Chapter 2
Enhancing *Rhizobium*–Legume Symbiosis Using Signaling Factors

Anna Skorupska, Jerzy Wielbo, Dominika Kidaj, and Monika Marek-Kozaczuk

Abstract Rhizobial symbiosis with leguminous plants affects the supply of organic nitrogen. Soil bacteria comprising members of the genera *Rhizobium, Bradyrhizobium*, *Mesorhizobium, Sinorhizobium*, and *Azorhizobium*, commonly referred to as rhizobia, are taxonomically diverse members of the α and β subclasses of the *Proteobacteria*. They possess the ability to induce root nodules on legume plants and provide these plants with fixed nitrogen, enabling them to grow in nitrogen-limited soils. Rhizobia colonize root nodules, fix nitrogen inside, transport usable form of N to plants, and concurrently facilitate the growth and grain yields of legumes. *Rhizobium*–legume symbiosis is a multi-step process requiring the exchange of numerous molecular signals between bacteria and the plant host. Precise fulfilling of all stages of this molecular dialogue is prerequisite to the effective symbiosis, allowing bacteria to invade the host and, conversely, enabling the host to derive benefits from the presence of bacteria. Individual legumes are often nodulated by multiple bacterial strains with varying symbiosis-establishing capabilities. Thus, selection of highly effective strains that successfully compete with less effective ones is required when developing legume inoculants. Various factors that influence symbiotic rhizobial interactions under competitive soil environment, including the exchange of plant and bacterial signaling molecules, such as flavonoids and nodulation factor (Nod factor), in the early stages of symbiosis is highlighted. Beneficial responses of rhizobial inoculants on to legumes, as well as manipulations of symbiotic signaling factors, is likely to increase their potential as biofertilizers for sustainable agriculture to promote growth and nodulation of legume plants.

A. Skorupska (✉), J. Wielbo, D. Kidaj, and M. Marek-Kozaczuk
Department of Genetics and Microbiology, M. Curie-Skłodowska University, Akademicka 19 st, 20-033 Lublin, Poland
e-mail: anna.skorupska@poczta.umcs.lublin.pl

M.S. Khan et al. (eds.), *Microbes for Legume Improvement,*
DOI 10.1007/978-3-211-99753-6_2,

2.1 Overview of *Rhizobium*–Legume Symbiosis

2.1.1 Ecological and Agricultural Importance of Symbiotic Nitrogen Fixation

The availability of reduced nitrogenous compounds is a major limiting factor in plant growth and agricultural productivity. The microbiological process that converts atmospheric dinitrogen (N_2) into a plant-accessible species is known as biological nitrogen fixation (BNF). BNF reduces the degree of the requirement for external input of chemical N fertilizers to replenish soil N and improve internal resources (Peoples et al. 1995a; Vance 2001; Herridge et al. 2008). Total global N_2 fixation from BNF has been estimated to 100–290 million tones N/year, with approximately 50–70 million tones N/year in agricultural systems, compared with 83 million tones N fixed industrially in fertilizer production.

Among the wide range of bacteria that have the ability to reduce N_2 to ammonia, the most important are the symbiotic systems of leguminous plants and rhizobial species belonging to β-proteobacteria of the genera *Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium*, and *Sinorhizobium (Ensifer)*, collectively called rhizobia (Perret et al. 2000; Jones et al. 2007; Franche et al. 2009). Recently, several new species of N_2-fixing microsymbionts, such as, *Methylobacterium* (Sy et al. 2001), *Devosia* (Rivas et al. 2002), *Herbaspirillum* (Valverde et al. 2003), *Ochrobactrum* (Zurdo-Piñeiro et al. 2007), *Phyllobacterium* (Valverde et al. 2005), and members of the β-proteobacteria such as *Burkholderia* (Moulin et al. 2001) and *Cupriavidus* (*Ralstonia)* (Chen et al. 2001) have been described. A successful interaction between legume plants and rhizobia leads to the formation of nodules on the roots or shoots. Bacteria in the form of bacteroids reside inside nodules and fix atmospheric N into ammonia (Perret et al. 2000; Gibson et al. 2008). The reduced nitrogenous compounds are transported into the host plant in exchange for organic acids. The symbiotic systems are a major source of nitrogen in most legumes with an average of 80% of N derived from BNF (Vance 2001; Graham and Vance 2003). There are estimates that the rhizobial symbioses with 18,000 legume species (Masson-Boivin et al. 2009), including more than 100 agriculturally important legumes spanning all the geographical regions, contribute nearly half of the annual quantity of BNF in soil ecosystems (Graham and Vance 2003). Rotations of legumes with other non-nitrogen-fixing plants enrich the soil with fixed N and increase the productivity and sustainability of agricultural systems. There is evidence that nitrogen derived from legume sources are less susceptible to losses than chemical fertilizer N, which in long term results in the build-up of a reserve of readily mineralizable organic N. The use of BNF in agriculture provides a renewable source of N to supplement or replace fertilizer N and arrests the decline of soil N fertility (Peoples et al. 1995a, b).

2.1.2 *Rhizobial Genomes: Background for BNF and Source of Biodiversity*

A common feature of the rhizobial genomes is that genes involved in nodulation and N_2-fixation are clustered on symbiotic plasmid (pSym), or incorporated into the chromosome as symbiotic islands (Palacios and Newton 2005). The architecture of rhizobial genomes may directly underlie the great genetic and physiological variation of rhizobial strains, resulting in a large diversity of populations. Rhizobial genomes are large (e.g., *R. leguminosarum* bv. *viciae*–7.7 Mb; *R. etli*–6.5 Mb; *Sinorhizobium meliloti*–6.7 Mb) and are composed of chromosomal core and plasmids, which comprise up to 50% of total genome (Galibert et al. 2001; Gonzalez et al. 2006; Young et al. 2006). Comparative analyses of rhizobial genomes revealed their mosaic structure: regions showing high degree of conserved synteny are separated by other sequences (Guo et al. 2003; Król et al. 2007). There is evidence that such genomes are dynamic structures, where recombination events are very frequent, permanently creating new versions of individual replicons (Brom et al. 1991; Guo et al. 2003). Moreover, not only the symbiotic plasmid but also considerable fractions of the nonsymbiotic plasmid pool present in rhizobial cells are necessary for establishing an effective *Rhizobium*–legume symbiosis (Brom et al. 1992; Mercado-Blanco and Toro 1996; Galibert et al. 2001). On the one hand, the transfer of symbiotic plasmids between strains in the rhizosphere was evidenced (Broughton et al. 1987), and the lateral transfer of genes was postulated to be a considerable force in rhizobial evolution and diversification (Souza et al. 1992). On the other hand, some data indicate that pSym plasmid transfer frequency is not as high in the field as under laboratory conditions (Wernegreen et al. 1997). Thus, the lateral transfer of genes by plasmid exchange is responsible for an emerging diversity within narrow genetic subdivisions, while main rhizobial genera are quite "reproductively isolated" (Wernegreen and Riley 1999; Bailly et al. 2007).

2.1.3 *Signaling in Rhizobium*–Legume Symbioses

Rhizobia nodulate wide range of legume plants. Some of them, such as *Rhizobium* sp. NGR234, are extremely promiscuous and are able to nodulate many different host plants (over 112 hosts) (Pueppke and Broughton 1999), while others, such as *R. leguminosarum* bv. *trifolii*, have a very narrow host range and nodulates only clover (*Trifolium* spp.) plants. Its close relative, *R. leguminosarum* bv. *viciae,* nodulates pea (*Pisum* spp.), vetch (*Vicia* spp), lentil (*Lens* spp.), and sweet pea (*Lathyrus* spp.) (Perret et al. 2000). The specificity of symbiotic interactions is achieved by exchange of molecular signals. In the early steps of symbiosis, a diverse array of compounds is exuded into the rhizosphere, including flavonoids, isoflavonoids, and non-flavonoid inducers (Fig. 2.1). These compounds are chemoattractants for rhizobia (Caetano-Anollés et al. 1988; Dharmatilake and Bauer 1992), influence

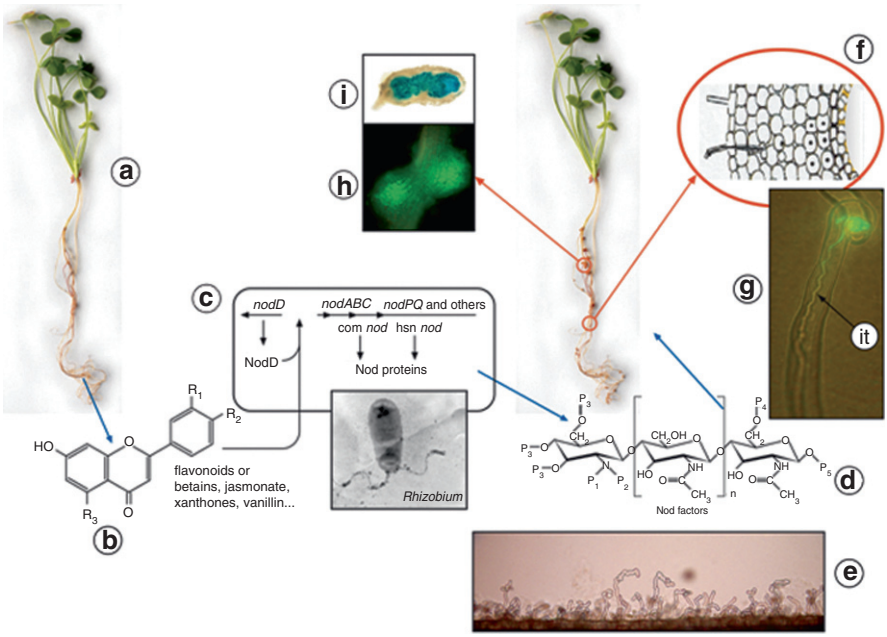

Fig 2.1 The events involved in *Rhizobium*–legume symbiosis. Roots of legume plant (**a**) exude flavonoid compounds (**b**), which are perceived by bacterial NodD regulatory protein (**c**) that activate set of *nod* genes resulting in the production of Nod factor (**d**). Nod factors secreted by rhizobia mediating root hair curling (**e**) and initiation of nodule primordium (**f**). Rhizobia attach to the root hairs and infection threads (it) are formed (**g**). Infection threads penetrate plant tissues, invade nodule primordium, and develops nodule (**h**, **i**). (**h**) Rhizobia tagged with *gfp* inside young nodules (**i**) rhizobia tagged with *gus* in mature nodule

bacterial growth, and induce the expression of nodulation genes (*nod* genes) (Peters et al. 1986; Hungria and Stacey 1997) (for detail see Sect. 2 and 3). As a result of *nod* genes expression, biosynthesis of specific lipochitin oligosaccharides called nodulation factors (Nod factors or LCOs) occurs (Lerouge et al. 1990). Nod factors are structurally diverse and a single rhizobial strain may produce a range of these metabolites (Spaink et al. 1991, 1995).

Nodulation genes have been classified into three categories. First, the common nodulation genes (*nodABC)* that are found in all bacteria including the β-proteobacteria (Moulin et al. 2001) with only one exception, that is, photosynthetic bradyrhizobia (Giraud et al. 2007). These are essential for nodulation and mutations in these genes lead to Nod$^-$ phenotype (Jacobs et al. 1985; Debellé et al. 1986). The encoded NodC is responsible for biosynthesis of β-1,4-linked *N*-acetyl-D-glucosamine trimeric to hexameric backbone, while NodB deacetylates glucosamine at the nonreducing end, following which NodA acylates free amino group of the terminal glucosamine (Spaink 2000; D'Haeze and Holsters 2002). Second, the host-specificity nodulation genes (*nodFE, nodH, nodG, nodPQ*, and several others) whose products modify the *N*-acylglucosamine backbone by adding species-specific

substituents, which are considered the main factors determining the host range of microsymbionts, and influence the rate of nodule formation (Debellé et al. 1986; Horvath et al. 1986; Schwedock and Long 1989). At the reducing-terminal residue, L-fucosyl, 2-*O*-Me-fucosyl, 4-*O*-Ac-fucosyl, acetyl, or sulphate esters are present, and at the nonreducing-terminal residues, *N*-methyl, *O*-acetyl, and *O*-carbamoyl are found, respectively. A fatty acyl chain of varying length and with varying degree of unsaturation is attached to the nonreducing end. These moieties controlled by the host-specificity *nod* gene products make the Nod factors specific for target plant hosts (Dénarié et al. 1996; Spaink 2000; D'Haeze and Holsters 2002). However, variation in Nod factor structure does not fully explain host-range specificity. For example, Nod factors produced by *R. etli* and *Mesorhizobium loti* have the same structure but nodulate distinct host legumes (e.g., *Phaseolus* spp., and *Lotus* spp.) as reported by Cardenas et al. (1995). This indicates that the elements of the molecular dialogue between the legume plant and rhizobia are more complex and that Nod factor is not the sole signal specifying the host range (Perret et al. 2000; Somers et al. 2004).

The third class of *nod* genes is a family of regulatory *nodD* genes (Spaink 2000). NodD proteins belong to the LysR family of transcriptional regulators (Hong et al. 1987; Fisher et al. 1988; Kondorosi et al. 1989). NodD, in complex with a flavonoid, binds conservative sequences upstream of *nod* operons, called *nod*-boxes, acting as transcriptional activator of several *nod, nol*, and *noe* gene promoters (Peck et al. 2006). *Sinorhizobium meliloti* synthesizes four NodD proteins (NodD1, NodD2, NodD3, and SyrM) that interact with different plant flavonoid signals. Some rhizobium species use other sensor-activator systems to control the host range. For instance, *B. japonicum* possesses *NodV–NodW,* a two-component system that is a positive regulator of *nod* genes responding to isoflavone signals. *NodV* and *nodW* are essential for the nodulation of *Macroptilium atropurpureum*, *Vigna radiata,* and *V. unguiculata*, but contribute only marginally to the symbiosis with *Glycine max* (Gőttfert et al. 1990; Sanjuan et al. 1994). Furthermore, several *nod* regulons, which are positively regulated by NodD protein, can be negatively regulated by NolR (Kondorosi et al. 1991; Cren et al. 1995). NolR binds to the promoter regions of *nod* genes and prevents their expression. Expression of *nolR* is negatively regulated by luteolin – a specific *nod* gene inducer in *S. meliloti* (Cren et al. 1995).

For optimal nodulation, proper level of *nod* gene expression is required and special mechanisms down-regulate the expression of *nod* genes in *S. meliloti* and *R. leguminosarum* bv. *viciae* (Somers et al. 2004; Perret et al. 2000; Hogg et al. 2002; Peck et al. 2006). In *R. leguminosarum* bv. *viciae* bacteroids, syntheses of NodA, NodI, NodE, and NodO proteins were reduced at least 14-fold compared with free-living bacteria, whereas the level of NodD protein was reduced only threefold (Schlaman et al. 1991). A decreased amount of NodD was also found in a strain harboring multiple copies of *nodD*. The in situ RNA hybridization of *Pisum sativum* and *Vicia hirsuta* nodules showed that transcription of inducible *nod* genes was switched off by unknown regulatory mechanism before the bacteria differentiated into bacteroids (Schlaman et al. 1991). Moreover, the concentration of Nod

factors in the rhizosphere is modulated by plant root hydrolases (Minic et al. 1998; Ovtsyna et al. 2000). Tetrameric NodD binds to 49 bp *nod*-boxes even in the absence of flavonoids (Feng et al. 2003). However, compatible flavonoids are required to induce the changes in DNA topology at the location of NodD binding in the promoter *nod* gene, thereby allowing RNA polymerase to initiate gene transcription (Chen et al. 2005). In *S. meliloti*, only luteolin is capable of activating in vivo *nod* gene transcription (Peck et al. 2006). Noninducing flavonoids, such as naringenin, eriodictyol, and daidzein, also stimulate an increase in the DNA-binding affinity of NodD1 to *nod* gene promoters but only luteolin is capable of promoting the topological changes necessary for *nod* gene induction. This is consistent with the hypothesis that noninducing flavonoids are acting as competitive inhibitors of inducing flavonoids, preventing NodD1 from activating *nod* gene transcription (Peck et al. 2006).

Flavonoids and *nod*-boxes also regulate other functions, such as: (1) N_2-fixation (Dombrecht et al. 2002), (2) synthesis and/or modification of polysaccharides (Mimmack et al. 1994; Wielbo et al. 2004a), (3) rhizopine catabolism (Rossbach et al. 1994), (4) synthesis of hopanoids (Kannenberg et al. 1995), or (5) synthesis of transcriptional regulators that modulate the synthesis of Tts1 and SyrM2 in *Rhizobium* sp. NGR234 (Kobayashi et al. 2004). The *nodDABC* genes, independently of flavonoids, are required for the establishment of the three-dimensional structure of a biofilm formed by *S. meliloti,* which is enhanced by the flavonoid luteolin – the inducer of *nod* genes (Fujishige et al. 2008). The core Nod factors facilitate bacterial adhesion to the roots until, in the presence of plant flavonoid inducers, a sufficient concentration of the host-specific Nod factors is reached and plant developmental processes are initiated (Fujishige et al. 2008; Faure et al. 2009).

Host-specific Nod factors are perceived by the plant via LysM-type receptor kinases and a complex signal transduction cascade that triggers early plant responses, such as intra and extracellular alkalinization, membrane depolymerization, calcium spiking, deformation of root hairs, initiation of cortical cell division, infection thread growth, and nodule primordia formation (D'Haeze and Holsters 2002; Oldroyd and Downie 2004; Jones et al. 2007). Recently, several genes of the Nod factor-signaling cascade have been identified and cloned from *Medicago truncatula* and *Lotus japonicus* model legumes (Geurts et al. 2005; Oldroyd and Downie 2004, 2006). Nod factors are biologically active at very low concentration (10^{-9}–10^{-12} M). Rhizobia enter the roots at the sites where root hair cell walls are hydrolysed and may produce either hydrolytic enzymes or use plant mechanisms (Perret et al. 2000). They invade the roots through tubular structures called infection threads. It has also been found that Nod factors reduce the salicylic acid level in the roots to help in the suppression of host defence responses and ensure successful infection by rhizobia (Martínez-Abarca et al. 1998).

Purified lipochitooligosaccharides (LCOs) are sufficient to induce root hair curling, reinitiation of cell division, and in some cases, elicitation of nodule-like structures (Dénarié and Cullimore 1993; Stokkermans et al. 1995; Heidstra and Bisseling 1996; Gibson et al. 2008). Furthermore, Nod factors and other chitin

oligosaccharides have been known to have developmental effects on nonleguminous plants, such as carrot (*Daucus carota*) (de Jong et al. 1993), tobacco (*Nicotiana tabacum*) (Schmidt et al. 1993), and Norway spruce *Picea abies* (Dyachok et al. 2000), in the absence of auxin and cytokinin. Global transcriptome analyses of host plants revealed significant changes in the expression of a wide range of genes involved in various aspects of the symbiotic interaction, such as bacterial infection, nodule formation and function, and defense responses (Fedorova et al. 2002; Mitra and Long 2004; Yahyaoui et al. 2004; Barnett and Fisher 2006).

Rhizobia multiply near the tip of growing infection threads, and after reaching the nodule primordium, they are released into the plant cells. Simultaneously, plant-derived peribacteroid membranes (PBM) forming symbiosomes encapsulate them. Inside symbiosomes, they differentiate into bacteroids, which reduces N to NH_4^+ (Brewin 2004; Gage 2004). Numerous rhizobial signaling molecules are required for initiation and elongation of the infection threads and nodule development. They include symbiotically relevant cell-surface polysaccharides such as capsular polysaccharides (CPS), exopolysaccharides (EPS), cyclic β-(1,2)-glucans, and lipopolysaccharides (LPS). Such polymeric compounds play an important role in plants forming indeterminate type of nodules with a persistent meristem such as *Vicia, Medicago, Pisum*, or *Trifolium* (Fraysse et al. 2003; Becker et al. 2005; Skorupska et al. 2006). However, plants that form determinate type of nodules do not have such a requirement (Hotter and Scott 1991). Several possible roles for bacterial polysaccharides in symbiosis are considered: (1) a mechanistic role in protecting bacteria against environmental stresses; (2) acting as signaling molecules triggering plant developmental response; (3) promotion of infection thread initiation and development and suppression of plant defense responses (Fraysse et al. 2003; Skorupska et al. 2006).

Recently, it has been reported that genomes of photosynthetic *Bradyrhizobium* strains (BTAi1 and ORS278) that induce stem and root nodules on aquatic *Aeschynomene* legume plants do not contain the common nodulation genes *nodABC* that are indispensable for Nod factor backbone synthesis, indicating that this group of rhizobia uses mechanism other than Nod factor strategy to enter into symbiosis (Giraud et al. 2007). Purine derivatives, such as cytokinins produced by some strains of *B. japonicum* and *R. leguminosarum*, have been considered as a potentially important signal triggering nodule formation (Giraud et al. 2007; Frugier et al. 2008). On the one hand, even though rhizobially derived cytokinins have not been found essential for Nod-factor-dependent symbiosis, yet they seem to be important for Nod-factor-independent nodulation in *Aeschynomene*. On the other hand, recent data indicate diverse functions of plant cytokinins in symbiosis, such as mediating root susceptibility to rhizobial infection and nodule organogenesis. Cytokinins act downstream of early Nod-factor signaling playing a crucial role in redifferentiation of cortical cells after the induction of Nod-factor-dependent pathways (Lohar et al. 2004; Frugier et al. 2008).

The host plant controls localization, shape, anatomy as well as the infection of the nodules. In general, there are two different kinds of nodules elicited by rhizobia: determinate and indeterminate. The elongated indeterminate nodules have

a persistent meristem that continually gives rise to new nodule cells that are subsequently infected by rhizobia. In this type of nodules, the developmental stages can be observed from the meristem at the nodule tip to the older senescent and saprophytic zones near the root (Vasse et al. 1990; Timmers et al. 2000). Bacteroids within indeterminate nodules undergo a terminal differentiation program and grow outside the host cells (Mergaert et al. 2006), while the bacteria released from the saprophytic zone are viable and grow in the rhizosphere and infect legume roots. Determinate nodules are formed generally by tropical legumes (e.g., *Glycine max, Vicia faba,* and *Lotus japonicus*) and are round due to lack of persistent meristem and do not display developmental zones (Perret et al. 2000; Gage 2004). Bacteroids within determinate nodules can dedifferentiate after release from nodules and grow in soil (Mergaert et al. 2006). These two types of nodules also differ significantly in their C and N metabolism (White et al. 2007).

2.1.4 Nitrogen Fixation

Inside nodules, rhizobia enclosed in PBM are transformed into bacteroids capable of N_2-fixation. This process is catalyzed by oxygen-sensitive molybdenum nitrogenase found in all rhizobia. The nitrogenase catalyzes the following reaction:

$$N_2 + 16\text{MgATP} + 8e^- + 8H^+ \rightarrow 2NH_3 + 16\text{MgADP} + \text{Pi} + H_2$$

In this reaction, 16–18 molecules of ATP are required for the reduction of N_2 molecule to two molecules of NH_3 and $2H^+$ to H_2. Nitrogenase is extremely O_2 sensitive and is rapidly inactivated in aerobic environment. The low O_2 concentration (3–22 nM) in the nodule is achieved by the high concentration of O_2-binding heme protein – leghemoglobin, and the symbiosome membrane diffusion barrier (Kaminski et al. 1998; Patriarca et al. 2002). Energetically, this is a very expensive process, which explains the inhibition of nodulation by the presence of fixed nitrogen (White et al. 2007).

Rhizobia are equipped with several *nif* genes responsible for the N_2 fixation process: structural gene (*nifH*) encoding dinitrogenase reductase, also designated Fe-protein, and *nifD, nifK* genes encoding α and β subunits of dinitrogenase, respectively, that form functional complexes with FeMo cofactor, also named FeMo protein. *nifB, nifE*, and *nifN* genes encode molecular scaffold for the assembly of FeMo cofactor. Prosthetic groups containing 4Fe–4S clusters are covalently bound to MoFe protein bridging the α and β subunits. The 4Fe–4S group is linked also to the Fe protein (Fisher 1994; Newton 2007). The *nifA* gene performs a regulatory role in expression of *nif* genes; NifA functions in low oxygen tension. In rhizobia, besides *nif* genes with significant homology to *Klebsiella pneumoniae nif* genes (Ruvkun and Ausubel 1980), several other *fix* genes involved in nitrogen fixation have been found. Generally, rhizobia show high plasticity not only in the

gene composition of *nif* regulon but also in the mode of *nif* genes regulation (Fisher 1994; Masson-Boivin et al. 2009).

Carbon sources for N_2-fixation are supplied by the host plant in the form of photosynthates (sucrose) that are catabolized to C_4 dicarboxylic acids and transported to the bacteroids to provide energy for N_2-fixation. Ammonium (and alanine) as a product is exported back to the plant through the symbiosome membrane and is further assimilated into glutamine or asparagine in the plant cytosol in indeterminate nodules. In determinate nodules, these amino acids are converted in the uninfected cells, specialized in further nitrogen assimilation into ureides or amides that are transported from the nodules to the shoots (Lodwig and Poole 2003; White et al. 2007).

2.2 Flavonoids Compounds Inducing Nod Factor Production and Enhancing Symbiosis

Plants exude large quantities of sugars (from mono and simple polysaccharides to high molecular weight polysaccharides), acids (aliphatic as well as aromatic acids), amino acids, amines, and many other low molecular weight compounds such as flavonoids, steroids, alkaloids, vitamins, and growth regulators (Gaworzewska and Carlile 1982; Knee et al. 2001; Bertin et al. 2003). All these compounds may affect bacterial soil populations. Some of them, such as sugars, acids, and aminoacids, serve as C and energy sources for microorganisms (Jaeger et al. 1999). Others such as flavonoids are involved in signal exchange between the two symbionts or in anti-pathogen plant defense system (Phillips and Kapulnik 1995). Moreover, some components of plant root exudates have been shown to interfere with quorum sensing-dependent bacterial communication systems (Teplitski et al. 2000). On the basis of these observations, it has been suggested that plants possess a great potential for "soil engineering" and may select for the most suitable bacteria (Simms and Taylor 2002). On the other hand, rhizosphere microorganisms can enhance root exudation of C and energy sources or flavonoids, suggesting the presence of "feedback" in plant–*Rhizobium* interactions related to bacterial nutrition (Lodwig and Poole 2003; Phillips et al. 2004).

Flavonoids are the most important components of plant root exudates for successful *Rhizobium*–legume relationships (Table 2.1). The flavonoid backbone is synthesized by condensation of 4-coumaryl-CoA provided by chalcone synthase (CHS) (Dixon and Paiva 1995). Several modifications of this structure yield different classes of flavonoids: flavanones, flavones, isoflavonoids, coumestans, chalcones, and anthocyanidines (Harborne and Williams 2000). More than 4,000 flavonoids are synthesized in vascular plants and released into the rhizosphere (Perret et al. 2000). The production spectrum of these substances may vary with the physiological state and age of the plant (Schlaman et al. 1998; Harborne and Williams 2001). Great amounts of flavonoids are released near the root hair zone;

Table 2.1 Substitution patterns of plant flavonoids. Deavours et al. (2006)

Flavonoid	Substitution pattern on ring positions				
	3	5	7	3′	4′
Flavones					
7,4′-dihydroxyflavone			OH		OH
Apigenin		OH	OH		
Luteolin				OH	
Isofavones					
Daidzein			OH		OH
Genistein		OH	OH		OH
Flavanones					
Hespertin		OH	OH	OH	OH
Naringenin		OH	OH		OH
Flavonols					
Quercetin	OH	OH	OH	OH	OH

OH hydroxyl

a site of rhizobium infection (Peters and Long 1988; Hartwig et al. 1990; Zuanazzi et al. 1998). Flavonoids play important roles in different stages of nodulation. First, they act as *nod* gene inducers by activation of NodD protein (Recourt et al. 1991; Hungria and Stacey 1997; Brencic and Winans 2005). For example, luteolin and 4, 7′-dihydroxyflavone (Dhf) are the inducers for *S. meliloti* (Caetano-Anollés et al. 1988; Hartwig et al. 1990; Peck et al. 2006), isoflavone genistein induces *nod* gene expression in *B. japonicum* but inhibits *S. meliloti nod* gene expression (Kosslak et al. 1987), and hespertin and naringenin are potent inducers of *R. leguminosarum* bv. *viciae* (Firmin et al. 1986; Begum et al. 2001). They induce Nod factor synthesis in the infection threads (Sharma and Signer 1990) and act in auxin transport regulation and initiation of nodule primordia cell division (Mathesius et al. 1998; Zhang et al. 2009). Recently, different roles of flavonoids in development of determinate and indeterminate nodules have been reported (Wasson et al. 2006; Subramanian et al. 2007; Zhang et al. 2009). For instance, total silencing of flavonoid biosynthesis in *M. truncatula* forming indeterminate nodules leads to a near complete loss of nodulation by *S. meliloti,* whereas flavone-deficiency results in reduced nodulation. Isoflavone-deficient roots are nodulated normally, indicating that isoflavones are not crucial in *M. truncatula* nodulation (Zhang et al. 2009). Addition of 7,4′-dihydroxyflavone but not flavonol kaempferol (an inhibitor of auxin transport) to flavones-deficient roots can completely restore nodulation. On the basis of these observations, Zhang et al. (2009) proposed the sequence of flavonoid involvement during interaction of *M. truncatula* with *S. meliloti*. It has been suggested that the essential role of Dhf is not only as the primary inducer but also in the sustained Nod-factor induction in the infection threads. The sustained Nod-factor induction in turn leads to the accumulation of flavonol kaempferol and accumulation of kaempferol subsequently leading to the localized auxin transport inhibition resulting in cell division, nodule initiation, and development (Zhang et al. 2009). In contrast, silencing of isoflavone biosynthesis in soybean roots forming

determinate nodules lead to reduced nodulation and increased auxin transport suggesting the essential role of isoflavones during soybean nodulation. In this case, the isoflavone might be responsible for the sustained induction of bacterial *nod* genes and Nod-factor biosynthesis inside the roots (Subramanian et al. 2006).

There are several examples of flavonoids, which have been used to increase *nod*-gene transcription and promote legume growth. For example, bean nodulation by *R. leguminosarum* bv. *phaseoli* or *R. tropici* was enhanced by the addition of quercetin and malvidin glucoside (Hungria and Phillips 1993); luteolin added to certain alfalfa cultivars significantly increased nodulation (Kapulnik et al. 1987); pretreatment of *B. japonicum* with genistein increased nodulation, total protein yield, and grain yield of soybean under laboratory (Zhang and Smith 1995) and field conditions (Zhang and Smith 1996); preinduction of *R. leguminosarum* with flavanones hespertin and naringenin, alone or in mixture, stimulated nodulation and plant dry matter accumulation of pea and lentil plants in comparison to uninduced *R. leguminosarum* cells in controlled environment growth chamber conditions (Begum et al. 2001).

Seed exudates, which are mixture of flavonoids, are economically more justified when used as exogenous *nod* genes inducers, although they also contain flavonoid-inhibitors of *nod* gene expression. Evidence exists that compounds sharing structural similarities with daidzein or genistein are the most effective inhibitors of *nod* gene induction in *B. japonicum*. The inhibitors of *nod* genes may act competitively against inducers at a common target site, such as the NodD protein (Kosslak et al. 1990). However, under laboratory conditions, the clover and bean exudates were more potent inducers of *nodA* gene of *R. leguminosarum* than the specific flavonoids alone, indicating the possibility of synergistic effects of *nod*-gene-activating compounds (Maj et al. 2010). Several authors demonstrated that stimulation of genes with combinations of multiple inducers resulted in better *nod* gene induction and might be advantageous for early symbiotic interactions (Cooper 2004, 2007). This would not exclude the specific interaction between NodD protein and individual flavonoid (Peck et al. 2006).

The potential of seed exudates as *nod*-gene inducers could be exploited in the symbiotic activation of inoculants before their use as biofertilizers. As an example, preincubation of *R. leguminosarum* bv. *trifolii* strains with clover seed exudate increased fresh mass of shoots and increased nodule numbers in a strain-specific manner under laboratory conditions (Maj et al. 2010). Preactivation of inoculant strains with flavonoids might increase competitiveness in the soil as well as legume productivity; in the case of *B. japonicum* preactivated with genistein, soybean yields increased by 10–40%, and the seasonal levels of N_2 fixation enhanced by 35% (Zhang and Smith 2002). Similarly, field pea and lentil plants displayed increased nodulation and biomass production when inoculated with *R. leguminosarum* pre-induced with hesperetin (Begum et al. 2001). Currently, flavonoids are used commercially to promote *Rhizobium*–legume symbioses and N_2 fixation in agricultural practices (Hungria and Stacey 1997; Mabood et al. 2008). For example, genistein and daidzein, inducers of *B. japonicum nod* genes, are used in commercial inoculants under the name SoyaSignal. Using SoyaSignal technology to early-planted

soybean increased yields by 10% depending on the soybean genotype potential (Leibovitch et al. 2001). These and other associated data thus suggest that the knowledge of the essential role of flavonoids in *Rhizobium*–legume symbioses could be exploited to enhance the nodulation of economically important legume crops by exogenous addition of synthetic or natural flavonoid compounds. However, the practical application of flavonoids or other signaling molecules in a complex soil environment is much more difficult, and hence, the anticipated results may be different in different ecological niches.

2.3 Non-Flavonoid Signaling Molecules Influencing Legume Productivity

Several non-flavonoid plant factors, such as jasmonates, aldonic acids (erythronic acid and tetronic acid), betains, xanthones, and simple phenolic compounds, can induce the expression of *nod* genes. The common features of such inducers are that they act at higher concentrations than flavonoids and can induce *nod* genes and enhance Nod factors production in several legume plants (Cooper 2007). Jasmonic acid (JA) and its ester, methyl jasmonate (MeJA), generally known as jasmonates, are derivatives of linolenic acid and are biosynthesized in plants via the octadecanoic pathway. They are important signal molecules involved in induced disease resistance and mediate many physiological activities, such as environmental stress responses, root growth promotion, or inter-plant communication in plants. Jasmonic acid exogenously applied to the growth medium at high concentration (100 μM) decreases the number of nodules on *S. meliloti* inoculated *Medicago truncatula* roots. At such dose rate, JA decreases the responsiveness of calcium spiking to Nod factor, whereas at low concentrations (10–50 μM), it modifies the calcium signal by decreasing the frequency of spiking. Modulation of calcium signaling might have an important role in the initiation of colonization where number of calcium spikes is critical for triggering the Nod factor-signaling pathway (Sun et al. 2006; Miwa et al. 2006; Gutjahr and Paszkowski 2009).

The direct effect of JA and MeJA on induction of *nod* genes leading to increased Nod factor production has been described for *R. leguminosarum* and *B. japonicum* (Rosas et al. 1998; Mabood et al. 2006). In the case of *B. japonicum*-soybean symbiosis, jasmonates and genistein at 50 μM and 20 μM, respectively, applied alone or together with a Brady*rhizobium* promoted nodulation and N fixation under controlled and field conditions, at both optimal and suboptimal root zone temperatures. In the absence of these compounds, at suboptimal root zone temperature, nodulation, nitrogen fixation, and plant growth were inhibited (Mabood and Smith 2005; Mabood et al. 2008). The synergistic effect of jasmonate and flavonoids (naringenin, genistein) on nodulation, N_2 fixation, and biomass production was also observed for *R. leguminosarum*-bean (*Phaseolus vulgaris* L.) symbiosis (Poustini et al. 2007). These results suggest that both inducers, jasmonates and flavonoids,

utilize different receptors for signal transduction, and a concomitant activation of different regulatory mechanisms enhances the transcription of *nod* genes and Nod factor production (Mabood et al. 2006).

Within the variety of flavonoids, isoflavonoids, and other compounds secreted by lupin (*Lupinus albus)* roots into the rhizosphere, major proportion is composed of aldonic acids, that is erythronic acid (4-C sugar acids) and its analog tetronic acid. These compounds act as *nod* gene inducers of *R. lupini, M. loti,* and *S. meliloti*. Both aldonic acids in mM concentrations stimulated the expression of *nodC* gene in rhizobia. In addition, lupiwighteone, a genistein monoprenyl, added to cultures together with either aldonic acids exerted a synergistic effect on *nod* genes induction of *R. lupini*. Synergistic effect of luteolin and tetronic acid (but not erytronic acid) on *nod* induction was also observed for *S. meliloti*. Concomitantly with *nod* gene induction, the increase in LCO production was observed in the presence of both aldonic acids in *R. lupini* cultures, and of tetronic acid in *M. loti* and *S. meliloti* (Gagnon and Ibrahim 1998).

The next group of non-flavonoid inducers studied to date is betaines (stachydrine and trigonelline), N-methylated derivatives of aspartic acid and ornithine, secreted in large amounts from germinating alfalfa *(Medicago sativa L.)* seeds (Goldmann et al. 1991; Phillips et al. 1992). Stachydrine and trigonelline transcriptionally activated only the gene encoding the NodD2 protein but no apparent activation was reported for NodD1, which was activated by luteolin (Phillips et al. 1992). Betaine inducers, similarly to aldonic acids, function at a 10^3-fold higher concentration than required for *nod* induction with flavonoids. In *S. meliloti*-alfalfa symbiosis, using non-flavonoid molecules that are synthesized via a metabolic pathway distinct from those for flavonoid synthesis could be beneficial in the case of flavonoid soil depletion. Also, the good solubility of stachydrine and trigonelline in water may allow them to diffuse more easily than flavonoids within the soil, increasing the availability of these inducers (Phillips et al. 1992). The *nod* genes of *B. japonicum* can also be induced by xanthones (Yuen et al. 1995).

2.4 Nod Factor-Enhancement of Bacterium–Plant Symbiosis

In early studies, the manipulation of a number of common *nod* genes led to a reduced nodulation of *Vicia faba* inoculated with *R. leguminosarum* harboring a multicopy plasmid carrying *nodABC* genes (Knight et al. 1986). Following this, attempts were undertaken to improve nodulation and N_2-fixation in alfalfa plants by amplifying specific regions of the symbiotic plasmids of *S. meliloti* strains 41 and 1021 (Castillo et al. 1999). Amplified DNA fragments containing *nodD1* regulatory gene, the common nodulation genes (*nodABC*) and *nifN* gene essential for N_2-fixation, were introduced into *S. meliloti* genome by homologous recombination. Derivatives of *S. meliloti* with a moderately increased copy number of symbiotic genes (2.5–3 copies) showed better symbiotic properties and promoted plant growth under controlled conditions. When the number of copies of symbiotic

genes was set to about seven, the nodulation and N_2-fixation decreased (Castillo et al. 1999). These results suggested that the manipulation of structural or regulatory *nod* genes in rhizobia to increase their symbiotic activity is possible but only to some extent.

The key role of the Nod factor in early steps of symbiosis and its mitogenic and morphogenic activity leading to the formation of nodule primordium sparked several attempts to employ it to enhance legume productivity (Spaink et al. 1991). "Hormone-like" effect of purified *B. japonicum* Nod factor (nod Bj-V $C_{18:1}$,MeFuc) was observed in legume (soybean) and nonlegume plants (corn) (Souleimanov et al. 2002). The application of Nod factor at concentrations from 10^{-7} to 10^{-9} M stimulated biomass accumulation and enhanced growth and architecture of roots in host and nonhost plants indicating that the perception of LCO signal is conserved among a variety of species. This was further confirmed by identifying the genes of Nod factor cascade essential for mycorrhiza or an endosymbiosis in many higher plant species (Geurts et al. 2005). Further experiments showed that *B. japonicum* Nod factor stimulated germination of soybean and a variety of economically important plants belonging to diverse families under laboratory and field conditions (Prithiviraj et al. 2003). Stimulation of seed germination and seedling growth of several members of angiosperms suggests that specific receptors might also exist on seed surface or the developing embryonic roots, and Nod factor-induced genes may be present in genomes of numerous leguminous or nonleguminous plants (Prithiviraj et al. 2003). The chitin pentamer did not elicit such responses, which demonstrated that the structure of Nod factor plays a role in specificity of its biological activity. For legumes forming indeterminate nodules, Macchiavelli and Brelles-Mariño (2004) observed a noticeable increase in nodule numbers after treating the seeds of *M. truncatula* with submicromolar concentration of *S. meliloti* LCOs before inoculation. Moreover, clover seeds treated with specific LCOs before planting displayed a significantly enhanced nodulation and clover growth under competitive conditions in the presence of a mixture of chemical signals in the soil. Under these conditions, the symbiotic activity and competitiveness of *R. leguminosarum* bv. *trifolii* test strain were not improved suggesting that mitogenic activity of LCOs was solely responsible for the observed effects (Maj et al. 2009). Similar effect was observed for *B. japonicum* and soybean, which produce determinate nodules. Commercially, the addition of Nod factors into inoculants of *B. japonicum* to promote nodulation and plant growth of soybean has been applied by Nitragin Inc (Mabood et al. 2008).

2.5 Competitiveness in Natural Populations and Its Effect on Legume Crops

From among the several desirable characteristics in rhizobial strains that can be used as biofertilizers, the most essential are: (a) the ability to form nodules and to fix nitrogen in the host plant in a range of environmental conditions, and (b) the

ability to compete for nodulation with indigenous rhizobial population (Brockwell et al. 1995). The soil is an exceedingly complex habitat inhabited by heterogenous microbial communities, which interact with each other *via* different chemical compounds secreted into the soil. Among these microbial populations, rhizobia constitute "common soil inhabitants" in all climatic zones, from arctic to the tropics, and are commonly found in different types of soils (Robertson et al. 1995; Andrade et al. 2002; Fagerli and Svenning 2005). The correlation between the type of soils and the quantity of rhizobia have shown that the number of *R. leguminosarum*, *Sinorhizobium*, or *Bradyrhizobium* in most cases was 10^2–10^5 cells/g soil (Andrade et al. 2002; Martyniuk et al. 2005). The extensive variability in the soil environment may induce the emergence of diversity in rhizobial populations. Moreover, rhizobial diversity may be influenced by soil properties, such as the availability of N, P, Ca, the acidity, chemical stresses, or by agricultural management regimes (Palmer and Young 2000; Andrade et al. 2002; Laguerre et al. 2006). However, most of the rhizobial biodiversity studies were done with strains collected from a single or a few neighboring geographical regions. They revealed differences in allele frequency of selected genes (e.g., 16S rRNA, *nodD*, *nodEF*, *nifDK*) and sequences (16–23S rRNA ITS), which go in parallel with metabolic differences, for example the enzymatic profile or carbon/energy substrate utilization (Louvrier et al. 1996; Mutch and Young 2004; Silva et al. 2007). Further analysis of these traits allowed clustering the strains and finding correlation between genetic content and origin of the strains. Still, there is evidence that large scale biodiversity also exist within populations of rhizobia colonizing single plants (Wielbo et al. 2010).

Unlike soil, root nodules of legumes form a microenvironment not accessible to all rhizobia inhabiting rhizosphere probably because of the following: (1) the relatively high plant host-microsymbiont specificity as the nodules can be induced and consequently colonized only by rhizobia, which recognize and exchange suitable molecular signals with the host; and (2) variation in secreted Nod factors (Perret et al. 2000; Jones et al. 2007). Moreover, such rhizobia are not exposed to plant defense mechanisms, which are activated if bacteria are not recognized as symbionts, for example as a result of changes in lipopolysaccharide or exopolysaccharide structure (Campbell et al. 2002; Wielbo et al. 2004b). Rhizobia recognized as "suitable microsymbionts" are subjected to a selection process during plant tissue invasion and colonization in two ways – they are exposed to intense competition from other strains, and they are under some selective pressure from the plant host.

The effect of the plant host on the structure and composition of rhizobial population is not conclusive, but there are reports that legumes favor particular symbiotic genotypes of rhizobia (Mutch and Young 2004; Rangin et al. 2008). This relationship seems to be more complex, and even slight differences in genotype or developmental stage of plants may have an influence on the constitution of rhizobial populations in root nodules (Depret and Laguerre 2008). The molecular adjustment of the microsymbionts to their host may be observed as prevalence of particular symbiotic genotypes (versions of *nod* genes cluster) in rhizobia isolated from root nodules (Laguerre et al. 2003) or dependence between the susceptibility

of rhizobia for plant-derived flavonoid induction and competitive abilities of microsymbionts (Maj et al. 2010). For this reason, hypotheses about coevolution in *Rhizobium*–legume symbiosis have been proposed (Doyle 1998) and recently modified, emphasizing the effect of plants on the evolution of bacteria (Martínez-Romero 2009).

Independently of the plant host selection pressure on rhizobial populations, a lot of bacterial traits are involved in the process of competition between the individual strains, which run a race to the nodules (Vlassak and Vanderleyden 1997). The "external" environmental conditions, such as soil acidity, salinity, and nutrient availability strongly affect the vegetative growth of rhizobia in soil, thus setting up the initial conditions for rhizobial competition. A lot of data are available concerning the effect of single, defined factors such as acid tolerance (Vinuesa et al. 2003), presence of small cryptic plasmid (Bromfield et al. 1985), production of vitamins (Streit et al. 1996), and rhizopines (Murphy et al. 1987) affecting the competitive properties of rhizobia. The ability for utilization of specific C and energy sources, such as rhamnose (Oresnik et al. 1999) and homoserine (Hynes and O'Connel 1990), as well as the ability to metabolize the most variable set of substrates (including acids and amino acids) (Wielbo et al. 2007) have been proved to be important determinants of competitiveness. Moreover, the direct strain-to-strain antagonistic effect should also be taken into consideration as the effect of bacteriocin production on strain competitiveness was also reported (Robleto et al. 1998; Oresnik et al. 1999).

The competition between rhizobial strains does not vanish following the relocation of the bacteria from the soil into plant and remain present after the root colonization stage, possibly inside the infection threads (Duodu et al. 2009). Because rhizobia are immobile in the infection thread, the rate of bacterial growth inside can determine the rate of infection thread proliferation and subsequent nodule occupancy (Hoang et al. 2008; Duodu et al. 2009). Presence of multiple bacterial strains inside a single infection thread was shown by Stuurman et al. (2000) and Gage (2004). The C and N exchange between bacteroids and plant cells suggests that metabolic traits of rhizobia may also be important during this endophytic part of bacterial life cycle (Prell and Poole 2006; White et al. 2007). From "bacterial point of view," the aim of competition is to reach nodule compartments, which later on serve as a place for growth and massive multiplication. In indeterminate nodules, such a compartment is called saprophytic zone and is an ecological niche where rhizobia take advantage of the interaction with their plant host, escape plant controls, and their morphology, and some of the physiological traits become similar to these characteristic for a saprophytic (nonsymbiotic) stage (Timmers et al. 2000; Wielbo et al. 2009). In summary, the better the competitive abilities of a strain relative to the autochthonous strains of a local population, the more are the chances for colonization and multiplication in the nodules. And consequently, for the "return to the soil" after plant's vegetative period, albeit in higher number, which may lead to a dominance in the population. The success of a strain is also dependent on the diversity and total viable cell number of the local population. In rhizobia-rich soils, an introduced strain may be quickly dissipated into the

autochthonic population, and its "half-life" may not exceed 1–2 years (Jensen and Sorensen 1987). On the other hand, when the soil is depleted for rhizobia, the persistence of the introduced strain may exceed a few years, and the strain's good competitive abilities may enable a progressive elimination of rivals (Svenning et al. 2001).

One of the strategies that have been postulated a century ago and that has been a common practice for years to enhance legume nodulation and N_2 fixation is the introduction of rhizobial inoculants into the cultivated soil (Martínez-Romero 2003). Inoculation has enhanced plant growth and yield in the cases where even the specific rhizobia were absent or inefficient (Streeter 1994; Brockwell et al. 1995; Giller and Cadisch 1995). On the other hand, strain effectiveness and competitiveness are traits not linked to each other, and bacteria introduced as biofertilizer for target host plants might be outcompeted by autochthonous rhizobia abundant in the fields with little end success (Vlassak and Vanderleyden 1997; Burgos et al. 1999). Therefore, there is a need to investigate the strain competitiveness for nodulation as part of the process of converting a "potentially useful strain" into a "commercial inoculant strain". As discussed earlier, numerous individual traits affecting the competitiveness were identified and some recommendations for improving strain competitiveness have been made (Maier and Triplett 1996), and even genetically engineered strains with competitive abilities targeted in a specific manner were constructed (van Dillewijn et al. 2001). Moreover, promising mathematical models describing nodulation competitiveness were formulated (Beattie et al. 1989). On the other hand, original optimistic attempt to identify and clone the "nodulation competitiveness genes" had to be rejected, with the idea of "spontaneous genetic changes," which render the strains competitive (Beattie and Handelsman 1993) gaining prominence. The advances in understanding about individual metabolic traits responsible for competitiveness (Murphy et al. 1987; Hynes and O'Connel 1990; Streit et al. 1996; Robleto et al. 1998; Oresnik et al. 1999) has replaced the previous simple or very inaccurate theories, and shed some light on the network of factors involved. In this context, the role of point mutations underlying diversity and thus increasing the adaptive potentials of microbial species was confirmed (de Weert et al. 2004), resulting in inclusion of this phenomenon in the list of factors affecting competitiveness. It is possible that such small-scale evolution and/or recently discovered factors may explain some unsolved problems, for example, why strains selected for a particular trait (e.g., acid tolerant) and competitive under laboratory conditions are outnumbered in the "appropriate" (i.e., low pH) soils by indigenous strains (Gemel and Roughley 1993). The practical consequence of such issue requires a continuous search for identifying effective and competitive strains. Nowadays, more efforts are made in countries with weak agricultural practice (Africa or Southerneastern Asia) or with poor fertility of soils (Australia) (Lupwayi et al. 1997; Slattery and Pearce 2002), while in North America and European Union, such work is still conducted despite the availability of numerous patented and industrially-made rhizobial inoculants. They are often coupled with the investigation of problems relating to agricultural use of the inoculants: bacterial carriers, terms of storage, time and method of application,

etc. (Brockwell and Bottomley 1995; Herridge et al. 2002), which may strongly affect the viability and metabolic status of bacteria, and thus exert considerable effect on inoculant competitiveness and legume improvement.

2.6 Conclusions

Research to date has shown that the productivity of *Rhizobium*–legume symbiosis can be enhanced by manipulating bacterial and plant signals, selecting well-adapted bacteria, so that they could be introduced into soil or by modifying plant and bacterial activities. However, the complexity of competitiveness of rhizobia and plethora of factors influencing this phenomenon, which allow them to multiply and out-compete autochthonous rhizobia, still remains to be elucidated. The advanced global analyses of cellular state through approaches such as genomics, transcriptomics, and metabolomics have however made it possible to investigate whole microbial communities, which in turn, has enhanced further the knowledge of diversity on the genetic and metabolic levels. These are likely to result in better formulation of the inoculants applied as biofertilizers in legume production across different ecological regions. All strategies that positively influence legume biomass and global fixed N are important for sustainable agriculture especially when soils lack specific rhizobia or when their number is low. Nowadays, despite a myriad of "traditional" rhizobial inoculants available in the market, new-formulated biofertilizers supplemented with flavonoids or Nod factors needs to be designed and developed and further tested for the promotion of legumes productivity.

Acknowledgments The research reported in this study was funded in part by a grant of Ministry of Sciences and Higher Education nr N N304 026734. The authors are grateful to Joanna Golus, Jolanta Kutkowska, and Jan Kuske for kindly providing the photographs presented in Fig. 2.1.

References

Andrade DS, Murphy PJ, Giller KJ (2002) The diversity of *Phaseolus*-nodulating rhizobial populations is altered by liming of acid soils planted with *Phaseolus vulgaris* L. in Brazil. Appl Environ Microbiol 68:4025–4034

Bailly X, Oliveri I, Brunel B, Cleyet-Marel JC, Bena G (2007) Host-specialization and speciation in the symbiotic nitrogen-fixing *Sinorhizobium* associated with *Medicago*. J Bacteriol 189:5223–5236

Barnett MJ, Fisher RF (2006) Global gene expression in the rhizobial-legume symbiosis. Symbiosis 42:1–24

Beattie GA, Clayton MK, Handelsman J (1989) Quantitative comparison of the laboratory and field competitiveness of *Rhizobium leguminosarum* biovar *phaseoli*. Appl Environ Microbiol 55:2755–2761

Beattie GA, Handelsman J (1993) Evaluation of a strategy for identifying nodulation competitiveness genes in *Rhizobium leguminosarum* biovar *phaseoli*. J Gen Microbiol 139:529–538

Becker A, Fraysse N, Sharypova L (2005) Recent advances in studies on structure and symbiosis-related function of rhizobial K-antigens and lipopolysaccharides. Mol Plant Microbe Interact 18:899–905

Begum AA, Leibovitch S, Migner P, Zhang F (2001) Specific flavonoids induced *nod* gene expression and pre-activated *nod* genes of *Rhizobium leguminosarum* increased pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.) nodulation in controlled growth chamber environments. J Exp Bot 152:1537–1543

Bertin C, Yang X, Weston LA (2003) The role of root exudates and allelochemicals in the rhizosphere. Plant Soil 256:67–83

Brencic A, Winans SC (2005) Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. Microbiol Mol Biol Rev 1:155–194

Brewin NJ (2004) Plant cell wall remodeling in the *Rhizobium*-legume symbiosis. Crit Rev Plant Sci 23:293–316

Brockwell J, Bottomley PJ (1995) Recent advances in inoculant technology and prospects for the future. Soil Biol Biochem 27:683–697

Brockwell J, Bottomley PJ, Thies JE (1995) Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment. Plant Soil 174:143–180

Brom S, de los Santos AG, de Lourdes Girard M, Davilla G, Palacios R, Romero D (1991) High-frequency rearrangements in *Rhizobium leguminosarum* bv. *phaseoli* plasmids. J Bacteriol 173:1344–1346

Brom S, de los Santos AG, Stepkowsky T, Flores M, Davilla G, Romero D, Palacios R (1992) Different plasmids of *Rhizobium leguminosarum* bv. *phaseoli* are required for optimal symbiotic performance. J Bacteriol 174:5183–5189

Bromfield ESP, Lewis DM, Barran LR (1985) Cryptic plasmid and rifampin resistance in *Rhizobium meliloti* influencing nodulation competitiveness. J Bacteriol 164:410–413

Broughton WJ, Samrey U, Stanley J (1987) Ecological genetics of *Rhizobium meliloti*: symbiotic plasmid transfer in the *Medicago sativa* rhizosphere. FEMS Microbiol Lett 40:251–255

Burgos PA, Castellanos J, Mora Y, Mora J (1999) Field inoculation of common bean (*Phaseolus vulgaris* L.) with high efficiency *Rhizobium* strains. In: Martínez E, Hernández G (eds) Highlights of nitrogen fixation research. Kluwer Academic/Plenum Publishers, New York, pp 255–257

Caetano-Anollés G, Crist-Estes DK, Bauer WD (1988) Chemotaxis of *Rhizobium meliloti* on the plant flavone luteolin requires functional nodulation genes. J Bacteriol 170:3164–3169

Campbell GRO, Reuhs BL, Walker GC (2002) Chronic intracellular infection of alfalfa nodules by *Sinorhizobium meliloti* requires correct lipopolysaccharide core. Proc Natl Acad Sci USA 99:3938–3943

Cardenas L, Dominguez J, Qiunto C, Lopez-Lara IM, Lugtenberg BJJ, Spaink HP, Rademarker GJ, Haverkamp J, Thomas-Oates JE (1995) Isolation, chemical structures and biological activity of lipo-chitin oligosaccharide nodulation signals from *Rhizobium etli*. Plant Mol Biol 29:453–464

Castillo M, Flores M, Mavingui P, Martinez-Romero E, Palacios R, Hernandez G (1999) Increase in alfafa nodulation, nitrogen fixation, and plant growth by specific DNA amplification in *Sinorhizobium meliloti*. Appl Environ Microbiol 65:2716–2722

Chen H-C, Feng J, Hou B-H, Li F-Q, Li Q, Hong G-F (2005) Modulating DNA bending affects NodD-mediated transcriptional control in *Rhizobium leguminosarum*. Nucleic Acid Res 33:2540–2548

Chen WM, Laevens S, Lee TM, Coenye T, de Vos P, Mergeay M, Vandamme P (2001) *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. Int J Syst Evol Microbiol 51:1729–1735

Cooper JE (2004) Multiple responses of rhizobia to flavonoids during legume root infection. Adv Bot Res 41:1–62

Cooper JE (2007) Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. J Appl Microbiol 103:1355–1365

Cren M, Kondorosi A, Kondorosi E (1995) NolR controls expression of the *Rhizobium meliloti* nodulation genes involved in the core Nod factor synthesis. Mol Microbiol 15:733–747

D'Haeze W, Holsters M (2002) Nod factor structures, responses and perception during initiation of nodule development. Glycobiol 12:79–105

De Jong AJ, Heidstra R, Spaink HP, Hartog MV, Meijer EA, Hendriks T, Schiavo FL, Terzi M, Bisseling T, Van Kammen A, De Vries SC (1993) *Rhizobium* lipooligosaccharides rescue a carrot somatic embryo mutant. Plant Cell 5:615–620

De Weert S, Dekkers LC, Kuiper I, Bolemberg GV, Lugtenberg BJJ (2004) Generation of enhanced competitive root-tip-colonizing *Pseudomonas* bacteria through accelerated evolution. J Bacteriol 186:3153–3159

Deavours BE, Liu CJ, Naoumkina MA, Tang YH, Farag MA, Sumner LW, Noel JP, Dixon RA (2006) Functional analysis of members of the isoflavone and isoflavanone O-methyltransferase enzyme families from the model legume *Medicago truncatula*. Plant Mol Biol 62: 715–733

Debellé F, Rosenberg C, Vasse J, Maillet F, Martinez E, Dénarié J, Truchet G (1986) Assignment of symbiotic developmental phenotypes to common and specific nodulation (*nod*) genetic loci of *Rhizobium meliloti*. J Bacteriol 168:1075–1086

Dénarié J, Cullimore J (1993) Lipo-oligosaccharide nodulation factors: New class of signaling molecules mediating recognition and morphogenesis. Cell 74:951–954

Dénarié J, Debellé F, Promé JC (1996) *Rhizobium* lipo-oligosaccharide nodulation factors: signalling molecules mediating recognition and morphogenesis. Annu Rev Biochem 65:503–535

Depret G, Laguerre G (2008) Plant phenology and genetic variability in root and nodule development strongly influence genetic structuring of *Rhizobium leguminosarum* biovar *viciae* populations nodulating pea. New Phytol 179:224–235

Dharmatilake AJ, Bauer WD (1992) Chemotaxis of *Rhizobium meliloti* towards nodulation gene-inducing compounds from alfalfa roots. Appl Environ Microbiol 58:1153–1158

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085–1097

Dombrecht B, Tesfay MZ, Verreth C, Heusdens C, Napoles MC, Vanderleyden J, Michiels J (2002) The *Rhizobium etli* gene *iscN* is highly expressed in bacteroids and required for nitrogen fixation. Mol Gen Genomics 267:820–828

Doyle JJ (1998) Phylogenetic perspectives of nodulation: evolving views of plants and symbiotic bacteria. Trends Plant Sci 3:473–478

Duodu S, Brophy C, Connolly J, Svenning MM (2009) Competitiveness of native *Rhizobium leguminosarum* bv. *trifolii* strain for nodule occupancy is manifested during infection. Plant Soil 318:117–126

Dyachok JV, Tobin AE, Price NPJ, von Arnold S (2000) Rhizobial Nod factors stimulate somatic embryo development in *Picea abies*. Plant Cell Rep 19:290–297

Fagerli IL, Svenning MM (2005) Arctic and subarctic soil populations of *Rhizobium leguminosarum* biovar *trifolii* nodulating three different clover species: characterization by diversity of chromosomal and symbiosis loci. Plant Soil 275:371–381

Faure D, Vereecke D, Leveau JHJ (2009) Molecular communication in rhizosphere. Plant Soil 321:279–303

Fedorova M, van de Mortel J, Matsumoto PA, Cho J, Town CD, VandenBosch KA, Gantt JS, Vance CP (2002) Genome-wide identification of nodule-specific transcripts in the model legume *Medicago truncatula*. Plant Physiol 130:519–537

Feng J, Li Q, Hu HL, Chen XC, Hong GF (2003) Inactivation of the *nod* box distal half-site allows tetrameric NodD to activate *nodA* transcription in an inducer-independent manner. Nucleic Acids Res 31:3143–3156

Firmin JL, Wilson KE, Rossen L, Johnston AWB (1986) Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. Nature 324:90–92

Fisher HM (1994) Genetic regulation of nitrogen fixation in rhizobia. Microbiol Rev 58:352–386

Fisher RF, Egelhoff TT, Mulligan JT, Long SR (1988) Specific binding of *Rhizobium meliloti* extracts containing *nodD* to DNA sequences upstream of inducible nodulation genes. Genes Dev 2:282–293

Franche C, Lindström K, Elmerich C (2009) Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant Soil 321:35–59

Fraysse N, Couderc F, Poinsot V (2003) Surface polysaccharide involvement in establishing the *rhizobium*-legume symbiosis. Eur J Biochem 270:1365–1380

Frugier F, Kosuta S, Murray JD, Crespi M, Szczygłowski K (2008) Cytokinin: secret agent of symbiosis. Trends Plant Sci 13:115–120

Fujishige NA, Lum MR, de Hoff PL, Whitelegge JP, Faull KF, Hirsch AM (2008) *Rhizobium* common *nod* genes are required for biofilm formation. Mol Microbiol 67:504–515

Gage DJ (2004) Infection and invasion of roots by symbiotic nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiol Mol Biol Rev 68:280–300

Gagnon H, Ibrahim RK (1998) Aldonic acids: a novel family of *nod* gene inducers of *Mesorhizobium loti*, *Rhizobium lupini*, and *Sinorhizobium meliloti*. Mol Plant-Microbe Interact 11:988–998

Galibert F, Finan TM, Long SR, Pühler A, Abola P, Ampe F, Barloy-Hubler F, Barnett MJ, Becker A, Boistard P, Bothe G, Boutry M, Bowser L, Buhrmester J, Cadieu E, Capela D, Chain P, Cowie A, Davis RW, Dréano S, Federspiel NA, Fisher RF, Gloux S, Godrie T, Goffeau A, Golding B, Gouzy J, Gurjal M, Hernandez-Lucas I, Hong A, Huizar L, Hyman RW, Jones T, Kahn D, Kahn ML, Kalman S, Keating DH, Kiss E, Komp C, Lelaure V, Masuy D, Palm C, Peck MC, Pohl TM, Portetelle D, Purnelle B, Ramsperger U, Surzycki R, Thébault P, Vandenbol M, Vorhölter FJ, Weidner S, Wells DH, Wong K, Yeh KC, Batut J (2001) The composite genome of the legume symbiont *Sinorhizobium meliloti*. Science 293:668–672

Gaworzewska ET, Carlile MJ (1982) Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. J Gen Microbiol 128:1179–1188

Gemel LG, Roughley RJ (1993) Field evaluation in acid soils of strains of *Rhizobium leguminosarum* bv. *trifolii* selected for their tolerance or sensitivity to acid soil factors in agar medium. Soil Biol Biochem 25:1447–1452

Geurts R, Fedorova E, Bisseling T (2005) Nod factor signaling genes and their function in the early stages of *Rhizobium* infection. Curr Opin Plant Biol 8:346–352

Gibson KE, Kobayashi H, Walker GC (2008) Molecular determinants of a symbiotic chronic infection. Annu Rev Genet 42:413–441

Giller KE, Cadisch G (1995) Future benefits from biological nitrogen fixation: an ecological approach to agriculture. Plant Soil 174:255–277

Giraud E, Moulin L, Vallenet D, Barbe V, Cytryn E, Avarre JC, Jaubert M, Simon D, Cartieaux F, Prin Y, Bena G, Hannibal L, Fardoux J, Kojadinovic M, Vuillet L, Lajus A, Cruveiller S, Rouy Z, Mangenot S, Segurens B, Dossat C, Franck WL, Chang WS, Saunders E, Bruce D, Richardson P, Normand P, Dreyfus B, Pignol D, Stacey G, Emerich D, Vermeglio A, Medigue C, Sadovsky M (2007) Legume symbioses: absence of *nod* genes in photosynthetic bradyrhizobia. Science 316:1307–1312

Goldmann A, Boivin C, Fleury V, Message B, Lecoeur L, Maille M, Tepfer D (1991) Betaine use by rhizosphere bacteria: genes essential for trigonelline, stachydrine and carnitine catabolism in *Rhizobium meliloti* are located on pSym in the symbiotic region. Mol Plant-Microbe Interact 4:571–578

Gonzalez V, Santamaria RI, Bustos P, Hernández-González I, Medrano-Soto A, Moreno-Hagelsieb G, Janga SC, Ramírez MA, Jiménez-Jacinto V, Collado-Vides J, Dávila G (2006) The partitioned *Rhizobium etli* genome: genetic and metabolic redundancy in seven interacting replicons. Proc Natl Acad Sci USA 103:3834–3839

Gőttfert M, Grob P, Hennecke H (1990) Proposed regulatory pathway encoded by the *nodV* and *nodW* genes, determinants of host specificity in *Bradyrhizobium japonicum*. Proc Natl Acad Sci USA 87:2680–2684

Graham PH, Vance CP (2003) Legumes: importance and constraints to greater utilization. Plant Physiol 131:872–877

Guo X, Flores M, Mavingui P, Fuentes SI, Hernandez G, Davila G, Palacios R (2003) Natural genomics design in *Sinorhizobium meliloti*: novel genomic architectures. Genome Res 8:1810–1817

Gutjahr C, Paszkowski U (2009) Weights in the balance: jasmonic acid and salicylic acid signaling in root-biotroph interactions. Mol Plant-Microbe Interact 22:763–772

Harborne JB, Williams CA (2000) Advances in flavonoid research since 1992. Phytochemistry 55:481–504

Harborne JB, Williams CA (2001) Anthocyanins and other flavonoids. Nat Prod Rep 18: 310–333

Hartwig UA, Maxwell CA, Joseph CM, Phillips DA (1990) Effects of alfalfa *nod* gene-inducing flavonoids on *nodABC* transcription in *Rhizobium meliloti* strains containing different *nodD* genes. J Bacteriol 172:2769–2773

Heidstra R, Bisseling T (1996) Nod factor-induced host responses and mechanisms of Nod factor perception. New Phytol 133:25–43

Herridge D, Gemell G, Hartley E (2002) Legume inoculants and quality control. In: Herridge D (ed) Inoculants and nitrogen fixation of legumes in Vietnam, ACIAR Proceedings 109e. PK Editorial Service, Australia, Brisbane

Herridge DF, Peoples MB, Boddey RM (2008) Global inputs of biological nitrogen fixation in agricultural systems. Plant Soil 311:1–18

Hoang HH, Gurich N, Gonzalez JE (2008) Regulation of motility by the ExpR/Sin quorum-sensing system in *Sinorhizobium meliloti*. J Bacteriol 190:861–871

Hogg B, Davies AE, Wilson KE, Bisseling T, Downie JA (2002) Competitive nodulation blocking of cv. Afghanistan pea is related to high level of nodulation factors made by some strains of *Rhizobium leguminosarum* bv. *viciae*. Mol Plant-Microbe Interact 15:60–68

Hong GF, Burn JE, Johnston AWB (1987) Evidence that DNA involved in the expression of nodulation (*nod*) genes in *Rhizobium* binds to the product of the regulatory gene *nodD*. Nucleic Acids Res 15:9677–9690

Horvath B, Kondorosi E, John M, Schmidt J, Török I, Györgypal Z, Barabas I, Wieneke U, Schell J, Kondorosi A (1986) Organization, structure and symbiotic function of *Rhizobium meliloti* nodulation genes determining host specificity for alfalfa. Cell 46:335–343

Hotter GS, Scott DB (1991) Exopolysaccharide mutants of *Rhizobium loti* are fully effective on a determinate nodulating host but are ineffective on an indeterminate nodulating host. J Bacteriol 173:851–859

Hungria M, Phillips DA (1993) Effects of a seed color mutation on rhizobial *nod*-gene-inducing flavonoids and nodulation in common bean. Mol Plant-Microbe Interact 6:418–422

Hungria M, Stacey G (1997) Molecular signals exchanged between host plants and rhizobia: basic aspects and potential application in agriculture. Soil Biol Biochem 29:819–830

Hynes MF, O'Connel MP (1990) Host plant effect on competition among strains of *Rhizobium leguminosarum*. Can J Microbiol 36:864–869

Jacobs TW, Egelhoff TT, Long SR (1985) Physical and genetic map of a *Rhizobium meliloti* nodulation gene region and nucleotide sequence of *nodC*. J Bacteriol 162:469–476

Jaeger CH, Lindow SE, Miller W, Clark E, Firestone MK (1999) Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. Appl Environ Microbiol 65:2685–2690

Jensen ES, Sorensen LH (1987) Survival of *Rhizobium leguminosarum* is soil after addition as inoculant. FEMS Microbiol Ecol 45:221–226

Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC (2007) How symbionts invade plants: the *Sinorhizobium-Medicago* model. Nat Rev Microbiol 5:619–633

Kaminski PA, Batut J, Boistard P (1998) A survey of symbiotic nitrogen fixation by rhizobia. In: Spaink HP, Kondorosi A, Hooykaas PJ (eds) The *Rhizobiaceae*. Molecular biology of model plant-associated bacteria. Kluver Academic Publisher, Dordrecht, The Netherland, pp 426–490

Kannenberg EL, Perzl M, Hartner T (1995) The occurrence of hopanoid lipids in *Bradyrhizobium* bacteria. FEMS Microbiol Lett 127:255–262

Kapulnik Y, Joseph CM, Phillips DA (1987) Flavone limitation to root nodulation and symbiotic nitrogen fixation in alfalfa. Plant Physiol 84:1193–1196

Knee EM, Gong FC, Gao M, Teplitski M, Jones AM, Foxworthy A, Mort AJ, Bauer WD (2001) Root mucilage from pea and its utilization by rhizosphere bacteria as a sole carbon source. Mol plant-Microbe Interact 14:775–784

Knight CD, Rossen L, Robertson JG, Wells B, Downie JA (1986) Nodulation inhibition by *Rhizobium leguminosarum* multicopy *nodABC* genes and analysis of early stages of plant infection. J Bacteriol 166:552–558

Kobayashi H, Naciri-Graven Y, Broughton WJ, Perret X (2004) Flavonoids induce temporal shifts in gene-expression of *nod*-box controlled loci in *Rhizobium* sp. NGR234. Mol Microbiol 51:335–347

Kondorosi E, Gyuris J, Schmidt J, John E, Hofmann DB, Schell J, Kondorosi A (1989) Positive and negative control of *nod* gene expression in *Rhizobium meliloti* is reqiured for optimal nodulation. EMBO J 8:1331–1340

Kondorosi E, Pierre M, Cren M, Haumann U, Buire M, Hoffmann B, Schell J, Kondorosi A (1991) Identification of NolR, a negative transacting factor controlling the *nod* regulon in *Rhizobium meliloti*. J Mol Biol 222:885–896

Kosslak RM, Bookland R, Berkei J, Paaren HE, Applebaum ER (1987) Induction of *Bradyrhizobium japonicum* common nod genes by isoflavones isolated from *Glycine* max. Proc Natl Acad Sci USA 84:7428–7432

Kosslak RM, Joshi RS, Bowen BA, Paaren HE, Applebaum ER (1990) Strain-specific inhibition of *nod* gene induction in *Bradyrhizobium japonicum* by flavonoid compounds. Appl Environ Microbiol 56:1333–1341

Król J, Mazur A, Marczak M, Skorupska A (2007) Syntenic arrangements of the surface polysaccharide biosynthesis genes in *Rhizobium leguminosarum*. Genomics 89:237–247

Laguerre G, Courde L, Nouaim R, Lamy I, Revellin C, Breuil MC, Chaussod R (2006) Response of rhizobial populations to moderate copper stress applied to an agricultural soil. Microbiol Ecol 52:426–435

Laguerre G, Louvrier P, Allard MR, Amarger N (2003) Compatibility of rhizobial genotypes within natural populations of *Rhizobium leguminosarum* biovar *viciae* for nodulating of host legumes. Appl Environ Microbiol 69:2276–2283

Leibovitch S, Migner P, Smith DL (2001) Evaluation of the effect of SoyaSignal technology on soybean yield [*Glycine max* (L.) Merr.] under field conditions over 6 years in Eastern Canada and the Northern United States. J Agron Crop Sci 187:281–292

Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Promé JC, Dénarié J (1990) Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature 344:781–784

Lodwig E, Poole P (2003) Metabolism of *Rhizobium* bacteroids. CRC Crit Rev Plant Sci 22:37–78

Lohar DP, Schaff JE, Laskey G, Kieber JJ, Bilyeu KD, Bird DM (2004) Cytokinins play opposite roles in lateral root formation, and nematode and rhizobial symbioses. Plant J 38: 203–214

Louvrier P, Laguerre G, Amarger N (1996) Distribution of symbiotic genotypes in *Rhizobium leguminosarum* biovar *viciae* populations isolated directly from soils. Appl Environ Microbiol 62:4202–4205

Lupwayi NZ, Haque I, Holl FB (1997) Effectiveness, competitiveness and persistence of inoculant *Rhizobium* in perennial African clovers in a highland. Vertisol J Agric Sci 129:429–437

Mabood F, Jung WJ, Smith DL (2008) Signals in the underground: microbial signaling and plant productivity. In: Nautiyal CS, Dion PE, Chopra VL (eds) Molecular mechanisms of plant and microbe coexistence. Springer-Verlag, Berlin, Heidelberg, pp 291–318

Mabood F, Smith DL (2005) Pre-incubation of *Bradyrhizobium japonicum* with jasmonates accelerates nodulation and nitrogen fixation in soybean (*Glycine max*) at optimal and suboptimal root zone temperatures. Physiol Plant 125:311–323

Mabood F, Souleimanov A, Khan W, Smith DL (2006) Jasmonates induce Nod factor production by *Bradyrhizobium japonicum*. Plant Physiol Biochem 44:759–765

Macchiavelli RE, Brelles-Mariño G (2004) Nod factor-treated *Medicago truncatula* roots and seeds show an increased number of nodules when inoculated with a limiting population of *Sinorhizobium meliloti*. J Exp Bot 55:2635–2640

Maier RJ, Triplett EW (1996) Toward more productive, efficient, and competitive nitrogen-fixing symbiotic bacteria. Crit Rev Plant Sci 15:191–234

Maj D, Wielbo J, Marek-Kozaczuk M, Skorupska A (2009) Pretreatment of clover seeds with Nod factors improves growth and nodulation of *Trifolium pratense*. J Chem Ecol 35:479–487

Maj D, Wielbo J, Marek-Kozaczuk M, Skorupska A (2010) Response to flavonoids as a factor influencing competitiveness and symbiotic activity of *Rhizobium leguminosarum*. Microbiol Res 165:50–60

Martínez-Abarca F, Herrera-Cervera JA, Bueno P, Sanjuan J, Bisseling T, Olivares J (1998) Involvement of salicylic acid in the establishment of the *Rhizobium meliloti*-alfalfa symbiosis. Mol Plant Microbe Interact 11:153–155

Martínez-Romero E (2003) Diversity of *Rhizobium-Phaseolus vulgaris* symbiosis: overview and perspectives. Plant Soil 252:11–23

Martínez-Romero E (2009) Coevolution in *Rhizobium*-legume symbiosis? DNA Cell Biol 28:361–370

Martyniuk S, Oroń J, Martyniuk M (2005) Diversity and numbers of root-nodule bacteria (rhizobia) in Polish soils. Acta Soc Bot Polon 74:83–86

Masson-Boivin C, Giraud E, Perret X, Batut J (2009) Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? Trends Microbiol 17:458–466

Mathesius U, Schlaman HR, Spaink HP, Sautter C, Rolfe BG, Djordjevic MA (1998) Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. Plant J 14:23–34

Mercado-Blanco J, Toro N (1996) Plasmids in rhizobia: the role of nonsymbiotic plasmids. Mol Plant-Microbe Interact 9:535–545

Mergaert P, Uchiumi T, Alunni B, Evanno G, Cheron A, Catrice O, Mausset AE, Barloy-Hubler F, Galibert F, Kondorosi A, Kondorosi E (2006) Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium*-legume symbiosis. Proc Natl Acad Sci USA 103:5230–5235

Mimmack ML, Hong GF, Johnston AWB (1994) Sequence and regulation of *psrA*, a gene on the Sym plasmid of *Rhizobium leguminosarum* biovar *phaseoli* which inhibits transcription of the *psi* genes. Microbiology 140:455–461

Minic Z, Brown S, De Kouchkovsky Y, Schultze M, Staehelin C (1998) Purification and characterization of a novel chitinase-lysozyme, of another chitinase, both hydrolysing *Rhizobium meliloti* Nod factors, and of a pathogenesis-related protein from *Medicago sativa* roots. Biochem J 332:329–335

Mitra RM, Long SR (2004) Plant and bacterial symbiotic mutants define three transcriptionally distinct stages in the development of the *Medicago truncatula*/*Sinorhizobium meliloti* symbiosis. Plant Physiol 134:595–604

Miwa H, Sun J, Oldroyd G, Downie JA (2006) Analysis of calcium spiking using a cameleon calcium sensor reveals that nodulation gene expression is regulated by calcium spike number and the developmental status of the cell. Plant J 48:883–894

Moulin L, Munive A, Dreyfus B, Boivin-Masson C (2001) Nodulation of legumes by members of the beta-subclass of proteobacteria. Nature 411:948–950

Murphy PJ, Heycke N, Banfalvi Z, Tate ME, de Brujin F, Kondorosi A, Tempe J, Schell J (1987) Genes for the catabolism and synthesis of an opine-like compound in *Rhizobium meliloti* are closely linked on the Sym plasmid. Proc Natl Acad Sci USA 84:493–497

Mutch LA, Young JPW (2004) Diversity and specifity of *Rhizobium leguminosarum* biovar *viciae* on wild and cultivated legumes. Mol Ecol 13:2435–2444

Newton WE (2007) Physiology, biochemistry and molecular biology of nitrogen fixation. In: Ferguson BH, SJ NWE (eds) Biology of nitrogen cycle. Elsevier, Amsterdam, pp 109–130

Oldroyd GED, Downie JA (2004) Calcium, kinases and nodulation signalling in legumes. Nat Rev Mol Cell Biol 5:566–576

Oldroyd GED, Downie JA (2006) Nuclear calcium changes at the core of symbiosis signalling. Curr Opin Plant Biol 9:351–357

Oresnik IJ, Twelker S, Hynes MF (1999) Cloning and characterization of a *Rhizobium leguminosarum* gene encoding a bacteriocin with similarities to RTX toxins. Appl Environ Microbiol 65:2833–2840

Ovtsyna AO, Schultze M, Tikhonovich IA, Spaink HP, Kondorosi E, Kondorosi A, Staehelin C (2000) Nod factors of *Rhizobium leguminosarum* bv. *viciae* and their fucosylated derivatives stimulate a nod factor cleaving activity in pea roots and are hydrolyzed in vitro by plant chitinases at different rates. Mol Plant Microbe Interact 13:799–807

Palacios R, Newton WE (2005) Genomes and genomics of nitrogen-fixing organisms. Springer, Dordrecht

Palmer KM, Young JPW (2000) Higher diversity of *Rhizobium leguminosarum* biovar *viciae* populations in arable soils than in grass soils. Appl Environ Microbiol 66:2445–2450

Patriarca EJ, Tate R, Iaccarino M (2002) Key role of bacterial NH_4^+ metabolism in *Rhizobium*-plant symbiosis. Microbiol Mol Biol Rev 66:203–222

Peck MC, Fisher RF, Long SR (2006) Diverse flavonoids stimulate NodD1 binding to *nod* gene promoters in *Sinorhizobium meliloti*. J Bacteriol 188:5417–5427

Peoples MB, Herridge DF, Ladha JK (1995a) Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production? Plant Soil 174:3–28

Peoples MB, Ladha JK, Herridge DF (1995b) Enhancing legume N_2 fixation through plant and soil management. Plant Soil 174:83–101

Perret X, Staehelin C, Broughton WJ (2000) Molecular basis of symbiotic promiscuity. Microbiol Mol Biol Rev 64:180–201

Peters NK, Frost JW, Long SR (1986) A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science 233:977–980

Peters NK, Long SR (1988) Alfalfa root exudates and compounds which promote or inhibit induction of *Rhizobium meliloti* nodulation genes. Plant Physiol 88:396–400

Phillips DA, Fox TC, King MD, Bhuvaneswari TV, Teuber LR (2004) Microbial products trigger amino acid exudation from plant roots. Plant Physiol 136:2887–2894

Phillips DA, Joseph CM, Maxwell CA (1992) Trigonelline and stachydrine released from alfalfa seeds activate NodD2 protein in *Rhizobium meliloti*. Plant Physiol 99:1526–1531

Phillips DA, Kapulnik Y (1995) Plant isoflavonoids, pathogens and symbionts. Trends Microbiol 3:58–63

Poustini K, Mabood F, Smith DL (2007) Preincubation of *Rhizobium leguminosarum* bv. *phaseoli* with jasmonate and genistein signal molecules increases bean (*Phaseolus vulgaris* L.) nodulation, nitrogen fixation and biomass production. J Agric Sci Technol 9:107–117

Prell J, Poole P (2006) Metabolic changes of rhizobia in legume nodules. Trends Microbiol 14:161–168

Prithiviraj B, Zhou X, Souleimanov A, Kahn WM, Smith DL (2003) A host-specific bacteria-to-plant signal molecule (Nod factor) enhances germination and early growth of diverse crop plants. Planta 21:437–445

Pueppke SG, Broughton WJ (1999) *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host ranges. Mol Plant-Microbe Interact 12:293–318

Rangin C, Brunel B, Cleyet-Marel JC, Perrineau MM, Bena G (2008) Effect of *Medicago truncatula* genetic diversity, rhizobial competition and strain effectiveness on the diversity of a natural *Sinorhizobium* species community. Appl Environ Microbiol 74:5653–5661

Recourt K, Schripsema J, Kijne JW, van Brussel AAN, Lugtenberg BJJ (1991) Inoculation of *Vicia sativa* subsp. *nigra* roots with *Rhizobium leguminosarum* biovar *viciae* results in release of *nod* gene activating flavonones and chalcones. Plant Mol Biol 16:841–852

Rivas R, Velazquez E, Willems A, Vizcaino N, Subba-Rao NS, Mateos PF, Gillis M, Dazzo FB, Martínez-Molina E (2002) A new species of *Devosia* that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume *Neptunia natans* (L.f.) Druce. Appl Environ Microbiol 68:5217–5222

Robertson BK, Dreyfus B, Alexander M (1995) Ecology of stem-nodulating *Rhizobium* and *Azorhizobium* in four vegetation zones of Senegal. Microb Ecol 29:71–81

Robleto EA, Kmiecik K, Oplinger ES, Nienhuis J, Triplett EW (1998) Trifolitoxin production increases nodulation competitiveness of *Rhizobium etli* CE3 under agricultural conditions. Appl Environ Microbiol 64:2630–2633

Rosas S, Soria R, Correa N, Abdala G (1998) Jasmonic acid stimulates the expression of *nod* genes in *Rhizobium*. Plant Mol Biol 38:1161–1168

Rossbach S, Kulpa DA, Rossbach U, de Bruijn FJ (1994) Molecular and genetic characterization of the rhizopine catabolism (*mocABRC*) genes of *Rhizobium meliloti* L5-30. Mol Gen Genet 245:11–24

Ruvkun GB, Ausubel FM (1980) Interspecies homology of nitrogenase genes. Proc Natl Acad Sci USA 77:191–195

Sanjuan J, Grob P, Gőttfert M, Hennecke H, Stacey G (1994) *NodW* is essential for full expression of the common nodulation genes in *Bradyrhizobium japonicum*. Mol Plant-Microbe Interact 7:364–369

Schlaman HRM, Horvath B, Vijgenboom E, Okker RJH, Lugtenberg BJJ (1991) Suppression of nodulation gene expression in bacteroids of *Rhizobium leguminosarum* biovar *viciae*. J Bacteriol 173:4277–4287

Schlaman HRM, Phillips DA, Kondorosi E (1998) Genetic organization and transcriptional regulation of rhizobial nodulation genes. In: Spaink HP, Kondorosi A, Hooykaas PJJ (eds) The *Rhizobiaceae*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 361–368

Schmidt J, Röhrig H, John M, Wieneke U, Stacey G, Koncz C, Schell J (1993) Alteration of plant growth and development by *Rhizobium nodA* and *nodB* genes involved in the synthesis of oligosaccharide signal molecules. Plant J 4:651–658

Schwedock J, Long SR (1989) Nucleotide sequence and protein products of two new nodulation genes of *Rhizobium meliloti*, *nodP* and *nodQ*. Mol Plant-Microbe Interact 2:181–194

Sharma SB, Signer ER (1990) Temporal and spatial regulation of the symbiotic genes of *Rhizobium meliloti* in planta revealed by transposon Tn5-*gusA*. Genes Dev 4:344–356

Silva C, Kan FL, Martinez-Romero E (2007) Population genetic structure of *Sinorhizobium meliloti* and *S. medicae* isolated from nodules *Medicago* spp. in Mexico. FEMS Microbiol Ecol 60:477–489

Simms EL, Taylor DL (2002) Partner choice in nitrogen-fixation mutualisms of legume and rhizobia. Integ Comp Biol 42:369–380

Skorupska A, Janczarek M, Marczak M, Mazur A, Król J (2006) Rhizobial exopolysaccharides: genetic control and symbiotic functions. Microb Cell Fact 5(7):1–19

Slattery JF, Pearce DJ (2002) Development of elite inoculant strains of *Rhizobium* in southeastern. In: Herridge D (ed) Inoculants and nitrogen fixation of legumes in Vietnam, Proceedings of a workshop proceedings, ACIAR proceedings 109:86–94

Somers E, Vanderleyden J, Srinivasan M (2004) Rhizosphere bacterial signalling: a love parade beneath our feet. Critic Rev Microbiol 30:205–240

Souleimanov A, Prithiviraj B, Smith DL (2002) The major Nod factor of *Bradyrhizobium japonicum* promotes early growth of soybean and corn. J Exp Bot 53:1929–1934

Souza V, Nguyen TT, Hudson RR, Pinero D, Lenski RE (1992) Hierarchical analysis of linkage disequilibrium in *Rhizobium* populations: evidence for sex? Proc Natl Acad Sci USA 89:8389–8393

Spaink HP (2000) Root nodulation and infection factors produced by rhizobial bacteria. Annu Rev Microbiol 54:257–288

Spaink HP, Bloemberg GV, van Brussel AAN, Lugtenberg BJJ, van der Drift KMGM, Haverkamp J, Thomas-Oates JE (1995) Host specificity of *Rhizobium leguminosarum* is determined by the hydrophobicity of highly unsaturated fatty acyl moieties of the nodulation factors. Mol Plant-Microbe Interact 8:155–164

Spaink HP, Sheeley DM, van Brussel AAN, Glushka J, York WS, Tak T, Geiger O, Kennedy EP, Reinhold VN, Lugtenberg BJJ (1991) A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature 354:125–130

Stokkermans TJ, Ikeshita S, Cohn J, Carlson RW, Stacey G, Ogawa T, Peters NK (1995) Structural requirements of synthetic and natural product lipo-chitin oligosaccharides for induction of nodule primordial on *Glycine soja*. Plant Physiol 108:1587–1595

Streeter JG (1994) Failure of inoculant rhizobia to overcome the dominance of indigenous strains for nodule formation. Can J Microbiol 40:513–522

Streit WR, Joseph CM, Philips DA (1996) Biotin and other water-soluble vitamins are key growth factors for alfalfa root colonization by *Rhizobium meliloti* 1021. Mol Plant-Microbe Interact 9:330–338

Stuurman N, Bras CP, Schlaman HRM, Wijfjes AHM, Bloemberg G, Spaink HP (2000) Use of green fluorescent protein color variants expressed on stable broad-host-range vectors to visualize rhizobia interacting with plants. Mol Plant-Microbe Interact 13:1163–1169

Subramanian S, Stacey G, Yu O (2007) Distinct, crucial roles of flavonoids during legume nodulation. Trends Plant Sci 12:282–285

Subramanian S, Stacey G, Yu O (2006) Endogenous isoflavones are essential for the establishment of symbiosis between soybean and *Bradyrhizobium japonicum*. Plant J 48:261–273

Sun J, Cardoza V, Mitchell DM, Bright L, Oldroyd G, Harris JM (2006) Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation and nodulation. Plant J 46:961–970

Svenning MM, Gudmundsson J, Fagerli IL, Leinonen P (2001) Competition for nodule occupancy between introduced strains of *Rhizobium leguminosarum* bv. *trifolii* and its influence on plant production. Ann Bot 88:781–787

Sy A, Giraud E, Jourand P, Garcia N, Willems A, de Lajudie P, Prin Y, Neyra M, Gillis M, Boivin-Masson C, Dreyfus B (2001) Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J Bacteriol 183:214–220

Teplitski M, Robinson JB, Bauer WD (2000) Plants secrete substances that mimic bacterial N-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. Mol Plant-Microbe Interact 13:637–648

Timmers AC, Soupène E, Auriac MC, de Billy F, Vasse J, Boistard P, Truchet G (2000) Saprophytic intracellular rhizobia in alfalfa nodules. Mol Plant-Microbe Interact 13:1204–121

Valverde A, Velázquez E, Fernández-Santos F, Vizcaíno N, Rivas R, Mateos PF, Martínez-Molina E, Igual JM, Willems A (2005) *Phyllobacterium trifolii* sp. nov., nodulating *Trifolium* and *Lupinus* in Spanish soils. Int J Syst Evol Microbiol 55:1985–1989

Valverde A, Velázquez E, Gutirrez C, Cervantes E, Ventosa A, Igual JM (2003) *Herbaspirillum lusitanum* sp. nov., a novel nitrogen-fixing bacterium associated with root nodules of *Phaseolus vulgaris*. Int J Syst Evol Microbiol 53:1979–1983

van Dillewijn P, Soto MJ, Villadas P, Toro N (2001) Construction and environmental release of a *Sinorhizobium meliloti* strain genetically modified to be more competitive for alfalfa nodulation. Appl Environ Microbiol 67:3860–3865

Vance C (2001) Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. Plant Physiol 127:390–397

Vasse J, de Billy F, Camut S, Truchet G (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J Bacteriol 172:4295–4306

Vinuesa P, Neumann-Silkow F, Pacios-Bras C, Spaink HP, Martínez-Romero E, Werner D (2003) Genetic analysis of a pH-regulated operon from *Rhizobium tropici* CIAT899 involved in acid tolerance and nodulation competitiveness. Mol Plant-Microbe Interact 16:159–168

Vlassak KM, Vanderleyden J (1997) Factors influencing nodule occupancy by inoculant rhizobia. Crit Rev Plant Sci 16:163–229

Wasson AP, Pellerone FI, Mathesius U (2006) Silencing the flavonoid pathway in *Medicago truncatula* inhibits root nodule formation and prevents auxin transport regulation by rhizobia. Plant Cell 18:1617–1629

Wernegreen JJ, Harding EE, Riley MA (1997) *Rhizobium* gone native: unexpected plasmid stability of indigenous *Rhizobium leguminosarum*. Proc Natl Acad Sci USA 94: 5483–5488

Wernegreen JJ, Riley MA (1999) Comparison of the evolutionary dynamics of symbiotic and housekeeping loci: a case for the genetic coherence of rhizobial lineages. Mol Biol Evol 16:98–113

White J, Prell J, James EK, Poole P (2007) Nutrient sharing between symbionts. Plant Physiol 144:604–614

Wielbo J, Golus J, Marek-Kozaczuk M, Skorupska A (2009) Symbiosis stage-associated alterations in quorum sensing autoinducer molecules biosynthesis in *Sinorhizobium meliloti*. Plant Soil. doi:10.1007/s11104-009-0166-z

Wielbo J, Marek-Kozaczuk M, Kubik-Komar A, Skorupska A (2007) Increased metabolic potential of *Rhizobium* spp. is associated with bacterial competitiveness. Can J Microbiol 53:957–967

Wielbo J, Marek-Kozaczuk M, Mazur A, Kubik-Komar A, Skorupska A (2010) Genetic and metabolic divergence within a *Rhizobium leguminosarum* bv. *trifolii* population recovered from clover nodules. Appl Environ Microbiol doi:10.1128/AEM.00667-10

Wielbo J, Mazur A, Król J, Marczak M, Kutkowska J, Skorupska A (2004a) Complexity of phenotypes and symbiotic behaviour of *Rhizobium leguminosarum* biovar *trifolii* exopolysaccharide mutants. Arch Microbiol 182:331–336

Wielbo J, Mazur A, Król J, Marczak M, Skorupska A (2004b) Environmental modulation of the *pssTNOP* gene expression in *Rhizobium leguminosarum* bv. *trifolii*. Can J Microbiol 50:201–211

Yahyaoui FE, Küster H, Amor BB, Hohnjec N, Pühler A, Becker A, Gouzy J, Vernié T, Gough C, Niebel A, Godiard L, Gamas P (2004) Expression profiling in *Medicago truncatula* identifies more than 750 genes differentially expressed during nodulation, including many potential regulators of the symbiotic program. Plant Physiol 136:3159–3176

Young JP, Crossman LC, Johnston AW, Thomson NR, Ghazoui ZF, Hull KH, Wexler M, Curson AR, Todd JD, Poole PS, Mauchline TH, East AK, Quail MA, Churcher C, Arrowsmith C, Cherevach I, Chillingworth T, Clarke K, Cronin A, Davis P, Fraser A, Hance Z, Hauser H, Jagels K, Moule S, Mungall K, Norbertczak H, Rabbinowitsch E, Sanders M, Simmonds M, Whitehead S, Parkhill J (2006) The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. Genome Biol 7:R34

Yuen JPY, Cassini ST, De Oliveira TT, Nagem TJ, Stacey G (1995) Xanthone induction of *nod* gene expression in *Bradyrhizobium japonicum*. Symbiosis 19:131–140

Zhang F, Smith DL (1995) Preincubation of *Bradyrhizobium japonicum* with genistein accelerates nodule development of soybean at suboptimal root zone temperatures. Plant Physiol 108:961–968

Zhang F, Smith DL (1996) Inoculation of soybean (*Glycine max* (L.) Merr.) with genistein-preincubated *Bradyrhizobium japonicum* or genistein directly applied into soil increases soybean protein and dry matter yield under short season conditions. Plant Soil 179:233–241

Zhang F, Smith DL (2002) Interorganismal signaling in suboptimum environments: the legume–rhizobia symbiosis. Adv Agron 76:125–61

Zhang J, Subramanian S, Stacey G, Yu O (2009) Flavones and flavonols play distinct critical roles during nodulation of *Medicago truncatula* by *Sinorhizobium meliloti*. Plant J 57:171–183

Zuanazzi JAS, Clergeot PH, Quirion JC, Husson HP, Kondorosi A, Ratet P (1998) Production of *Sinorhizobium meliloti nod* gene activator and repressor flavonoids from *Medicago sativa* roots. Mol Plant-Microbe Interact 11:784–794

Zurdo-Piñeiro JL, Rivas R, Trujillo ME, Vizcaino N, Carrasco JA, Chamber M, Palomares A, Mateos PF, Martínez-Molina E, Velásquez E (2007) *Ochrobactrum cytisi* sp. nov., isolated from nodules of *Cytisus scoparius* in Spain. Int J Syst Evol Microbiol 57:784–788