the neutral soils and the cool climate where they were isolated.

An investigation on the genetic diversity and phylogeny of rhizobia isolated from agroforestry legume species in Southern Ethiopia was carried out by Wolde-meskel et al., (2005). The genetic diversity within 195 rhizobial strains isolated from root nodules of 18 agroforestry species (15 woody and three herbaceous legumes) growing in different eco-climatic zones in southern Ethiopia was investigated during the study by using PCR–RFLP of the ribosomal operon [16S rRNA gene, 23S rRNA gene and the internal transcribed spacer (ITS) region between the 16S rRNA and 23S rRNA genes] and 16S rRNA gene partial sequence (800 and 1350 bp) analyses. All of the isolates and the 28 reference strains were segregated by using these methods. The size of the ITS were found to bedifferent among test strains (500–1300 bp), and 58 strains contained double copies. UPGMA (Un-weighted Pair Group Method with Arithmetic Mean) dendrograms produced from cluster analyses of the 16S and 23S rRNA gene PCR–RFLP data were in good agreement, and the combined distance matrices delineated 87 genotypes, signifyingsubstantial genetic diversity among the isolates. The phylogenetic analyses in the studysuggested that strains native to Ethiopia belonged to the genera Agrobacterium, Bradyrhizobium, Mesorhizobium, Methylobacterium, Rhizobium and Sinorhizobium. Documentation have been done where many of the rhizobia isolated from previously un-investigated indigenous woody legumes had novel 16S rRNA gene sequences and were phylogenetically diverse. The study clearly indicates thatthe characterization of symbionts of unexplored legumes growing in previously unexplored bio-geographical areas will in fact reveal additional diversity.

Aserse et al., (2012) carried out an investigation which showed that Bradyrhizobium strains isolated from root nodules of Crotalaria spp., Indigofera spp., Erythina brucei and soybean (Glycine max) growing in Ethiopiarepresented genetically diverse phylogenetic groups of the genus Bradyrhizobium. During the study Amplified Fragment Length Polymorphism fingerprinting technique (AFLP) and Multilocus Sequence Analysis (MLSA) of core and symbiotic genes were used for the characterization of the strains. Bradyrhizobium strains were distributed into fifteen phylogenetic groups under B. japonicum and B. elkanii super clades in the study through the phylogenetic analyses of concatenated recA–glnII–rpoB–16S rRNA genes sequences. Severalof the isolates were found belonging to the species *B. yuanmingense, B. elkanii* and *B. japonicum*type I. Majority of the isolates howeverrepresented unnamed Bradyrhizobium genospecies and from which, two distinctive lineages that most likely represent novel Bradyrhizobium species were identified among Ethiopian strains.Sequence analysis of nodulation nodA gene revealed that all Ethiopian Bradyrhizobium isolates belonged to nodA sub-clade III.3. Further classification of the strains into 14 groups was also done together with strains from Africa, as well as some originating from the other tropical and subtropics regions. The nifH phylogenies of the Ethiopian Bradyrhizobium were also found to be generally congruent with the nodA gene phylogeny, supporting the monophyletic origin of the symbiotic genes in Bradyrhizobium. The phylogenies of nodA and nifH genes were also partially congruent with that inferred from the concatenated core genes sequences, giving the conclusion of the investigation that the strains obtained their symbiotic genes vertically from their ancestor as well as horizontally from more distantly related Bradyrhizobium species.

A study on the genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang province of China was done by Li et al., (2008). A total of 98 non-symbiotic endophytic bacterial strains isolated from soybean root nodules were classified into eight rDNA types inARDRA analysis and 21 BOX types in BOX-PCR in the study. The strains were recognised as Pantoea, Serratia, Acinetobacter, Bacillus, Agrobacterium, and Burkholderia by the phylogenetic analysis of 16S rDNA. The study revealed that most

of the strains (85.7%) were found in three very similar rDNA types conforming to Pantoea agglomerans, and many strains shared the same BOX-PCR patterns indicating a very limited genetic diversity among these bacteria. No significant effects were observed on the growth and nodulation of soybean after inoculation with nodule endophytes, but most of the strains produced indole-acetic acid (IAA), could solubilize mineral phosphate, and could fix nitrogen, implying that they are a valuable pool for discovering plant growth promoting bacteria. Results of the study demonstrated that the nodule endophytes were common in soybean and the plant's character and the soil conditions have a huge impact on their diversity. The study revealing 99% similarities in the nifH genes of Bradyrhizobium japonicum and of the endophytic Bacillus strains also strongly suggested that horizontal transfer of symbiotic genes happened between the symbiotic bacteria and the endophytes.

Isolation of a putative new endophytic nitrogen-fixing bacterium Pantoea sp. from sugarcane was performed by Loiret et al., (2004). Two N_2-fixing isolates, 9C and T2, were obtained during the study from surface-sterilized stems and roots, respectively, of sugarcane variety ML3-18. Acetylene reduction and H_2 production in nitrogenfree media were observed on both the isolates. Nitrogenase activity measured by H_2 production was found to be about 15 times higher for isolate 9C than for T2 or for *Gluconoacetobacter diazotrophicus* (PAL-5 standard strain, ATCC 49037). Amplification of nifH gene segment was donefor both isolates using specific primers. Both T2 and 9C was classified on the basis of morphological, biochemical, PCR tests and 16S rDNA sequence analysis. From its 16S rDNA, isolate 9C was identified as a Pantoea species but showed considerable differences in physiological properties from previously reported species of this genus. It was observed that 9C can be cultured over a wide range of temperature, pH and salt concentration, and showed high H_2production (up to 67.7 nmol H_2 h^{-1} 10^{-10} $cell^{-1}$). The study indicates that isolate T2 conformed toa strain of *Gluconacetobacter diazotrophicus*. On the other hand 9C designated as a new N_2-fixing endophyte, i.e. Pantoea, able to produce $H_2$and to grow in a wide range of conditions, was isolated from sugarcane stem tissue and characterized. The study concludedthat strain with these attributes could well be valuable for agriculture.

A work on the Molecular Characterization of *Pseudomonas* spp. Isolatedfrom Root Nodules of Various Leguminous Plants of Shekhawati Region, Rajasthan, India was carried out by Issar et al., (2012). During the study plant growth promontory *Pseudomonas* strains were isolated from root nodules of five leguminous plant species, viz., *Trifolium pretense*, *Cicerarietinum*, *Amaranthus polygamus*, *Vignamungo*, and *Trigonellafoenum*; that plants were denizen of Shekhawati region of Rajasthan. By using PGP propertiesa total of 8 (eight) bacterial isolates were evaluated for plant growth promotion. Partial 16S rDNA sequencing was also incorporated in the study and the data showed that these 8 bacterial isolates belonged to genus *Pseudomonas.* Neighbour joining tree by employing boot strap method was constructed by using MEGA 4.0.2, software. The result of the studydemonstrated significant diversity among recovered *Pseudomonas* strains.

A study on the Endophytic bacterial diversity in Rice plant cultivated on soil of eight sites (districts) of Phu Yen province, Vietnam was done by Nhu and Diep, (2014). Endophytic bacteria were isolated in three kinds of medium (LGI, NFb, RMR) together with 16S rRNA gene amplification using eubacterial universal primers (p515FPL and p13B). A total of 561 isolates were isolated during the studyand all of them were found having nitrogen fixing ability and phosphate solubilization together with IAA biosynthesis. However, 73 isolates having the best characteristics were identified as rice endophytes having nifH gene. The 73 selected endophytic bacteria showed high degrees of similarity to those of the GenBank references strains (between 97% and 100%). Out of 73 isolates, 23 isolates were found to belonged to Bacillus (31.54%), 44 isolates were Proteobacteria (60.24%), while 6 isolates were Bacteroides (8.22%). Based on Pi value (nucleotide diversity) and Theta values (per sequence) calculation for DNA polymorphism, it was found that Bacteroides group had the highest values in comparison of three group.These results from the study indicated that thirteen strains (*Bacillus megaterium* TANa5, *Bacillus methylotrophicus* TAN17, *Bacillus megaterium* TALa14, *Pseudomonas putida* TAL1, *Bacillus subtilis* TAL4, *Burkholderia kururiensis* TAL22, *Azospirillum amazonense* SHL70, *Bacillus subtilis* DXL 136, *Burkholderia kururiensis* PHL87, *Burkholderia vietnamiensis* PHL103, *Bacillus megaterium* PHL105, Bacillus megaterium DHL154

and *Bacillus subtilis* SHIM60) revealed encouraging candidates with numerous beneficial characteristics having the potential for application as inoculants or bio-fertilizer adapted to poor soils and high-yielding rice.

Isolation and identification of nitrogen-fixing*Enterobacter cloacae* and*Klebsiella planticola* associated with the roots of rice plants was carried out by Ladha et al., (1983). Initially the acid and gas producingnitrogen-fixing bacteria isolated from rice roots and leafsheaths along with reference Enterobacteria strains were characterized biochemically and serologically. By the use of selected cultural and biochemical tests, all isolates were found to conformed to*Enterobacter cloacae,*howevertwo strains were found to be similar with *Klebsiella pneumoniae***.**Further biochemical tests indicated that the *Klebsiella* isolates were *K.planticola.*After preparation of Antisera and fluorescent antibodies (FA) against K. *pneumoniae*M5a1, *K. planticola*DWUL2, and *E. cloacae*EnSs, results of FA cross-reactions of 28 strains isolated from rice plants and of other type cultures showed a separation into two different serogroups: *E. cloacae*and *K. planticola*.In contrast, the cross-reactions in gel immunodiffusion were found to be extensive and widespread, where strains of *Enterobacteriaceae*tested produced at least one immunodiffusible precipitin band with any one or all antisera. Theend result of the study indicates the population ofN_2-fixingEnterobacteria associated with the root and stem of rice fall within the range of 10^3 and 10^5per gram dry weight. The percentage occurrence of N_2-fixingEnterobacteria among the aerobic heterotrophic bacteria in the root and stem was however found to be less than 1%.