Chapter 9
Legume–Rhizobia Symbiosis and Interactions in Agroecosystems

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Abstract In the present scenario, when the population of the world is expected to become 8–9 billion by 2040, the major concern is to maintain sustained food supply. Production of high-quality protein-rich food is extremely dependent on the availability of sufficient nitrogen. Nitrogen though abundant on Earth is unavailable to plants. Indiscriminate use of nitrogenous chemical fertilisers has significantly increased food production and quality but at the same time affected ecosystem sustainability. Hence, the process of biological nitrogen fixation (BNF) has gained considerable significance. BNF is both free-living as well as symbiotic. Symbiotic N_2 fixation accounts for about 65 % of the total biologically fixed nitrogen. *Frankia* and rhizobia are two groups that fix atmospheric nitrogen symbiotically. Out of these, rhizobia–legume symbiosis accounts for about 45 % of nitrogen being used in agriculture. Rhizobia and legumes both are diverse. Currently 98 species of legume-nodulating bacteria have been identified within 13 bacterial genera, 11 in α-proteobacteria, whereas 2 in β-proteobacteria. Similarly, 13,000 species have been identified in 700 legume genera. Specificity of nodulation is an important attribute of legume–rhizobia symbiosis and is governed by both legume and rhizobial signals. For any successful legume–rhizobia symbiosis, interaction with other belowground microbes like AM fungi is also important. Here we give an account of rhizobial diversity and systematics, signals governing legume–rhizobia symbiosis, genes regulating nodulation and nitrogen fixation and legume–rhizobia–AM interactions.

Rhizobia?

Rhizobia are classically defined as soil bacteria capable of eliciting and invading root and stem tissue forming nodules on leguminous plants. Inside the nodules, the rhizobia convert dinitrogen into ammonia and ammonium compounds, and this process is known as nitrogen fixation. Legume–rhizobium association is both ‘symbiotic’ as well as ‘mutualistic’. It is symbiotic because bacteria live in intimate association with the plant and mutualistic because both partners gain.

‘Rhizobium’ *Versus ‘Rhizobium’*

The word ‘rhizobium’ is actually derived from two Greek words ‘rhizo’ meaning root and ‘bium’ meaning home, together conveying the meaning ‘root dweller’; ‘rhizobium’ is single bacterium and ‘rhizobia’ several bacteria. ‘*Rhizobium*’ is the

formal taxonomic name of a bacterial genus, and this certainly cannot be written as Rhizobia. Since the late nineteenth century (Frank 1889), all legume root-nodule bacteria were placed in the genus '*Rhizobium*'. Gradually it was realised that they were rather diverse. A few slow-growing rhizobia were split off into a new genus '*Bradyrhizobium*'. In the 1984 edition of Bergey's *Manual of Systematic Bacteriology* (Krieg and Holt 1984), all rhizobia were placed in the family *Rhizobiaceae* which included *Bradyrhizobium* and *Rhizobium*. Since then, the number of bacterial genera representing rhizobia has increased rapidly (Sy et al. 2001); presently, rhizobia are placed in genera that have been created to describe other non-nodulating bacteria as well (Willems 2006). Thus, the genus name is no longer a good criterion to describe whether a bacterium will be a rhizobium.

Importance of Legume–*Rhizobium* Symbiosis

In the present scenario, the population of the world stands at 6 billion and is projected to increase and stabilise at 8–9 billion by 2040; the major concern is to maintain sustained food supply to feed an ever-increasing global population. The adequate food production is possible using intensive agricultural practices, that is, increased use of chemical fertilisers and irrigation. As currently practised, agriculture will require an additional 40 and 20×10^6 million tonnes of N and P, respectively, to meet food production needs in the year 2040. The use of chemical fertilisers has increased agricultural production, but it is accompanied by deteriorating soil health and environmental quality (Tilman et al. 2001; Trewavas 2001).

Although nitrogen is amongst the most abundant element on Earth, it is the critical limiting element for growth of plants due to its unavailability (Graham and Vance 2000). Production of high-quality protein-rich food is extremely dependent upon availability of sufficient nitrogen. Plants acquire nitrogen from two principal sources: (a) the soil, through commercial fertilisers and manure/mineralisation of organic matter, and (b) biological fixation of atmospheric nitrogen (BNF). The first option that is the intense use of chemical fertilisers has been practised since 1960s and accounts for about 25 % of Earth's fixed nitrogen. About 50 % of the nitrogenous chemical fertilisers that are applied to agricultural fields are leached, and this has led to contamination of soil, increased concentration of toxic nitrates in drinking water and eutrophication of lakes and rivers. This has adversely affected biodiversity and ecosystem sustainability. Thus, in the present scenario, BNF has gained importance.

BNF is estimated to add nearly 90 % of 180×10^6 metric tonnes of the total nitrogen fixed annually in the terrestrial environment (Sahgal and Johri 2003; Gage 2004) which is equivalent to generation of resources equivalent to US $160–180 billion. This process is catabolised by prokaryotes only. Prokaryotes fixing atmospheric nitrogen are diverse. These include 2 genera of archaea, 38 genera of bacteria and 20 genera of cyanobacteria. The process of biological nitrogen fixation is both free-living as well as symbiotic. Symbiotic nitrogen fixation is restricted to a limited number of bacterial

groups, i.e. *Frankia* and rhizobia. *Frankia* is a filamentous Gram-positive actinomycete that induces nodules on a variety of woody plants in the families *Betulaceae*, *Casuarinaceae*, *Coriariaceae*, *Datiscaceae*, *Elaeagnaceae*, *Myricaceae*, *Rhamnaceae* and *Rosaceae* (Benson and Clawson 2000). Rhizobia are Gram-negative bacteria that induce nodules on stem and roots of plants belonging to family *Leguminosae*. They represent 13 genera spread over α- and β-proteobacteria.

There are approximately 700 genera and about 13,000 species of legumes, only 20 % of which have been examined for nodulation and shown to have the ability to fix nitrogen. Symbioses of rhizobia with 100 agriculturally important legumes contribute about 70 million tonnes of nitrogen year^{-1}. Legume–rhizobia symbiosis, apart from reducing the use of chemical nitrogen fertilisers, also contributes to carbon sequestration. The biological nitrogen fixation of 45×10^6 metric tonnes of nitrogen per year by legume–rhizobia symbiosis is equivalent to sequestering an additional 770 to 990×10^6 metric tonnes of carbon year^{-1} (Vance 2001). Thus, in conclusion it can be said that BNF is of substantial economic importance in low-input sustainable agriculture, agroforestry and land reclamation.

Diversity of Rhizobia Versus Taxonomy

Rhizobial Classification Based on Specificity of Symbiotic Plant Range

Rhizobia have legume host preferences for nodulation and nitrogen fixation. Nobbe and co-workers (1891, 1895) observed that bacteria isolated from legume *Pisum sativum* were very specific and were unable to nodulate plants belonging to the legume tribes Genisteae and Hedysareae. Thus, earliest classification of rhizobia was based on the hosts it nodulated and fixed nitrogen (Hiltner and Störmer 1903). Fred et al. (1932) recognised six species in the genus *Rhizobium*, namely, *R. japonicum* (*Lathyrus*, *Lens*, *Pisum* and *Vicia*), *R. lupini* (*Lupinus*), *R. meliloti* (*Melilotus*, *Medicago*, *Trigonella*), *R. phaseoli* (*Phaseolus*) and *R. trifolii* (*Trifolium*) based on their host range for nodulation. A few years later, Wilson (1939), while testing the host ranges of rhizobia isolated from 31 different genera of legumes on 160 different legume species, observed that on an average a particular rhizobial isolate nodulated 33 % of the total legume species. He also reported that *Rhizobium* sp. strain NGR234 nodulated 112 out of 160 legume genera tested, and *R. fredii* USDA257 nodulated 77 genera, whereas *Vigna* was a promiscuous host that was nodulated by several rhizobial species. Now it is well established that a single rhizobial species is able to nodulate different legume genera, and that many legumes can be nodulated by several rhizobial species (for review see Sahgal and Johri 2003; Perret et al. 2000). It is only six decades later in the early 1960s that rhizobia were separated into different groups based on extensive microbiological criteria (Graham 1964; Moffett and Colwell 1968). At the same time Norris (1965) observed differences in growth rate of rhizobia and proposed that it was associated with their symbiotic affinity. Slow

growers were largely associated with tropical legumes and fast growers with temperate legumes (Allen and Allen 1981; de Lajudie et al. 1994). But several workers (Dreyfus and Dommergues 1981; Scholla and Elkan 1984; Jenkins et al. 1987; Fulchieri et al. 1999) reported the presence of both fast- and slow-growing rhizobia in tropical legumes. The roots of tropical legume *Phaseolus vulgaris* were nodulated by ten different *Rhizobium* species. These species include *Bradyrhizobium japonicum*, *Mesorhizobium loti*, *Rhizobium etli*, *R. tropici*, *R. leguminosarum* bvs. *trifolii* and *viciae*, *Rhizobium* spp. NGR234 and GRH_2, *Sinorhizobium fredii* and *S. meliloti* (Michiels et al. 1998). Similarly, there are *Rhizobium* strains which are relatively non-specific for their legume partner, e.g. *Rhizobium* sp. strain NGR234 that has broad host range and is able to elicit nodules on 50 % of the known legumes (Pueppke and Broughton 1999). Hence, classification of rhizobia on the basis of host range and biological and physiological properties has serious shortcomings.

Polyphasic Approach for Taxonomy

In the 1990s emerged the concept of polyphasic taxonomy. Polyphasic taxonomic approach includes characterisation based on biochemical, physiological and genetic fingerprinting methods along with host range for nodulation in case of rhizobia. This has led to the description of the new genera and reorganisation of the existing genera. PCR-based genetic fingerprinting methods and base sequence comparisons of 16S rRNA genes as well as other housekeeping genes have been used extensively for characterising and classifying rhizobia (Willems and Collins 1993; Chen et al. 1995; Wang et al. 1999a; Willems et al. 2001; Zeigler 2003). Several bacterial isolates located outside traditional rhizobial genera in class α-proteobacteria have been reported from legume nodules that are capable of nitrogen fixation. In the year 2001 β-proteobacteria were reported in legume nodules for the first time when *Burkholderia* spp. were described from the nodules of the South African legume *Aspalathus carnosa* (Moulin et al. 2001) and *Ralstonia taiwanensis* in *Mimosa* nodules from Taiwan (Chen et al. 2001). Tripathi (2002) has reported *Ralstonia* from *Mimosa* nodules from India and how a good science was left behind in the publication race. Other new lines that contain N_2-fixing legume symbionts include *Methylobacterium* (Jourand et al. 2004), *Devosia* (Rivas et al. 2002), *Ochrobacterium* (Trujillo et al. 2005) and *Phyllobacterium* (Valverde et al. 2005), all α-proteobacteria. Till 2003, 36 rhizobial species distributed amongst seven genera were recognised (Sahgal and Johri 2003). In the subsequent 3 years, eight new rhizobial species were described. By 2006, 44 species of nodule bacteria on legumes were recognised within 11 genera (Sahgal and Johri 2006). With the use of genetic characteristics (DNA–DNA, DNA–rRNA hybridisations, rRNA catalogues, rDNA sequencing) and sequence analysis-based systematics, more diversity has been discovered amongst rhizobia, their relationships recognised and relationships with other groups of bacteria became apparent. In α-proteobacteria, a single species *Allorhizobium undicola* (de Lajudie et al. 1998) was reported within genus *Allorhizobium*. *Sinorhizobium* is now *Ensifer* with two species (Young 2003). Amongst β-proteobacteria single species, *Ralstonia*

taiwanensis within genus *Cupriavidus* (Chen et al. 2001; Vandamme and Coenye 2004) has been identified. Other species described were *Devosia neptuniae* for strains from *Neptunia natans* from India (Rivas et al. 2002) and *Methylobacterium nodulans* for strains from *Crotalaria* (Jourand et al. 2004; Sy et al. 2001). *Ochrobacterium lupines* was described from *Lupinus* species (Trujillo et al. 2005), *Phyllobacterium lupinii* for isolates nodulating *Trifolium* and *Lupinus* (Valverde et al. 2005) and *Shinella kummerovia* from *Kummerowia stipulacea* (Lin et al. 2008). All these new nodulating bacteria have 16S rDNA distinct from traditional rhizobial genera but carry *nod* genes similar to those of rhizobia. Thus, currently 98 species of legume-nodulating bacteria have been identified within 13 bacterial genera, 11 in α-proteobacteria and 2 in β-proteobacteria (Weir 2012). The above-mentioned number is severalfold less than the expected number considering the great number and vast distribution of leguminous hosts. Approximately 19,700 legume species are present globally, and rhizobia characterised and described are mainly from a small portion of legumes, mainly crops. A few bacterial isolates have been characterised and described from wild annuals and woody tree legumes. Out of 43 rhizobial species known till 2005, only ten were from tree legumes.

The present-day classification of rhizobial species is based on 16S rDNA sequence comparisons and physiological and biochemical properties. It does not reflect symbiotic features of rhizobia particularly host plant range. Although it is widely agreed that phylogenies based on stable chromosomal genes are necessary to establish biologically meaningful rhizobial taxonomy, a proper definition of broad host range should consider the diversity of symbiotic (sym) genes rather than the diversity of species that carry them. Thus, characterisation and the phylogenetic classification of sym genes must be included in the minimal standards for the description of new rhizobia (Laguerre et al. 2001).

Legume–Rhizobia Interactions

Legume–*Rhizobium* symbiosis is a marriage between two vastly different genomes. Rhizobial genome totals about 6–9 Mbp (Perret et al. 2000). In contrast, genome of legumes is larger with total DNA contents in the range of 450–4,500 Mbp per haploid genomes (Arumuganathan and Earle 1991). Legume genomes are thus at least 50 times larger than those of their microsymbionts. Nevertheless their respective contributions are almost similar. Rhizobia provide fixed nitrogen to the plants and bacteria are supplied with nutrients (Lodwig and Poole 2003) as well as protected inside nodule structure (van Rhijin and Vanderleyden 1995).

Nodule Development

Nodule development is a multistep process. It consists of recognition of host plants by bacteria, attachment of bacteria to root hair, root hair curling and formation of

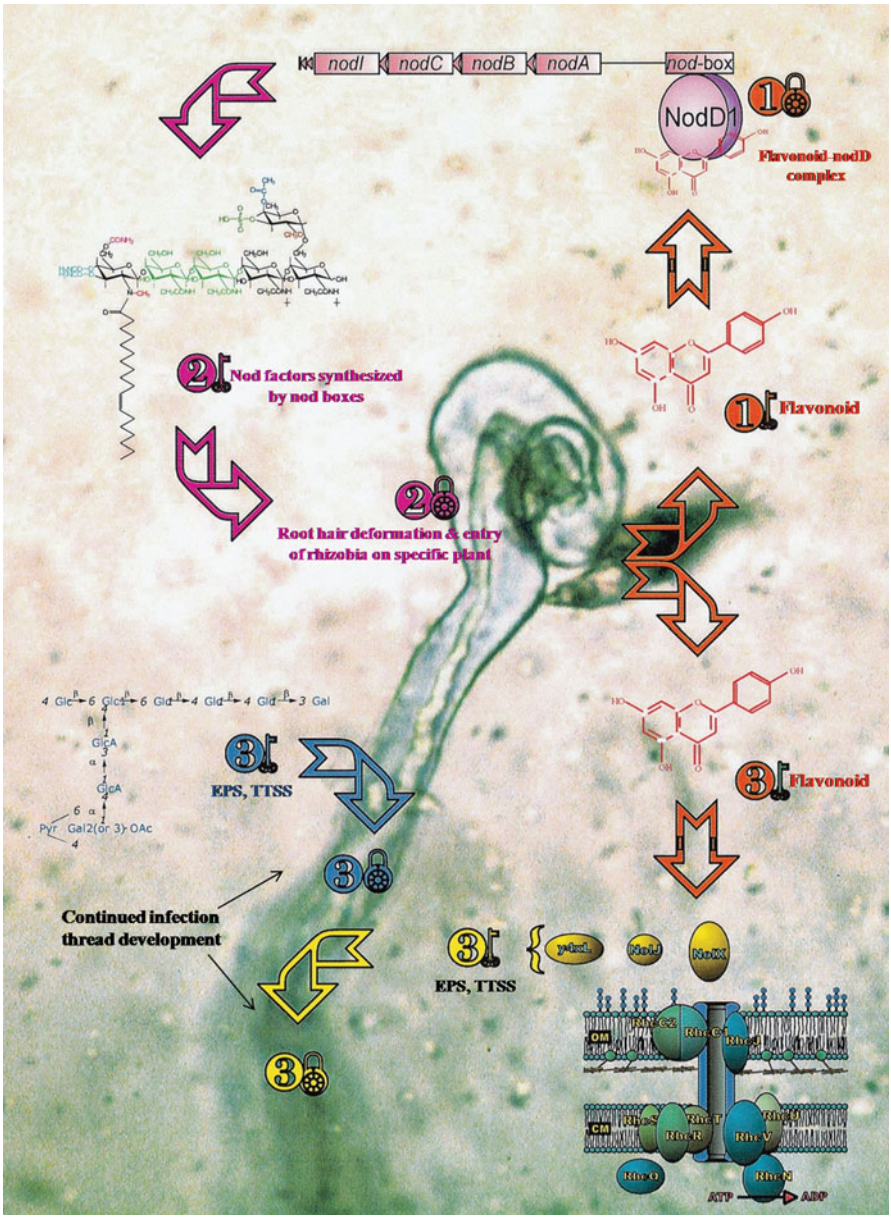

Fig. 9.1 Signals involved in legume–*Rhizobium* symbiosis (*Source*: Broughton et al. 2000)

infection thread and nodule development. Inside the nodules, bacteria differentiate into bacteroids, the site of biological nitrogen fixation.

At least three different set of symbiotic signals are exchanged between legumes and rhizobia during nodule development (Fig. 9.1). Legume roots secrete flavonoids and betaines that accumulate in the host plant rhizosphere. Since legumes are

nonmotile, so bacterial partner (rhizobia) senses the flavonoids and betaines secreted by legume host and advances into its rhizosphere and courtship begins. These flavonoids activate rhizobial Nod D proteins and form a flavonoid–Nod D complex. This complex acts as transcriptional regulator of nodulation genes (Broughton and Perret 1999). As a result nodulation genes (*nod*, *nol* and *noe* genes) secrete Nod factors that are chemically lipochitooligosaccharides (LCOs) (Spaink 2000). Nod factors are a second set of signals that trigger root hair curling and allow rhizobia to enter through infection thread. Infection thread reaches nodule primordium and releases the bacteria into cytoplasm. The meristematic activity of root cortex and active multiplication of rhizobia lead to nodule formation. The third set of signals necessary for the completion of nodule development are extracellular polysaccharide (EPS), lipopolysaccharides, K antigens, cyclic glycans, lectins and proteins exported by type three secretion system (TTSS).

Host Specificity

The interaction between rhizobia and legume is host specific. It means that rhizobial species are specific for nodulating a legume or that not all rhizobia nodulate all legumes. Based on host specificity, rhizobia are classified as broad and narrow host range rhizobia. For example, *Rhizobium leguminosarum* bvs. *viciae* and *trifolii*, though closely related, are specific for their legume partner. *R. leguminosarum* bv. *viciae* nodulates *Lathyrus*, *Lens*, *Pisum* and *Vicia*, whereas *R. leguminosarum* bv. *trifolii* nodulates *Trifolium* spp. NGR234 is a broad host range rhizobia and nodulates at least 35 different legumes. Hence, the degree of host specificity varies tremendously amongst rhizobia. The amount and the structural variation of Nod factors are important in determining the host specificity in rhizobia–legume symbiosis (Bladergroen and Spaink 1998).

All the Nod factors consist of a backbone of two to six β-1,4-linked *N*-acetyl-d-glucosamine residues. The nonreducing terminal end of *N*-acetyl-d-glucosamine is substituted on the C-2 position with a fatty acid whose structure is variable, and the reducing end may be substituted with a sulphate group or with a d-arabinose, l-fucose or 2-*O*-methyl fucose. The Nod factor synthesis is induced by flavonoids secreted by legumes. Thus, composition and concentration of flavonoid mixture liberated into the rhizosphere by the legume host are important in determining the nodulation preferences of rhizobia. The flavonoids produced via phenylpropanoid biosynthetic pathways are strongest inducers of nod gene expression (Stafford 1997; Werner 1998), whereas those related glycosides or related conjugates are less active in inducing nod genes (Hartwig and Phillips 1991). Compositions of flavonoids in seeds, roots and root exudates of *Glycine max*, *P. vulgaris*, *Medicago sativa*, *Trifolium repens* and *Vicia sativa* are significantly different from each other, and this determines their preferences for nodulation with rhizobial species (Perret et al. 2000). It was observed that *G. max*, *P. vulgaris*, *Robinia pseudoacacia* and *Sesbania rostrata* were nodulated by a broad host range *Rhizobium* sp. strain NGR234, in addition to their homologous *Rhizobium*, and thus were highly

non-selective for rhizobia, whereas *M. sativa* and *Vicia* sp. have restricted host ranges and are thus highly selective for rhizobial partners.

Symbiotic Genes in Rhizobia

Several rhizobial and symbiotic genes are required for legume–rhizobia symbiosis. Rhizobial genes include those involved in Nod factor synthesis (van Rhijin and Vanderleyden 1995), nodule development, synthesis of nitrogen-fixing apparatus and bacteroid metabolism. Amongst these are nodulation (*nod*, *nol*, *noe*) and nitrogen fixation (*nif*, *fix*) genes, whereas plant genes expressed in root tissues as a consequence of the interaction with rhizobia are nodulin genes (Verma et al. 1992). Symbiotic genes are located on either plasmid or chromosome. In the genus *Rhizobium*, *nod* genes are located on a large plasmid known Sym plasmid, pSymA, pSymB in *Rhizobium meliloti* (now *Sinorhizobium meliloti*) (Galibert et al. 2001), whereas in *Azorhizobium* spp., *Bradyrhizobium* and *Mesorhizobium loti* on the chromosomes (Kaneko et al. 2000). The organisation of these genes in operons is very similar in *Rhizobium* and *Bradyrhizobium* (Fig. 9.2).

The Nodulation Genes

In all there are 13 *nod* genes. The nodulation genes have been classified into two groups: common and host specific (hsn). The common *nod* genes are *nod*A,-B,-C,-I,-J. The common nodulation genes *nod* ABC are found in all rhizobial isolates studied so far (Martínez et al. 1990; Goethals et al. 1992) and are structurally conserved and functionally interchangeable between the rhizobial species without altering host range; another essential gene is nod D, which is present in one or more alleles depending on the rhizobial species. The nod D gene behaves as a common *nod* gene for nodulation on some host plants, while in other cases it represents an important determinant of host specificity (Gyorgypal et al. 1991; Schlaman 1992). Nod D gene is present in a single allele in *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii*; in four alleles *nod* D, *nod* D_2, *nod* D_3 and syr M in *R. meliloti*; and two alleles *nod* D_1 and *nod* D_2 in *B. japonicum*. The *nod* hsn genes are specific and determine its host for nodulation and are not conserved amongst rhizobia. The host-specific *nod* genes include *nod* FE, *nod* L and *nod* M common to all *Rhizobium* sp., *nif* W (formerly *nod* O) in *R. leguminosarum* bv. *viciae*, *nod* H and *nod* PQ in *S. meliloti* (formerly *R. meliloti*) and nod Z in *B. japonicum*, respectively.

The Nitrogen Fixation Genes

These are *nif* and *fix* genes. Rhizobial *nif* genes are structurally homologous to 20 *Klebsiella pneumoniae nif* genes (Arnold et al. 1998), but their organisation in rhizobia is different than those in *K. pneumoniae*, in which 20 adjacent *nif* genes

Table 9.1 The list of *nif* and *fix* genes of *S. meliloti*, *B. japonicum* and *A. caulinodans* and their functions

S. no.	Gene	Product and/or (proposed) function
a.	***nif* genes**	
i.	*nif* H	Fe protein of nitrogenase
ii.	*nif* D	α-subunit of MoFe protein of nitrogenase
iii.	*nif* K	f3 subunit of MoFe protein of nitrogenase
iv.	*nif* N	Involved in FeMo cofactor biosynthesis
v.	*nif* B	Involved in FeMo cofactor biosynthesis
vi.	*nif* S	Cysteine desulphurase activation of sulphur for metallocluster synthesis?
vii.	*nif* W	Unknown function; required for full activity of FeMo protein
viii.	*nif* X	Unknown function
ix.	*nif A*	Positive regulator of *nif*, *fix* and additional genes
b.	***fix* genes**	
i.	*fix* ABCX	Unknown function; required for nitrogenase activity; Fix X shows similarity to ferredoxins
ii.	*fix* NOQP	Microaerobically induced, membrane-bound cytochrome oxidase
iii.	*fix* GHIS	Redox process-coupled cation pump?
iv.	*fix* LJ	Oxygen-responsive two-component regulatory system involved in positive control of *fix* K (*Sm*, *Bj*, *Ac*) and *nif* A (*Sm*)
v.	*fix* K/*fix* K_2	Positive regulator of *fix* NOQP (*Sm*, *Bj*, *Ac*), *nif* A (*Ac*), *rpo*Nj, and 'nitrate respiration' (*Bj*); negative regulator of *nif* A and *fix* K (*Sm*)
vi.	R_m*fix*K′	Reiterated, functional copy of *fix* K
vii.	*Bj fix* K_1	*Fix* K homolog of unknown function; not essential for nitrogen fixation
viii.	*fix* R	Unknown function; not essential for nitrogen fixation
ix.	*Nfr* A	Regulation of *nif* A

Source: Fischer (1994)
Sm: *S. meliloti*, *Bj*: *B. japonicum*, *Ac*: *A. caulinodans*

are organised in eight operons within 24 kb of DNA. At least nine different *nif* genes have been identified so far in *S. meliloti* (formerly *R. meliloti*), *B. japonicum* and *A. caulinodans* (Table 9.1). These are *nif* HDK and *nif* E, N, B, S, W, X and A. These *nif* genes play a similar role in rhizobia as in *K. pneumoniae*. The *nif* HDK is a good marker for nitrogen fixation as it is not constitutively expressed and is regulated in response to factors that control nitrogen fixation. Amongst *nif* HDK, *nif* H gene is widely used as a nitrogen fixation marker (Haukka et al. 1998) because large sequence data is available for this gene. The 'fix' genes play an important role in nitrogen fixation but do not have a homologous counterpart in *K. pneumonia*. The 'fix' genes represent genes originally involved in the development and metabolism of bacteroids but at the same time may also play an important role in other processes not related to nitrogen fixation or may even be present in non-diazotrophs.

S. meliloti carries two megaplasmids pSymA of 1,400 kb and pSymB of 1,700 kb (Young 2000). In *S. meliloti*, *nif* and *fix* genes are organised into two clusters (Fig. 9.2a). Cluster I includes *nif* HDKE, *nif* N, *fix* ABCX, *nif* A, *nif* B and *frd* X and cluster II includes *fix* LJ, *fix* K, *fix* NOQP *and fix* GHIS, and both

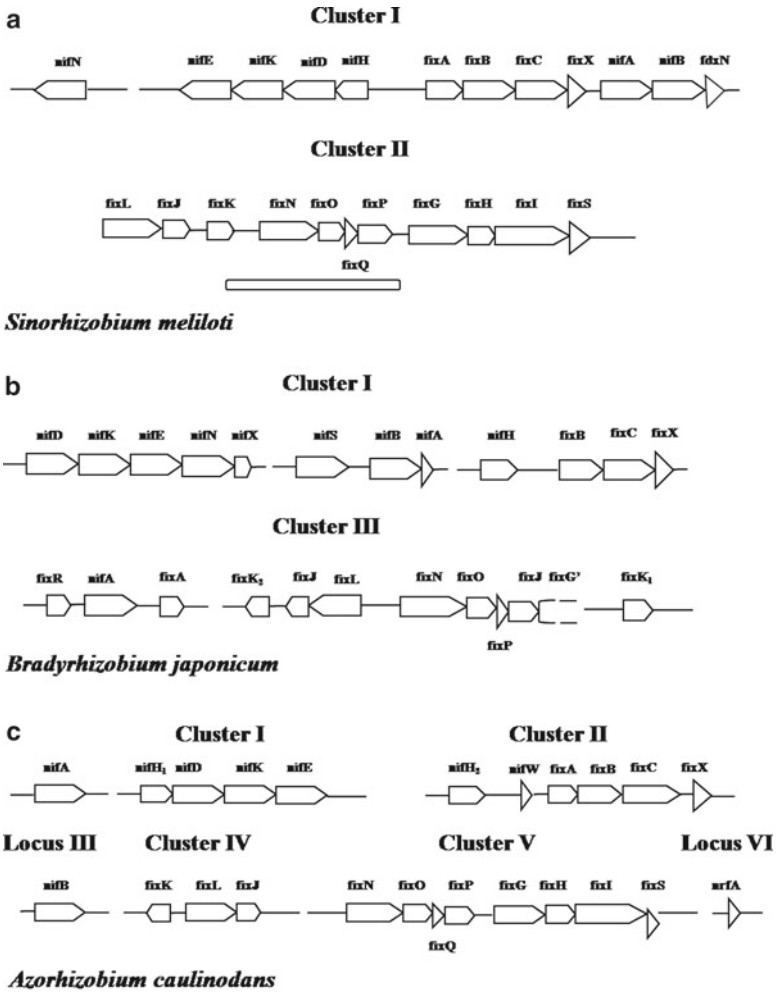

Fig. 9.2 Organisation of *nif* and *fix* gene clusters in (**a**) *S. meliloti*, (**b**) *B. japonicum* and (**c**) *A. caulinodans* (*Source*: Fischer 1994)

the clusters are present on megaplasmid pSymA. The cluster II genes are located 220 kb downstream of the *nif* HDKE operon and are transcribed in opposite orientation to it. Additional genes that are required for effective symbiosis are located on megaplasmid pSymB. Rhizobial species *B. japonicum* and *A. caulinodans* do not have plasmids. Hence, *nif* and *fix* genes are located on chromosomes and organised as shown in Fig. 9.2b and c, respectively. In *B. japonicum*, *nif* and *fix* genes are organised into four clusters and along with common nod genes are located within 100 kb on chromosome. Thus, it can be presumed that symbiotic gene region of *B. japonicum* was located originally on a plasmid and became part of chromosome by integration. Alternatively, the symbiotic

plasmids of *S. meliloti* (or other rhizobia) might have evolved by excision of a chromosomal region. In *A. caulinodans* four clusters of *nif* or *fix* genes and two additional loci carrying nitrogen-fixing genes *nif* B and *nif* A are present. Moreover, additional gene regions that are located in nodulation (nod UV) (Göttfert et al. 1990) or expressed under symbiotic conditions (*rpo*N) (Kullik et al. 1991), *gro* ESL_3 (Fischer et al. 1993) and *ndp* (Weidenhaupt et al. 1993), are present close to the segment harbouring essential *nif* and *fix* genes (Kündig et al. 1993).

nif Genes

The nitrogenase enzyme complex is composed of two multisubunit metalloproteins component I and II. Component I is composed of two heterodimers encoded by *nif* K and *nif* D genes and has active sites for nitrogen reduction. The *nif* D and *nif* K genes specify α- and β-subunits, respectively, of $\alpha_2\beta_2$FeMo protein (component I or dinitrogenase, Mr ≈ 220,000). Component II is composed of two identical subunits encoded by nif H and transfer electrons and protons to component I. *nif* H encodes homodimeric Fe protein (component II or dinitrogenase reductase, Mr ≈ 60,000). In *S. meliloti*, the *nif* HDK genes are organised in an operon along with *nif* E, whereas in *A. caulinodans*, *nif* HDK and *nif* E form two separate transcriptional units. The *nif* H gene is present in two alleles, *nif* H and *nif* H_2, differing in two nucleotides. In *A. caulinodans*, *nif* H_2 is found in cluster II along with *fix* ABCX genes, and in *R. leguminosarum* bv. *phaseoli*, three identical and functional copies of *nif* H genes are present. The products of *nif* genes *nif* E, *nif* N and *nif* B are required for the synthesis of FeMo cofactor of component I.

fix ABCX Genes

These are present in *S. meliloti*, *B. japonicum*, *A. caulinodans*, *R. leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *phaseoli*. They are organised in a single operon in all species except *B. japonicum*, in which *fix* A and *fix* BCX form distinct transcriptional units present in clusters II and I, respectively. The products of *fix* ABCX genes are involved in electron transport to nitrogenase. Mutation in any one of the *fix* ABCX genes of *S. meliloti*, *B. japonicum* and *A. caulinodans* completely stops nitrogen fixation. These include genes encoding MoFe protein and Fe protein as well as accessory genes for electron transfer proteins, metal cluster synthesis and regulation (Dean and Jacobsen 1992).

fix NOQP Genes

The products of *fix* NOQP genes constitute a membrane-bound cytochrome oxidase (Kahn et al. 1993; Mandon et al. 1994; Preisig et al. 1993). This oxidase complex

supports bacteroid respiration under low oxygen conditions present in root nodules (Hennecke 1993; Preisig et al. 1993). These were first described in *S. meliloti* and are expressed under symbiotic conditions. These were limited to regulatory genes *fix* LJ and *fix* K. Subsequently, they have been identified in *B. japonicum* (Preisig et al. 1993), *A. caulinodans* (Mandon et al. 1994) and *R. leguminosarum* bv. *viciae*. The *B. japonicum* and *S. meliloti* fix NOQP mutants are defective in symbiotic nitrogen fixation, whereas a corresponding mutant of *A. caulinodans* showed 50 % wild-type nitrogenase activity.

fix GHIS Genes

These are present downstream of the *fix* NOQP operon in cluster II of *S. meliloti*. All four *fix* GHIS gene products are transmembrane proteins. *fix* G is likely to be involved in redox process, and *fix* I is homologous to the catalytic subunit of bacterial and eukaryotic ATPases involved in cation pumping.

fix R

This is present in *B. japonicum* and is located downstream of the regulatory *nif* A gene. The product of *fix* R is involved in redox-dependent activation and inactivation of the Nif A protein.

Regulation of nif *and* fix *Genes*

S. meliloti, *B. japonicum* and *A. caulinodans* all use largely identical regulatory elements (FixL, FixJ, FixK, NifA and RpoN); however, these are integrated into different species-specific networks.

Intracellular Oxygen Tensions

Oxygen concentration controls the expression of *nif* and *fix* genes (Soupene et al. 1995). Enzyme nitrogenase is extremely sensitive so inside nodule oxygen concentration has to be very low. However, the colonising rhizobia require oxygen to generate ATP, which is required in large amounts for the energy-intensive process of nitrogen fixation. Tightly packed plant cortical cells adjacent to the surface of the nodule form an oxygen diffusion barrier and leghaemoglobin present in the nodule cytoplasm tightly binds to oxygen. Hence, diffusion of oxygen to actively respiring bacteroids is prevented. Rhizobia sense oxygen concentration through two proteins, Fix L and Nif A. At low oxygen concentrations, these proteins are active and are responsible for the induction of genes involved in fixation of atmospheric nitrogen.

FixL–FixJ

In *S. meliloti*, the FixL–FixJ two-component system is the master regulator of all nif and fix genes (Agron and Helinski 1995). The FixL is a membrane-bound histidine kinase which at low levels of oxygen autophosphorylates and then transfers the phosphoryl group to FixJ (Gilles-Gonzalez and Gonzalez 1993; Lois et al. 1993). Phosphorylated FixJ activates transcription of regulatory *fix* K and *nif* A genes. The products of *fix* K and *nif A* genes regulate transcription of the rest of the nitrogen fixation genes. The FixL–FixJ system is one of the few two-component systems whose signal-responsive autophosphorylation and phosphotransfer have been reconstituted in vitro. Anoxic conditions enhance FixL autophosphorylation, whereas phosphorylation of FixJ is independent of oxygen status.

FixK

It is a regulatory protein whose expression is activated by FixJ in response to low concentrations of oxygen (Kaminski et al. 1998). It is homologous to the regulator Fnr except that cysteine residues are not present at N-terminal domain. Fix K can act either as an activator or as a repressor depending on the position of its binding site within the target promoter. In *S. meliloti*, Fix K activates the transcription of *fix* NOQP and *fix* GHIS operons and negatively regulates its own expression as well as the expression of *nif* A (Waelkens et al. 1992; Foussard et al. 1997).

Nif A

It is a transcriptional regulator whose expression and activity are inhibited by high oxygen concentrations. It does not belong to a family of two-component systems because it does not contain a receiver domain. Nif A protein is a homolog of Ntr C. It acts in conjugation with sigma 54 and requires hydrolysis of an ATP molecule to activate transcription. In the absence of oxygen, Nif A activates the expression of its own gene as well as that of nif HDKE and fix ABCX operons (Fischer 1994, 1996). It also induces transcription of genes involved in the synthesis of rhizopines.

Rhizopines in Sinorhizobium*–Plant Interaction*

Rhizopines are nutritive compounds produced by bacteroids of certain strains of rhizobia, i.e. *S. meliloti* and *R. leguminosarum* bv. *viciae*. They are synthesised by 11 % of *S. meliloti* and 12 % of *R. leguminosarum* bv. *viciae* strains. Structurally, rhizopines are 3-*O*-methyl-scyllo-inosamine (3-O-MSI) and scylloinosamine (SI) (Dessaux et al. 1998). In *S. meliloti*, genes involved in rhizopine synthesis (*mos* genes)

and rhizopine catabolism (*moc* genes) are located on the symbiotic megaplasmid pSymA, along with nitrogen fixation genes. The *mos* locus is regulated by symbiotic nitrogen fixation regulator NifA; hence, it is co-ordinately regulated with nitrogen fixation and controlled by low oxygen levels. Rhizopine catabolic gene (*moc*) is not expressed in bacteroid (Saint et al. 1993), but catabolic products of rhizopines affect intraspecies competition for nodulation (Murphy et al. 1995). Although very few rhizobia synthesise rhizopines, it is possible that new classes of rhizopines might be discovered and this phenomenon may be more universal amongst rhizobia (Brencic and Winans 2005).

Regulation of Bradyrhizobium*–Soya Bean Symbiosis*

Symbiotic interaction of *Bradyrhizobium* with soya bean (*G. max*) is influenced by both the bacterial and host genotypes. Soya bean genotypes, including cultivars and plant introductions (PI), have been shown to be differentially nodulated by specific stains or genotypes of *B. japonicum* (Cregan and Keyer 1986; Sadowsky et al. 1987). The nodulation of *Glycine max* by *B. japonicum* USDA 110 and USDA 123 is controlled by legume host genotype and bacterial population density (Jitacksorn and Sadowsky 2008). Nodulation was enhanced when soya bean plants received low cell diversity inocula (10^5 cell ml^{-1}), whereas it was suppressed when plants received high diversity inocula (10^9 cell ml^{-1}). The regulation of nod gene expression in the *Bradyrhizobium* occurs via three regulatory pathways involving *nod* D, *nod* VW and *nol* A (Loh and Stacey 2001). *B. japonicum* produces two Nod D proteins (Nod D_1 and Nod D_2). Nod D1, a LysR-type regulator, is a positive transcriptional activator and responds to plant-secreted isoflavones (Göttfert et al. 1992), whereas $NodD_2$ represses nod D1 expression (Loh and Stacey 2003). Although initial studies by Göttfert and colleagues (1992) showed that there was no role of $nodD_2$ gene in inoculation of soya bean plants, subsequent studies by the same group have shown that nodulation of soya bean plants was delayed in $nodD_2$ deletion mutant of *B. japonicum* as compared to wild-type stain. Nod VW is essential for the nodulation of cowpeas, siratro and mung bean but not for soya bean and provides an alternative pathway for nod gene activation in NodD mutants that are able to nodulate soya bean. The third pathway is regulated by NolA, a MerR family of regulatory proteins, and was identified as the product of genotype-specific nodulation gene. NolA activates the expression of $NodD_2$ which in turn represses nod gene expression in *Bradyrhizobium*. *B. japonicum* strain USDA 110 grown to high cell density secretes an extracellular quorum-responsive signal molecule, bradyoxetin. Bradyoxetin induces NolA which in turn leads to nod gene repression. The production of bradyoxetin is regulated in a population-density-dependent manner; the greatest production occurs in high population density and iron-depleted conditions. Thus, expression of nod genes in the *Bradyrhizobium* is modulated by quorum-responsive signal molecules. The functional copy of the nodD1 gene is required for diversity-dependent

enhanced nodulation of soya bean, and that *B. japonicum* strain with mutation in *nol* A and *nod* D_2 can be used to enhance the nodulation of soya bean at high inoculum densities. In nitrogen-fixing bacteroids, carboxylic acids are a major source of carbon and energy, necessary for the generation of ATP and reducing power needed for nitrogenase activity (Kaminski et al. 1998). However, dicarboxylic acids also inhibit the expression of nod genes, e.g. *B. japonicum* (Yuen and Stacey 1996).

Rhizobia Associated with Annual Legumes

Agricultural soils often contain diverse indigenous rhizobial populations. Rhizobia have great potential for improving growth of host plants (Becki et al. 2004; Bogino et al. 2008). Their performance in field is affected by host plant specificity, environmental factors as well as soil conditions (Diouf et al. 2007). Correlations between the rhizobial genomic groups and their geographic origins have been detected amongst symbionts of faba bean (*Vicia faba*) (Tan et al. 2007) and epidemic legumes growing on the Qinghai–Tibet plateau (Hou et al. 2009). Several other studies have shown that both abiotic (pH, rainfall, soil, temperature) and biotic (genotypes of host plants and their distribution) conditions might affect the diversity of the rhizobial species in soil (Hagen and Hamrick 1996; Handley et al. 1998; Bromfield et al. 2001).

Host plant plays a central role in site-specific selection of rhizobia. Wang et al. (1999a) observed that *R. etli* from root nodules of *Mimosa affinis* growing in Mexico was different in *nif* H gene and host specificity as compared with *R. etli* strains nodulating *P. vulgaris* L. They proposed new biovariety for *R. etli* strains nodulating *M. affinis*. Thus, repeated cultivation of legumes like *M. affinis* is likely to reduce rhizobial diversity to a marked strain than repeated cultivation of a promiscuous legume like *P. vulgaris* which is nodulated by genetically diverse rhizobia, namely, *Bradyrhizobium* spp., *R. leguminosarum* bv. *phaseoli* (Andrade et al. 2002), *R. tropici* (Martínez-Romero et al. 1991), *R. etli* (Graham et al. 1982), *R. giardinii* and *R. gallicum* (Amarger et al. 1997). Nodulation of rhizobia on heterologous host (cross-nodulation pattern) is an important trait in defining their diversity. But association between rhizobia and their host under laboratory conditions is less important than in natural environment because such species of rhizobia can form nodules with legumes under laboratory conditions from which they have never been isolated in the field, e.g. nodulation of *R. huautlense* on *Leucaena leucocephala* in in vitro studies (Wang et al.1998).

Geographical locales can also influence genetic diversity amongst rhizobial populations. Han et al. (2008) characterised genetic and symbiotic rhizobial diversity from three introduced (*Lathyrus odoratus*, *Robinia pseudoacacia* and *V. faba*) and nine wild legumes, *Astragalus* spp., *Alhagi sparsifolia*, *Caragana jubata*, *Halimodendron halodendron*, *Lotus* sp., *Oxytropis glabra*, *Sophora alopecuriodes*, *Vicia hirsuta* and *Orobus* (Lathyrus) *luteus*, growing in the Xinjiang region of

China. They identified nine genomic species amongst 111 rhizobial strains associated with 25 legume species within 12 legume genera. Regardless of the composition of sampled legumes, *Rhizobium* was the most predominant bacteria (genomic sp. I and II), *Mesorhizobium* (genomic sp. V and VI) second largest and *Bradyrhizobium* populations were least dominant. This implied that highly alkaline and saline soils in Xinjiang were dominant in acid-producing strains of *Rhizobium*, *Mesorhizobium* and *Ensifer* than alkaline-producing *Bradyrhizobium* strains. The characterisation of nodule bacteria from unexplored legumes will reveal additional diversity and novel species are likely to be described (Wolde-Meskel et al. 2005). Moreover, an introduced legume in an area might trap rhizobial populations that exist locally as a minority in the soil. Consequently, both sampled legumes and local environment may affect the composition of rhizobial community. Chen and co-workers (1988, 1995) have reported that soya bean plants in Xinjiang region have been nodulated by *Mesorhizobium tianshanense* and *Sinorhizobium fredii*, whereas in other regions with bradyrhizobia. Similarly, Velázquez et al. (2001) observed that bean isolates recovered from León (France) belonged to *R. leguminosarum* bvs. *viciae* and *trifolii*, whereas those from Andalucia were more diverse and belonged to *R. etli*, *R. gallicum*, *R. giardinii*, *R. leguminosarum* bv. *viciae* and bv. *trifolii* and *S. fredii*. Similarly, Bernal and Graham (2001), while studying bean rhizobia in Ecuador and Northern Peru, observed that *R. etli* strains from the Mesoamerican region were phenotypically and phylogenetically separated from those associated with beans in the Andean region. Physical properties of soil also affect the genetic diversity amongst rhizobial populations. Andrade et al. (2002) reported higher rhizobial diversity in limed soils in the *P. vulgaris*-growing region of Brazil. Shifts observed in genetic diversity amongst the population of *S. meliloti* (formerly *R. meliloti*) and *R. leguminosarum* nodulating *M. sativa* growing in Italy have been attributed to chemical and physical differences between soil (Paffeti et al. 1996), history of N fertilisation (Caballero-Mellado and Martínez-Romero 1999) and land management practices (Palmer and Young 2000). The observation that there is a correlation between geographical regions and rhizobial diversity has been strengthened by studies of rhizobia from legume-growing regions in China. Lu et al. (2009) studied the rhizobial diversity associated with endemic *Caragana* species, *C. bicolor*, *C. erinacea*, *C. franchetiana*, *C. intermedia* and *C. jubata*, growing in three ecoregions of China, ecoregion A (Eastern Inner Mongolia having prairie with sandy soils), ecoregion B (Northern Shanxi hills with saline/alkaline soil) and ecoregion C (hillside/forest land with fertile soil in north-western Yunnan). Ecoregions A and B represented temperate condition, whereas ecoregion C, a tropial soil and climatic conditions. Rhizobial communities associated with *Caragana* species were different in the three ecoregions of China. *Caragana* species in region A were nodulated by *Mesorhizobium* genospecies I, II, IV, VI and VII, and in region B by genospecies *M. temperatum*, *M. tianshanense*, *M. septentrionale*, *M.* genospecies III, *R. yanglingense* and *Rhizobium* sp. IV, whereas with *M. plurifarium*, *M.* genospecies V and VII and *Rhizobium* sp. IV in region C. In conclusion, the above study demonstrated that *Caragana* species could be nodulated with distinctive populations mainly with *Mesorhizobium* spp. (82.8 %) and occasionally with

Rhizobium and *Bradyrhizobium*. The same group of workers observed identical results while studying *Caragana* isolates in another ecoregion in Northeastern China (Yan et al. 2007) but different from those in which *Rhizobium/Agrobacterium-related* strains were predominant in *C. intermedia*-associating rhizobia (Gao et al. 2002).

Most of the *Mimosa* species are native to Central and South America (Barneby 1991) with Cerrado region of Central Brazil being the major centre of diversification (Barneby 1991; Simon and Proenca 2000). It has long been known that *Mimosa* plants are nodulated by diverse rhizobial species. Prior to year 2000, all had been ascribed to known α-rhizobial genera (Barret and Parker 2005; Wang et al. 1999a; Moreira et al. 1993; Oyaizu et al. 1993). Since the first report of β-rhizobia from legume nodules (Moulin et al. 2001), β-rhizobia belonging to genera *Ralstonia* (now *Cupriavidus*) and *Burkholderia* have been reported from legumes, and a majority of them have been reported from *Mimosa* spp. (Chen et al. 2001, 2003; Verma et al. 2004). Chen et al. (2005), while investigating the diversity of nodule isolates from *Mimosa* spp. in South America, observed that most of the nodule isolates belonged to *Burkholderia* and none belonged to *Cupriavidus*, which appears strange considering that *Cupriavidus taiwanensis* is dominant in Taiwan (Chen et al. 2003) and possibly India (Verma et al. 2004). The possible explanation for this could be that *C. taiwanensis* is an Asian bacterium that has acquired its symbiosis genes from *Burkholderia* strains resident within *Mimosa* nodules that were introduced in Asia from tropical America and Caribbean by European colonists. The study of genetic diversity of rhizobia in medicinal legumes, namely, *Abrus precatorius*, *Mucuna pruriens*, *Melilotus officinalis*, *Trigonella foenum-graecum* and *Vicia angustifolia*, growing in the sub-Himalayan tract of Uttarakhand defined six rDNA genotypes within these rhizobia, and their phylogenetic relationships were intertwined within *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium* (Pandey et al. 2004).

Traditionally chickpea-nodulating rhizobia were rather host specific with two described species, *Mesorhizobium ciceri* (Nour et al. 1994) and *M. mediterranean* (Nour et al. 1995). However, Romdhane et al. (2009), while studying nodulation of chickpea in Tunisia under water-deficient conditions, reported that its nodulation by *M. mediterranean* was reduced, while with *Ensifer meliloti* was favoured. *E. meliloti* has also been reported from chickpea growing in the Terai and Almora regions of Uttarakhand Himalayas. When characterised, rhizobial isolates recovered from the nodules of various annual legumes, *Lens culinaris*, *Cicer arietinum*, *T. foenum-graecum*, *P. sativum* and *Trifolium* species, were genetically diverse, and symbiosis of *E. meliloti* with chickpea was effective. An interesting finding from the above study is the presence of *Rhizobium*, *Sinorhizobium* and *Burkholderia* from *Lens culinaris* nodules. This is the first report of *Burkholderia* from *Lens culinaris* nodules in India (Fig. 9.3).

The extensive survey of rhizobial diversity from various legumes, *Amorpha fruticosa*, *Astragalus*, *Glycyrrhiza* spp., *Gueldenstaedtia* spp. and *Lespedeza* spp., in the Northwestern region of China has led to the recovery of novel forms within the *Bradyrhizobium* (Yao et al. 2002), *Mesorhizobium* (Wang et al. 1999b), *Rhizobium* (Tan et al. 2001; Wei et al. 2002, 2003) and *Sinorhizobium* (Wei et al. 2002). From

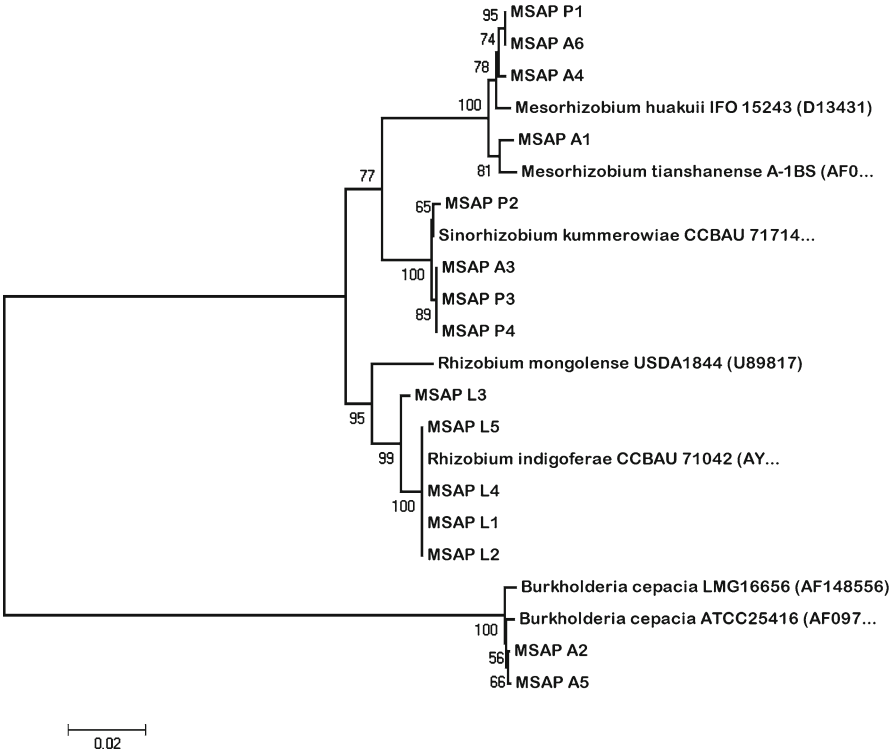

Fig. 9.3 Phylogenetic relationships based on full 16S rDNA sequences amongst rhizobial isolates from annual legumes of Uttarakhand (*Source*: unpublished)

these studies it has emerged that rhizobia in temperate regions are as diverse as those in tropical regions. Moreover, genetically diverse rhizobia are present at any single site and closely related strains could be found in varied geographic locations (Zhang et al. 1999). *Bradyrhizobium* strains nodulating genistoid legumes (brooms) in Canary Islands, Morocco, Spain and the Americas were highly diverse. Phylogenetic analysis of *Bradyrhizobium* strains using ITS, *atp*D, *gln* II and *rec*A sequences revealed that these belonged to four distinct evolutionary lineages, one representing *B. japonicum*, another representing *B. canariense* and the other two representing unnamed genospecies. Strains of *B. canariense* did not nodulate *Glycine max* but nodulated diverse legumes in tribes Genisteae and Loteae (Vineusa et al. 2005). Bacterial strains from nodules of *Genista tinctoria* were similar to slow-growing bradyrhizobia and genetically heterogenous. They did not nodulate *G. max*, *Lupinus corniculatus*, *M. sativa*, *P. vulgaris*, *T. repens* and *Vigna sativa* (Kalita and Malik 2004). Rodríguez-Navarro et al. (2004) reported that *Bradyrhizobium* strains nodulating legume *Pachyrhizus* were highly diverse and related to *B. elkanii*, *B. japonicum*, *B. liaoningense*, *B. yuanmingense* and *B. betae*. Nodule isolates from *Macrotyloma uniflorum* growing in the Almora region of Uttarakhand formed two genetic lineages: lineage I, representing fast-growing strains, and lineage II, very

slow-growing strains. The bacterial isolates from lineage I did not form nodules on homologous host but nodulated *G. max*, whereas slow-growing isolates nodulated *M. uniflorum* but not *G. max* (Agarwal 2009).

Rhizobia Associated with Tree Legumes

Ecological interaction between tree legumes and rhizobacteria is beneficial from three angles: increased biomass and amelioration of degraded sites on the account of improved water and nutrient uptake, prevention of soil erosion and increased soil fertility through N_2 fixation and greater organic matter production and recycling of nutrients. The leguminous trees are well nodulated under drought stress conditions. Species of *Acacia* are prevalent in Africa, Asia, Australia and Central America, and with the exception of *A. brevispica* from Africa, all nodulate effectively (Odee and Sprent 1992; Masutha et al. 1997; Tissue et al. 1997) with both fast- and slow-growing rhizobia (Barnet and Catt 1991). Other leguminous trees forming effective symbiosis with rhizobia are *Albizia* and *Leucaena*. A few leguminous trees can fix about 43–581 kg of N ha^{-1}, as compared with 15–210 kg of N ha^{-1} (Dakora and Keya 1997). Rhizobia of *Acacia senegal* and *Prosopis chilensis* are phenotypically and genotypically diverse (Zhang et al. 1991; Haukka and Lindström 1994; Haukka et al. 1996; Nick 1998; Dhabhai and Batra 2012). Zhang et al. (1991) placed *Acacia* rhizobial strains from Sudan in nine different clusters based on numerical analysis. Genetic characterisation based on 16S rRNA gene analysis (Haukka et al. 1996) showed that most Sudanese and Kenyan strains belonged to the genus *Sinorhizobium* and a few to *Mesorhizobium*. Nick and co-workers (1999) subsequently utilised DNA–DNA hybridisation on Sudanese and Kenyan isolates and grouped them into two clusters which showed low similarity with already described species of other tree legumes. Lafay and Burdon (2001) grouped nodule isolates from Australian acacias into nine genomospecies represented in genera *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium*, eight representing novel forms. He also proposed that majority of strains represented *Bradyrhizobium* spp. Hoque and co-workers (2011) genetically characterised the nodule symbionts of *A. salicina* and *A. stenophylla* growing across South-eastern Australia and reported the presence of *Burkholderia*, *Devosia*, *Ensifer*, *Mesorhizobium*, *Phyllobacterium* and *Rhizobium*. Dhabhai and Batra (2012) identified two genospecies inside the nodules of *Acacia nilotica* L., one showing homology to *Mesorhizobium loti* and second intermediate between *R. leguminosarum* and *Rhizobium hainanense*.

Rhizobia nodulating a diverse pool of forest legume species in Brazil were investigated by Moreira et al. (1998) who found six novel sequences amongst 44 strains from 29 leguminous tree species belonging to 13 tribes of *Leguminosae*. Studies undertaken with *Dalbergia sissoo*, *L. leucocephala*, *Mimosa* and *Prosopis* reveal that rhizobial isolates recovered from them are also diverse (Dupuy et al. 1994; de Lajudie et al. 1998; Nick et al. 1999). The long-term association between the symbionts allows gradual differentiation and diversity in compatible rhizobial

Table 9.2 Rhizobia described from tree legumes

Microsymbiont	Tree species	Reference
Mesorhizobium chacoense	*Prosopis alba*	Velázquez et al. (2001)
M. plurifarium	*Acacia*, *Leucaena*	de Lajudie et al. (1998)
R. tropici	*Leucaena* sp.	Martínez-Romero et al. (1991)
R. huautlense	*Sesbania herbacea*	Wang et al.(1998)
Ralstonia taiwanensis	*Mimosa* sp.	Chen et al. (2001)
Sinorhizobium arboris	*Acacia senegal*, *Prosopis chilensis*	Nick et al. (1999)
S. kostiense	*Acacia senegal*, *P. chilensis*	–do–
S. saheli	*Sesbania* sp.	de Lajudie et al. (1994)
S. terangae	*Acacia* sp.	de Lajudie et al. (1994)
S. morelense	*Leucaena leucocephala*	Wang et al. (2002)

populations resident in native soils. Rhizobial strains isolated from root nodules of native and exotic woody legumes (*Albizia gummifera*, *Erythrina brucei* and *Millettia ferruginea*) growing in Ethiopia showed very little metabolic and genomic relatedness to reference strains, hence representing probably novel forms. Phenotypic characterisation of the above gene pool showed a large diversity including very-fast- and extraslow-growing forms (Wolde-Meskel et al. 2004). Molecular systematics of *Sesbania* microsymbionts from Venezuelan wetlands using *rrs*, *atp*D, *rec*A and *nif* H sequence analysis identified them as *Mesorhizobium plurifarium* and *Rhizobium huautlense* (Vineusa et al. 2005). Amongst 98 rhizobial species known so far from legumes, 10 are from tree legumes (Table 9.2).

We observed considerable variability in rhizobia isolated from *Dalbergia sissoo* growing in various ecozones of Northern India (Sahgal 2002; Sahgal and Johri 2003). Out of 35 isolates, all were able to nodulate the homologous host, *D. sissoo*, while only 22 nodulated heterologous host *Sesbania aculeata*; only three nodulated *L. leucocephala* and *Vigna mungo* (Sahgal et al. 2004). Based on amplified rDNA restriction analysis of 16S and IGS, these isolates were grouped into seven rDNA types wherein none was identical to reference strains representing *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium*. Further extension of this work by Samant (2003) showed that six isolates from *D. sissoo* clones CPT5 and CPT6 were genetically different from those of the previous study (Sahgal 2002) and did not match any of the reference strains. The geographical origin appears to have considerable influence on the heterogeneity of rhizobia that nodulate wild tree legumes and those microsymbionts with restricted host ranges are limited to specific niches and represent specialisation of widespread and more ancestral promiscuous symbiosis.

Legume–Rhizobia–Mycorrhiza: A Tripartite Relationship

Legumes form tripartite symbiotic associations with nodule-inducing soil bacteria of the genera *Rhizobium*, *Bradyrhizobium* or *Azorhizobium* (Caetano-Anollés and Gresshoff 1991; Hirsch 1992) and with arbuscular mycorrhizal (AM) fungi

(Koide and Schreiner 1992). AM fungi and rhizobia are two of the most important plant symbionts to assess the capacity of plants to acquire nutrients. Mycorrhiza benefits the host through mobilisation of phosphorus from non-labile sources, whereas rhizobia fix N_2 (Scheublin and Vander Heijden 2006). Both the rhizobial and fungal microsymbionts improve the mineral nutrition of the host plant in exchange for assimilates provided by the latter. The nitrogenase enzyme of rhizobia fixes atmospheric nitrogen in the nodules (Thorneley 1992), and fungal hyphae facilitate the uptake of ions, mainly phosphate, in mycorrhizal roots (Smith and Gianinazzi-Pearson 1988). There are many similarities between rhizobial and AM symbioses, which suggest common properties in interactions with plants. Both are surrounded by plant-derived membranes in the established stage of the symbiosis: the peribacteroid membranes in the infected nodule cells and the perihaustorial membranes around arbuscules in the mycorrhizal roots, respectively. These interfaces are characterised by symbiosis-specific proteins (Perotto et al. 1994).

When soya bean (*G. max* [L.] Merr.) roots were co-inoculated with *B. japonicum* 61-A-1O1, considerable enhancement of colonisation by the mycorrhizal fungus *Glomus mosseae* was observed. In association with AM fungi, the rhizobia–bean symbiosis is benefitted by a better supply of phosphorus (Sanginga et al. 2000). Plants do not acquire phosphorus in organic form but AM is also able to help in this process (Bucher et al. 2001). Bargaz and colleagues (2011) reported that nitrogen fixation was significantly limited by P deficiency, and plants deficient in P show decreased nodule number and biomass. When compared with the control treatments, it was found out that dual inoculation with AM and rhizobia decreased the harmful influence of sulphate salinity on plant growth and nutrient accumulation (P and N) in *Lathyrus sativus* (Jin et al. 2010). Xie et al. (1995) described that highly purified Nod factors also increased the degree of mycorrhizal colonisation. Nod factors differed in their potential to promote fungal colonisation on the basis of their acetylation and sulphation. The acetylated factor NodNCR-V (MeFuc, Ac), added at concentrations as low as 10^{-9} M, promoted AM colonisation of plant roots, whereas the sulphated factor NodNCR-V (MeFuc, S) did not. The plant flavonoids mediate the Nod factor-induced stimulation of mycorrhizal colonisation in soya bean roots similar to determining host specificity in rhizobia–legume symbiosis. Thus, both symbioses share parts of signalling pathways, indicating intimate interactions between all three partners during co-evolution (Demir and Akkopru 2007; Xiao et al. 2010).

Mycorrhiza and Rhizobia: Common Signalling Factors

For the establishment of *Rhizobium* symbiosis, elucidation of the Nod factor structure was a major step to unravel the signalling pathway in legumes. Rhizobial Nod factors are lipochitooligosaccharides (LCOs) consisting of three to five

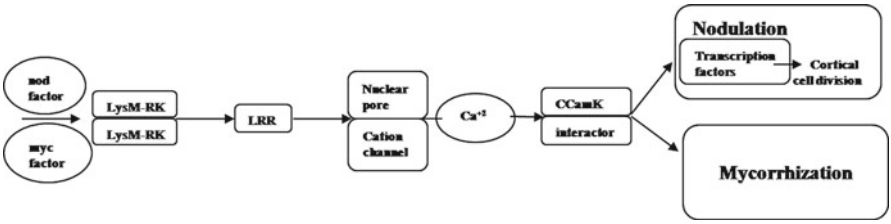

Fig. 9.4 Schematic representation of rhizobia–mycorrhiza symbiosis common signalling pathway (*Source*: Streng et al. 2011)

N-acetyl-glucosamines; the amino group of the nonreducing glucosamine is acylated with a fatty acid of 16–20 C-atoms in length (C16 to C20). Furthermore, terminal glucosamines contain species-specific substitutions, thereby determining that specific Nod factor structure is determined by the specific legume host plant. The modifications may be glycosylation, sulphation, acetylation and methylation, for which a particular *Rhizobium* species harbours specific nodulation (*nod*) genes (Gardes and Bruns 1993; Parniske 2008). Hence, it is assumed that the perception of Nod factors by legume host plants has co-evolved with their corresponding rhizobial symbionts. The calmodulin-binding domain and calcium-binding motifs of CCaMK (calcium–calmodulin-dependent kinase) allow the protein to sense calcium, which makes it a prime candidate for the response to calcium signatures induced by AM fungi (Kosuta et al. 2008) or the Nod factor that induces calcium spiking. The legume–rhizobia symbiosis and legume–mycorrhizal symbiosis pathways have interrelated factors. A deregulated version of CCaMK can trigger spontaneous nodule formation in the absence of rhizobia, which indicates that deregulation of CCaMK alone is sufficient to trigger the nodule formation. Also, in *Medicago truncatula*, three genes, called *DMI* (for *Does Not Make Infection*)-1, *DMI*-2 and *DMI*-3, are needed for infection by AM. These encode a protein that is a receptor-like kinase present on the cell membrane. Their one region extending to the outside of cell can bind to signal molecules such as growth factors, whereas an interior segment regulates other proteins by adding phosphate groups to them. This can conclude that DMI protein might be part of recognition machinery for Nod factors.

It is well known that in legumes, mycorrhizal and rhizobial symbioses share some common symbiotic genes. This has been first of all unravelled in pea (*P. sativum*) and the model species of legume, *M. truncatula* (medicago) and *Lotus japonicus* (lotus), respectively (Kouchi et al. 2010). In both the model species, the common symbiotic signalling pathway comprises a conserved set of six genes, encoding a plasma membrane receptor kinase (MtDMI2 and LjSYMRK), several components in the nuclear envelope including a cation channel (MtDMI1, LjCASTOR and LjPOLLUX), a nuclear localised calcium–calmodulin-dependent kinase (CCaMK; MtDMI3 and LjCCaMK) and a CCaMK interacting protein (MtIPD3 and LjCYCLOPS) (Fig. 9.4). Nod factor perception and signal transduction in the plant involve calcium spiking and

lead to induction of nodulation gene expression; mycorrhizal symbiosis bifurcation also takes place from this step. Mycorrhizae and rhizobia induced signalling bifurcates downstream of CCaMK, possibly due to a different nature of the calcium signal (Kosuta et al. 2008). Rapid calcium influxes are induced by both Nod factors (Oldroyd and Downie 2004) and AM fungal exudates (Kosuta et al. 2003). SYMRK perceives both mycorrhizal and rhizobial signals, probably at the junction of the common pathway (Parniske 2008; Oldroyd and Downie 2004). It encodes a leucine-rich repeat (LRR) receptor-like kinase.

Conclusion and Future Scenario

Rhizobial or fungal (AM) invasions of plant roots are decidedly beneficial for both their host plants and the world's agricultural systems. Plant–AM symbiosis helps plant to acquire phosphate from the soils, whereas legume–rhizobia symbiosis converts atmospheric nitrogen into the form required for plant growth. Unravelling the molecular underpinnings of these symbiosis shows that these associations share some common signalling factors concluding that both the associations are interrelated. For legume–rhizobia interactions, nodule development is an important event. Legume–rhizobia symbiotic control is exercised at three points: flavonoids in Nod D proteins, Nod factors in Hac or bacterial entry, as well as EPS and/or TTSS proteins in infection thread. Legume roots secrete flavonoids and betaine which are sensed by rhizobial partner that aids in the activation of Nod proteins and in turn secretion of Nod factors which assist in nodule development. The nodulation genes (*nod*, *nol*, *noe*) and nitrogen fixation genes (*nif*, *fix*) are key symbiotic genes in rhizobia, whereas nodulin genes that are expressed in root tissues as a consequence of interaction with rhizobia are symbiotic genes in plants. Host preferences of rhizobial partner are due to Nod D protein that can be activated by a large variety of flavonoids, production of more than 80 different types of Nod factors and the fact that its Nod D protein can be activated by both EPS and TTSS proteins. Diverse AM fungi produce small, diffusible factors that trigger the activity of one of the same genes activated by Nod factors. Hence, fungal and rhizobial Nod factors may play an analogous role. In conclusion, legume–rhizobia interactions are incomplete without mycorrhizae. The chemical nature of various Nod factors is known. It is expected that in the near future, the chemical nature of fungal factor and flavonoid–Nod factor association is elucidated. We must investigate rhizobial partners of yet unexplored legumes, their natural variations and responsiveness with biodiversity collections of important crop plants. The long-term aim is to identify or design crop–rhizobia–fungus combinations with optimised performance so that fertiliser and energy input can be reduced.

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