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MOLECULAR SIGNALS EXCHANGED BETWEEN HOST PLANTS AND RHIZOBIA: BASIC ASPECTS AND POTENTIAL APPLICATION IN AGRICULTURE

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Summary—Rhizobia have the ability to infect and establish a N_2-fixing symbiosis with many leguminous and a few nonleguminous plants. The result of this interaction is the formation of a novel plant organ, the nodule, where N_2 fixation occurs. Research has shown that the establishment of this symbiosis requires coordinate bacterial and plant gene expression that is regulated through the mutual exchange of diffusible signal molecules. For example, each legume host exudes signals, mostly flavonoids, that induce the transcription of bacterial genes (i.e. *nod*, *nol* or *noe* genes), whose protein products are required for the infection process. It is now known that some of these bacterial nodulation genes encode proteins involved in the biosynthesis of novel lipo-chitin oligosaccharide (LCO) nodulation signals active on the roots of the plant host. Both the induction of bacterial nodulation gene expression and the activity of the LCO nodulation signals are host specific. These two communication steps are likely determine to a large extent the specificity of rhizobia–legume host range. At present, only limited efforts are being made to put this basic information concerning the mechanisms of rhizobia–legume communication to practical use. However, there are promising results that suggest that nodulation of economically important legume crops, such as soybean and bean, can be enhanced by the exogenous application of nodulation gene-inducing compounds. It may be possible to modify commercial inoculant preparations to include nodulation gene-inducing compounds. Alternatively, it may be possible to select for legume host varieties that produce large amounts of nodulation gene-inducing compounds. Additional results provide clues that additional complexities are likely to be discovered in the communication networks that control rhizobial–host interaction. These complexities are further compounded by the myriad of interactions that occur in the plant rhizosphere. Yet, the speed of research advances in this area leads one to be optimistic concerning the advance in our basic understanding of rhizobial–host interaction and the eventual application of this information for agronomic benefit. © 1997 Elsevier Science Ltd

INTRODUCTION

Bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Azorhizobium*, collectively called here as rhizobia, associate with roots and stems of many leguminous and a few nonleguminous plants, forming a highly specialized structure, the nodule, where biological N_2-fixation occurs. Nodulation is a multi-step process that involves specific plant and bacterial gene expression. The process starts with multiplication of bacteria in the rhizosphere, followed by chemotaxis to plant exudates, adhesion of rhizobia to the root and infection. The initiation of this infection process requires the mutual exchange of molecular signals between the bacterium and host plant. Although the basic aspects of this molecular communication are now known, a more comprehensive view of how this knowledge can be used to improve nodulation and N_2 fixation is lacking. The purpose of this paper is to review the most important aspects of legume–rhizobia molecular signaling and to discuss potential applications in agriculture. Due to a limitation of space, only recent reviews and a few original papers are cited.

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INDUCTION OF RHIZOBIAL NOD GENES BY HOST EXUDATES

Nodulation genes

Rhizobial genes essential to the infection process and nodulation are called nodulation genes (*nod*, *nol* and *noe*) and, when mutated, result in alteration of the infection process. Since 1982, when the first *nod* genes of *Rhizobium meliloti* (now *Sinorhizobium meliloti*) were isolated, 56 nodulation genes have been identified and the biochemical functions of some of these genes are now known.

Nodulation genes are usually classified in categories, which include the following:

(1) The common nodulation genes, *nodA*, *nodB* and *nodC*, which are host inducible and essential for nodulation. These genes show a high degree of sequence and functional homology among species of rhizobia. They are involved in the biosynthesis of substituted lipo-chitin oligosaccharides (LCOs, i.e. *nod* signal).

(2) *nodD*, a regulatory gene that positively regulates nodulation gene transcription. Other regulatory genes have also been identified (e.g. *nolR* in *S. meliloti*, *nolA* and *nodVW* in *B. japonicum*).

(3) Genes that determine host range specificity (*hsn*, host-specific nodulation). Some of these genes (e.g. *nodFE*, *nodL* and *nodM*) show sequence homology between rhizobial species and some do not (e.g. *nodO*, *nodH*, *nodPQ*). Some of these genes encode enzymes that modify the basic lipo-chitin *nod* signal molecule.

(4) Genotypic specific nodulation (GSN) genes, which appear to specify the ability to nodulate selected genotypes within a legume species (e.g. *nodX* of *R. leguminosarum* bv. *viceae* strain TOM; *nolA* of *B. japonicum*). The nodulation genes are localized on a megaplasmid (pSym) in fast-growing *Rhizobium*, whereas in *Bradyrhizobium* and *Azorhizobium* they are located on the chromosome (Peters and Verma, 1990; Phillips, 1992; Schlaman *et al.*, 1992; Hungria, 1994; Stacey, 1995).

nod *gene-inducers released by plants*

Mulligan and Long (1985) used a *nodDABC–lacZ* fusion to demonstrate the presence of a compound in alfalfa (*Medicago sativa*) exudates that induced *nod* gene expression. This induction was shown to require the *nodD* gene product. Subsequently, the inducer, isolated from alfalfa seed extracts, was identified as a flavonoid: luteolin (Peters *et al.*, 1986). This was surprising, since although more than 4000 flavonoids have been identified within the plant kingdom, few physiological functions have been attributed to these compounds. One current model predicts that flavonoid inducers bind to NodD at the cytoplasmic membrane, causing a conformational change. Although NodD binds to DNA in the absence of inducer, inducer binding is required to activate gene transcription. NodD binds to a specific 47 bp promoter sequence, the "*nod*-box", that precedes the inducible genes and shows similarity among rhizobial species.

NodD–flavonoid interaction plays an important role in determining host-range specificity. For example, transfer of *nodD1* from the broad host-range *Rhizobium* sp. strain NGR234 (now assigned to *Sinorhizobium fredii*) to *R. leguminosarum* bv. *trifolii* enlarged the nodulation host range of this organism (Bassam *et al.*, 1988). Mutations in the *nodD* gene of *R. leguminosarum* bv. *trifolii* extended the host range to the nonlegume *Parasponia* (McIver *et al.*, 1989). Also, the transfer of the *nodD* gene from a strain that nodulated siratro (*Macroptilium atropurpureum*) to *S. meliloti* resulted in *nod* gene-induction in the presence of siratro exudates (Rossen *et al.*, 1985). Although all rhizobia have a *nodD* gene, these genes are not always interchangeable. For example, some *nodD* mutations are not complemented by the *nodD* of other rhizobial species (Spaink *et al.*, 1987). It is also possible to construct strains with hybrid *nodD* genes that nodulate other hosts (Spaink *et al.*, 1987). Some rhizobial species have more than a single *nodD* gene, which likely serves to expand the host range, but may also have other biological meaning (Peters and Verma, 1990; Schlaman *et al.*, 1992; Hungria, 1994; Stacey, 1995).

After the identification of luteolin, *nod* gene-inducers released by seeds and roots of other host plants were identified as flavonoids and, in addition, two betaines were shown to activate *nod* gene expression in *S. meliloti* (Table 1). The chemical specificity of the flavonoid inducers has been investigated with suggestions that the C-7 (Zaat *et al.*, 1989; Le Strange *et al.*, 1990), or C-5 and C-7 (Hungria *et al.*, 1992) are essential for activity. However, little consistent similarity in structure was found between various inducer or inhibitor molecules of *nod* gene expression in *B. japonicum* (Cunningham *et al.*, 1991). A direct interaction between NodD and any inducer molecule has not been shown biochemically.

Plant inducers and host specificity

Studies using either *S. meliloti*, *R. leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *viceae* or *B. japonicum* have suggested that host plants exude different groups of inducers which control, at least to some degree, host specificity (Peters and Verma, 1990; Hungria, 1994; Phillips *et al.*, 1994). However, when investigations were extended to other rhizobia, such as the broad host strain NGR234, bean (*Phaseolus vulgaris*) rhizobia, or *S. fredii*, promiscuous *nod* gene-inducing systems were discovered. For example, the *nodD1* gene product of NGR234 was found to respond to a variety of compounds, including simple phenolics, such as vanillin and isovanillin, present in wheat (*Triticum aestivum*) seedling extracts (Le Strange *et al.*, 1990). Bean plants exude many inducers from seeds and a different set from roots (Hungria *et al.*, 1991a,b). Since bean rhizobia can be genetically unstable, bacteria could have evolved to respond to several inducers with the three *nodD* genes (Hungria *et al.*, 1992). Furthermore, bean seed coat colors often result from mixtures of flavonoids (Hungria *et al.*, 1991a), and these compounds may have been selected by the plant for effects on plant pathogens or sym-

Table 1. Rhizobial *nod* gene inducers isolated from leguminous hosts under sterile conditions

Host × rhizobia	Flavonoid *nod* gene inducer	Common name	Source	Reference
Medicago sativa × *S. meliloti*	5,7,3′,4′-Tetrahydroxyflavone	Luteolin	Seed extract	Peters *et al.* (1986)
	5,3′,4′-Trihydroxyflavone-7-*O*-glucoside	Luteolin-7-*O*-glucoside	Seed exudate	Hartwig *et al.* (1990)
	5-Methoxy-7,3′,4′-trihydroxyflavone	5-Methoxyluteolin	id.	id.
	3′,5-Dimethoxy-7,4′-dihydroxyflavone	3′,5-Dimethoxyluteolin	id.	id.
	3′-Methoxy-5,7,4′-trihydroxyflavone	Crysoeriol	id.	id.
	7,4′-Dihydroxyflavanone	Liquiritigenin	Root exudate	Maxwell *et al.* (1989)
	7,4′-Dihydroxyflavone	—	id.	id.
	4,4′-Dihydroxy-2′-methoxychalcone	Methoxychalcone	id.	id.
	Nonflavonoids			
	Stachydrine		Seed exudate	Phillips *et al.* (1992)
	Trigonelline		id.	id.
Pisum sativum × *R. leguminosarum* bv. *viceae*	5,7,4′-Trihydroxyflavone-7-*O*-glucoside5,7,3′,4′-tetrahydroxyflavonone	Apigenin-7-*O*-glucosideeriodictyol	Seed exudate and root exudate	Firmin *et al.* (1986) id.
Vicia sativa × *R. leguminosarum* bv. *viceae*	3,5,7,3′-Tetrahydroxy-4′-methoxyflavanone	—	Root exudate	Zaat *et al.* (1989)
	7,3′-Dihydroxy-4′-methoxyflavanone	—	id.	
Trifolium repens × *R. leguminosarum* bv. *trifolii*	7,4′-Dihydroxyflavone	—	Root exudate and	Redmond *et al.* (1986)
	7,4′-Dihydroxy-3′-methoxyflavone	Geraldone	seedling extract	
	4′-Hydroxy-7-methoxyflavone	—	id.	
Glycine max × *B. japonicum* and ×*S. frediiG. max* × *B. japonicum*	7,4′-Dihydroxyisoflavone	Daidzein	Seed extract and	Kosslak *et al.* (1987)
	5,7,4′-Trihydroxyisoflavone	Genistein	seedling extract	id
	4,2′,4′-Trihydroxychalcone	Isoliquiritigenin	Root exudate	Kape *et al.* (1992)
Phaseolus vulgaris × *R. leguminosarum* bv. *phaseoli* and *R. etli*	3-*O*-Glycosides of	3-*O*-Glycosides of		
	3,5,7,3′4′,5′-Hexahydroxyflavylium	Delphinidin	Seed exudate	Hungria *et al.* (1991a)
	3,5,7,4′,5′-Pentahydroxyflavylium	Petunidin	id.	id.
	3,5,7,4′-Tetrahydroxyflavylium	Malvidin	id.	id.
	3-*O*-Glycosides of	3-*O*-Glycosides of		
	3,5,7,3′4′5′-Hexahydroxyflavone	Myricetin	id.	id.
	3,5,7,3′,4′-Pentahydroxyflavone	Quercetin	id.	id.
	3,5,7,4′-Tetrahydroxyflavone	Kaempferol	id.	id.
	5,4′-Dihydroxyisoflavone-7-*O*-glycoside	Genistein-7-*O*-glycoside	Root exudate	Hungria *et al.* (1991b)
	5,7,3′,4′-Tetrahydroxyflavanone	Eriodictyol	id.	id.
	5,7,4′-Trihydroxyflavanone	Naringenin	id.	id.

bionts. *S. fredii nod* genes can also be induced by several flavonoids, including flavones, isoflavones, a flavanone and a coumestan (Kosslak *et al.*, 1987). Recently, the *B. japonicum nod* genes were found to be strongly induced by two xanthones isolated from a nonhost plant, *Haploclathra* species (Yuen *et al.*, 1995).

Biosynthesis and release of flavonoids

Flavonoids are produced through chalcone synthase (CHS) by condensation of 4-coumaryl-CoA, which is derived from phenylalanine, and three malonyl-CoA molecules. Plants exude inducers in the absence of rhizobia, but the pattern clearly changes with the addition of bacteria. Inoculation of *Vicia sativa* subsp. *nigra* roots with an infective *R. leguminosarum* bv. *viceae* strain increased CHS mRNA, but noninfective rhizobia had no effect (Recourt *et al.*, 1992). The homologous (but not the heterologous rhizobia) also increased by 10–20 times the total *nod* gene-inducing activity in root exudates with the discharge of seven new compounds (Recourt *et al.*, 1991). This rhizobial-inducing effect, called Ini (increased *nod* gene-inducing activity) was also observed after inoculation of host plants with *R. leguminosarum* bv. *trifolii*, *R. loti* and *S. meliloti* (Phillips *et al.*, 1994). It has been proposed that this plant response resembles that of pathogen attack in which phytoalexins are produced. Since *nod* gene-inducers and phytoalexins share common precursors, the suggestion has been made that the rhizobial symbiosis evolved from a pathogenic relationship. The idea is reinforced by reports that phytoalexins can be produced after the inoculation with *S. meliloti* or *B. japonicum* (Schmidt *et al.*, 1992; Phillips *et al.*, 1994).

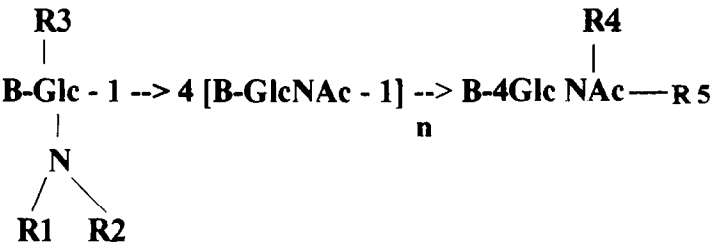
In alfalfa (Maxwell and Phillips, 1990) and bean (Hungria and Phillips, 1993), *nod* gene-inducing activity during the first hours was found to be dependent on flavonoids stored in the seeds. In contrast, flavonoid release from alfalfa roots was affected by inhibitors of the key enzymes of flavonoid metabolism, CHS and phenylalanine ammonia lyase (PAL), indicating a need for *de novo* synthesis (Maxwell and Phillips, 1990; Schmidt *et al.*, 1992). Nodule number was also dramatically decreased in root regions that had been in contact with the inhibitor (Schmidt *et al.*, 1992). Bean, an annual crop, discharged 6000–7000 times more *nod* gene-inducers than that released from seeds and roots, respectively, of the perennial alfalfa (Maxwell *et al.*, 1989; Hartwig *et al.*, 1990; Hungria *et al.*, 1991a,b). These differences could be due to the higher I_{50} for bean compounds (Hungria *et al.*, 1992), but are also probably due to the short cycle of bean growth, that requires an early and fast establishment of nodules. The transcription levels of CHS and chalcone isomerase and the release of flavonoids were higher in root tips and emerging root hairs, which are more susceptible to infection (Redmond *et al.*, 1986; McKhann and Hirsch, 1994). In both alfalfa and bean, a synergistic effect was observed between the main seed and root inducers. This pattern of *nod* gene-inducer production likely contributes to the fact that the root crown is highly susceptible to nodulation and the region where most nodules occur (Hungria *et al.*, 1992; Phillips *et al.*, 1994).

The same flavonoid inducers released by young alfalfa and bean roots continue to be released in later stages of growth (Maxwell and Phillips, 1990; M. Hungria, unpubl.). However, *in planta*, *nod* gene expression in *R. leguminosarum* bv. *viceae* bacteroids is switched off. This switch off is not due to an absence of NodD, inducing compounds or the presence of anti-inducers. Furthermore, constitutive expression of inducible *nod* genes in bacteroids results in Fix$^-$ nodules (Schlaman *et al.*, 1992), raising the question of why the plant continues to synthesize *nod* gene-inducers without apparent benefit to the symbiosis. It may be that these compounds play a broader, presently unknown, function in nodulation.

NODULATION SIGNALS PRODUCED BY RHIZOBIA

LCO Nod signals

The induction of *nod* gene transcription leads to the synthesis of bioactive molecules responsible for the earliest stages of the root infection process. The presence of bacterial nodulation signals was known from studies of the legume root response to sterile *Rhizobium* culture filtrates. Subsequently, Lerouge *et al.* (1990) were able to purify the active compound from *S. meliloti* culture filtrates and identify the chemical structure as a substituted lipo-chitin molecule. Following this first report, several laboratories reported similar molecules produced by other (*Brady*) *Rhizobium* species (Fig. 1). Since all of these molecules were found to be acylated forms of small chitin fragments, they have been called lipo-chitin oligosaccharides (LCO). These LCO are composed of three to five 1,4-β-linked *N*-acetylglucosaminosyl (GlcNAc) residues, *N*-acylated at the terminal nonreducing glucosaminosyl residue (GlcN). A variety of additional substitutions can be present, such as fucosyl, arabinosyl, sulfate, carbamyl, acetyl or glycerol. The *nodABC* gene products are involved in the synthesis of the basic acylated chitin backbone. This structure is then further modified via enzymes encoded by the host specific nodulation genes. For example, the *nodHPQ* genes of *S. meliloti* encode enzymes that sulfate the LCO. The genetics and biochemistry of the Nod signal (i.e. LCO) biosynthesis and transport has recently been reviewed, but several steps and key enzymes remain to be identified (Carlson *et al.*, 1994). The



Lipo-Chitin Nodulation Signals

Species	R1	R2	R3	R4	R5	n	References (see below)
R. meliloti	H	C16:2 C16:3	Ac(O-6) H	Sulfate	H	1/2/ 3	a,b,c
R.l. bv. viciae	H	C18:4 C18:1	Ac(O-6)	H Ac(O-6)	H	2/3	d
R.l. bv. trifolii	H	C18:0 C18:1 C18:2 C18:3 3OH-C14:0 3OH-C16:0	H Ac	H	H	1/2/ 3	e,f
B. japonicum	H	C18:1 C16:0 C16:1	Ac(O-6) H	MeFuc	H	3	g,h
B. elkanii	H; Me	C18:1	Ac(O-6) H Cb	MeFuc Fuc	H Gro	2/3	h
R. sp. NGR23	Me	C18:1 C16:0	Ac(O-6) H Cb(1,2)	MeFuc AcMeFuc MeFucS	H	3	i
A. caulinodans	Me	C18:1 C18:0	Ac(O-6) Cb	D-ara H	H	2/3	j
R. tropici	Me	C18:1	H	Sulfate	H	3	k
R. fredii	H	C18:1	H	MeFuc Fuc	H	1/2/ 3	l
R. loti	Me	C18:0 C18:1	Cb(1,2)	AcFuc	H	3	m

Fig. 1. Lipo-chitin nodulation signals of rhizobia. References for the table are: (a) Lerouge *et al.* (1990); (b) Roche *et al.* (1991); (c) Schultze *et al.* (1992); (d) Spaink *et al.* (1991); (e) Orgambide *et al.* (1995); (f) Spaink *et al.* (1995); (g) Sanjuan *et al.* (1992); (h) Carlson *et al.* (1993); (i) Price *et al.* (1992); (j) Mergaert *et al.* (1993); (k) Poupot *et al.* (1993); (l) Bec-Ferté *et al.* (1993); (m) Lopez-Lara *et al.* (1995a).

plant responses to LCO addition are host specific and resemble those normally associated with bacterial inoculation (summarized in Table 2). These plant responses occur in the presence of extremely low concentrations of purified LCO, in the range of 10^{-9} to 10^{-12} M (Dénarié *et al.*, 1992; Spaink *et al.*, 1993; Carlson *et al.*, 1994; Stacey, 1995). On a few plant species, the application of the appropriate LCO has been found to induce a nodule primordium which closely resembles, both anatomically and histologically, a bacterial-induced nodule (e.g. alfalfa; Truchet *et al.*, 1991).

Nod signals and host specificity

Analysis of the structure–function relationships of various LCO molecules has shown that specificity is determined by the various residues present on the core lipo-chitin backbone. For example, in the case of *R. leguminosarum* bv. *viceae*, specificity is determined, at least in part, by the hydrophobicity of the highly unsaturated fatty acyl moiety (Spaink *et al.*, 1993). In the case of *B. japonicum*, specificity is determined, in part, by the presence of a 2-*O*-methylfucose residue attached to the terminal, reducing sugar (Stacey *et al.*, 1994). However, it is becoming evident that LCO Nod signals are not as specific as first thought. For example, Nod factors with a broad variety of structures are active on bean roots. The common LCO produced by bean rhizobia, a pentameric LCO with an *N*-methyl substituent, is also produced by rhizobia that do not nodulate bean. Therefore it is likely that host specificity is determined by a number of factors (e.g. NodD-flavonoid specificity, LCO structure, etc.). Additional signal molecules may also be important. For example, a conjugated auxin, whose production is encoded by genes on the pSym, appears to be an additional nodulation signal produced by *R. tropici* type A strains (Martínez *et al.*, 1995).

A full understanding of the mechanisms controlling host specificity may allow, in the near future, the exciting possibility of extending nodulation ability to other plant species. There is also evidence that the restriction of nodulation by certain legume genotypes may be determined by LCO structure (Firmin *et al.*, 1993). This work opens the possibility of managing species–genotype interaction by

Table 2. First reports of the effects of rhizobial LCO Nod signals on host-root responses

Root phenotype	Rhizobia	Host plant	Reference
Tsr (thick and short root)	*R. l.* bv. *viceae* and *S. meliloti*	*Vicia, Medicago*	Zaat *et al.* (1987)
		Melilotus	Spaink *et al.* (1991)
Had (root hair deformation)	*R. l.* bv. *viceae*	*Vicia*	Spaink *et al.* (1991)
	S. meliloti	*Vicia, Medicago Melilotus*	Banfalvi and Kondorosi (1989), Lerouge *et al.* (1990)
	B. japonicum	*Glycine*	Sanjuan *et al.* (1992)
Hai (hair induction)	*S. meliloti*	*Medicago, Vicia*	Roche *et al.* (1991)
	A. caulinodans	*Sesbania*	Mergaert *et al.* (1993)
	Rhizobium GRH2	*Acacia, Phaseolus*	Lopez-Lara *et al.* (1995b)
Coi (cortical cell division induction)	*S. meliloti*	*Medicago, Vicia*	Lerouge *et al.* (1990)
	R. l. bv. *viceae*	*Vicia*	van Brussel *et al.* (1992)
	R. l. bv. *phaseoli*	*Phaseolus*	Martínez *et al.* (1993)
	R. tropici	*Phaseolus*	Martínez *et al.* (1993)
	B. japonicum	*Glycine*	Sanjuan *et al.* (1992)
	A. caulinodans	*Sesbania*	Mergaert *et al.* (1993)
Mitosis of plant protoplasts	*S. meliloti*	*Medicago*	Schmidt *et al.* (1988)
Membrane depolarization	*S. meliloti*	*Medicago*	Ehrhardt *et al.* (1992)
Pit (pre-infection threads)	*R. l.* bv. *viceae*	*Vicia*	van Brussel *et al.* (1992)
Noi (nodule induction)	*S. meliloti*	*Medicago*	Truchet *et al.* (1991)
	R. l. bv. *viceae*	*Vicia*	Spaink *et al.* (1991)
	R. tropici	*Phaseolus*	Martínez *et al.* (1995)
	B. elkanii	*Glycine* (*G. soja*)	Stokkermans and Peters (1994)
	Rhizobium GRH2	*Acacia, Phaseolus*	Lopez-Lara *et al.* (1995b)
	Rhizobium etli	*Phaseolus*	Martínez *et al.* (1995)
ENOD (early nodulation, nodulins)	*S. meliloti*	*Medicago*	Truchet *et al.* (1991)
	R. l. bv. *viceae*	*Vicia*	Scheres *et al.* (1990)
	B. japonicum	*Glycine soja*	Minami and Stacey (unpubl.)

plant breeding. Such an approach may offer a practical way in which to overcome nodulation competition from inferior, indigenous soil rhizobia.

Molecular signals and rhizobia evolution

No correlation has been found between rhizobia phylogeny, based on the analysis of 16S rRNA gene sequences, and the LCO Nod signal modifications, such as addition of sulfate, fucose or acetate, produced by the bacteria (Martínez *et al.*, 1995). However, in this study, the LCO lipid moiety was not considered. It has been suggested that the specific acyl moiety present on the LCO Nod signal may be designed to match the fatty acid composition of host plant membranes. In temperate legumes, fatty acid saturation is less prevalent. This correlates with the presence of unsaturated lipids on the LCO Nod signals of rhizobia that infect temperate legumes, such as *S. meliloti* and *R. leguminosarum* bv. *viciae*. Likewise, saturated lipids are more prevalent in the membranes of tropical legumes, and this correlates with saturated or mono-unsaturated fatty acids present in the LCO Nod signals produced by rhizobia that infect such legumes (Martínez *et al.*, 1995). Indeed, this correlation extends to DNA sequence comparisons of the common nodulation genes of various rhizobia. Dobert *et al.* (1994) concluded on the basis of such comparisons that there are two groups of *nod* genes among *Rhizobium* and *Bradyrhizobium* species, those which nodulate temperate and those which nodulate tropical legumes.

Practical application of basic research: what happens in the soil?

The mechanisms that are usually so clear under optimal and sterile conditions in the laboratory are often less clear when tested under field conditions where microorganisms and plants interact in a complex environment. Exudates of several plants may be available and have the potential to interact to induce *nod* gene expression. For example, exudates of both nodulating and non-nodulating *Acacia* species are able to induce *nod* genes of several tree rhizobia, with surprisingly high inducing-activity found for exudates of the non-nodulating *A. brevispica* (Sprent, 1994). The *nod* genes of the broad host strain NGR234 have been shown to be induced by seed exudates of the nonhost wheat (Le Strange *et al.*, 1990). Expression of the *nod* genes of bean rhizobia can also be stimulated by seed exudates of several nonhost leguminous and nonleguminous plants (M. Hungria, unpubl.), including maize (*Zea mays*) (Table 3). Therefore, there is considerable potential for both host and nonhost plants to contribute to *nod* gene expression. What actual roles such interactions play in the field are presently unknown, but such a phenomenon may contribute to situations in which high rates of nodulation are found in previously uninoculated soil. For example, high spontaneous bean nodulation was observed in a field never inoculated before and where pasture had been growing for 40 y (Araujo, 1994).

Recognition of the importance of sustainable agriculture is driving the increasing utilization of

Table 3. Effects of bean and maize exudates on the rhizosphere population of *Rhizobium tropici* and *Azospirillum lipoferum* and on *Rhizobium nod* gene-inducing activity, Hai phenotype, nodulation and total N accumulated in bean (*Phaseolus vulgaris*) plants, cv. Carioca

Cultivars	Population (log)[2]		β-gal.[3]	Hai phenotype[4]		NDW[5]	TNS[5]
	R. tropici	*A. lipoferum*		Primary	Secondary	(mg pl^{-1})	(mg N pl^{-1})
			Phaseolus vulgaris				
EMGOPA-201	6.40 ab[1]	6.80 A	1,101 b	3.2 ABb	4.2 a	80ABC	105 ab
ESAL-580	6.45 ab	6.85 A	1,312 ab	3.4 AB	4.3 a	90 AB	110 ab
Carioca	7.20 a	6.90 A	1,514 a	3.8 A	4.5 a	100 A	120 a
Carioca-80	6.45 ab	5.75 B	802 b	2.5 BC	3.8 ab	73 BCD	95 bc
Negro Argel	7.20 a	6.80 A	1,520 a	3.8 A	4.5 a	98A	124a
Rio Tibagi	6.02 b	5.50 C	622 b	1.5 C	3.0 bc	70 BCD	86 c
IAPAR-20	6.00 b	5.50 C	687 b	1.5 C	2.8 cd	66 CD	80 c
			Zea mays				
Centralmex	6.50 b	7.30 A	624 b	1.8 C	3.2 b	76 BCD	95 bc
IPEAX	4.80 c	6.75 AB	380 c	1.0 CD	2.0 d	68 CD	80 c
AG162	4.80 c	7.25 A	630 b	1.5 C	2.8 cd	60 D	82 c
Agroman 2001	5.00 c	6.75 A	270 c	1.0 CD	2.0 d	48 CD	80 c
Control	3.82 d	4.25 D	—	0.4 d	1.0 e	40 D	85 c
CV (%)	18	23	25	5	9	18	12

[1]Mean of four replicates and, when followed by the same letter, did not show statistical differences ($P \leq 0.05$, Tukey's test).
[2]Population of *R. tropici* CIAT 899 and *Azospirillum lipoferum* Sp. 242 in the rhizosphere of several bean and maize cultivars 20 d after the inoculation with 5 ml of 10^5 cells ml^{-1} of each species. Experiment performed in Leonard jars and control treatment consisted of jars without plants.
[3]*nod* gene-inducing activity (U of β-galactosidase seed^{-1} h^{-1}, obtained with the *nodA–lacZ R. leguminosarum* strain RBL1283) of bean and maize sterile aqueous seed exudates obtained during 24 h of inbibition.
[4]Root Hai (hair induction) phenotype of bean, cv. Carioca, inoculated with sterile supernatant of *R. topici* CIAT 899 grown in minimum medium supplied with 1 ml of aqueous bean or maize seed exudates or without exudates (control). Notes from 0 (low) to 5 (high) number of root hairs.
[5] Nodule dry weight (NDW) and total N in shoots (TNS) of bean, cv. Carioca, inoculated with strain *R. tropici* CIAT 899 and supplied with 5 ml of bean or maize seed exudates during the inoculation procedure. Plants harvested at 30 d after emergence and control consisted of inoculated plants without extra-exudates.

intercrop, crop rotation and no-tillage cropping systems. Fields growing under a maize–bean intercrop rotation can represent up to 90% of the bean cultivated in some agriculturally important areas of Brazil. Under such conditions, symbiotic N_2 fixation effects have been seen on both the bean and maize (Table 3). Exudates of some maize cultivars were able to increase the growth rate of *R. tropici* in minimum medium (MM) at rates comparable to some bean cultivars (M. Hungria, unpubl.), and there was a reciprocal stimulation of both microorganisms in the rhizosphere (Table 3). Effects of *nod* gene-inducers on growth in MM, not related to C nutrition, were previously reported for *S. meliloti* (Phillips, 1992) and, since soil is likely a nutrient-limited ecosystem, such effects may be very important for microbial survival. *R. tropici* cells also show chemotaxis toward bean and maize exudates (M. Hungria, unpubl.) and, although the agent of the chemotaxis has not been identified, there are reports of chemotaxis of rhizobia to flavonoids (Caetano-Anollés *et al.*, 1988). Maize exudates also have the ability to induce *R. tropici nod–lacZ* expression and, when such exudates were applied to bean seeds at the time of inoculation, they were found to contribute to an increase in nodule dry weight (Table 3). Furthermore, sterile culture supernatants of *R. tropici* cells grown in minimal medium supplied with maize or bean exudates induced root hair deformation when applied to bean seedlings (Table 3).

Phenolic compounds accumulate in soils (Siqueira *et al.*, 1991). Indeed, *nod* gene-inducing activity was detected in soil around alfalfa plants (León-Barrios *et al.*, 1993). Phenolic compounds and *nod* gene-inducing activity were found in the rhizospheres of bean and soybean (*Glycine max*) plants grown under different tillage and no-tillage systems (Table 4; M. Hungria, unpubl.). The activity found was surprisingly high, since soil microorganisms, including rhizobia, have the ability to degrade phenolic compounds (Siqueira *et al.*, 1991).

Other soil microorganisms can also stimulate nodulation. For example, nodulation enhancement in the presence of *Agrobacterium tumefaciens* (Caetano-Anollés and Bauer, 1988) and *Bacillus subtilis* (Araújo and Hungria, 1995) has been attributed to the production of additional bioactive molecules by these bacteria. Likewise, flavonoids have been shown to promote spore germination and hyphal growth of mycorrhizal fungi (Siqueira *et al.*, 1991). Improved plant growth due to mycorrhizal infection would likely result in better nodulation and higher N_2-fixation rates. One important observation from studies of different cropping systems is that soils with a higher concentration of *nod* gene-inducers appeared to have a higher microbial diversity (Hungria *et al.*, 1995; Table 4). Therefore, one can conclude that the complex interactions between different plants and microbial species that occur in the rhizosphere can significantly affect the nodulation response. Additional research is needed in this

Table 4. Content of phenolic compounds, β-galactosidase activity and rhizobial population in two Brazilian soils under different crop systems (Hungria *et al.*, 1995)

Crop system	Phenolic content[4] (μg C g^{-1} soil)	β-gal[5] (U g^{-1} soil)	*Bradyrhizobium*[6] (log g^{-1} soil)	*Rhizobium* (log g^{-1} soil)[7] *Phaseolus*	*Phas./Leu.*	*Leucaena*
			Crop rotation[2]			
Soybean/wheat	35.33 a[1]	65.18 A	5.06 a	1.51 A	1.99 a	2.35 A
Maize/wheat	29.49 b	40.15 B	4.05 c	0.72 B	0.83 b	2.21 A
Soybean/wheat/maize	35.33 a	60.49 A	4.91 b	1.60 A	1.86 a	2.40 A
			Monoculture/intercrop[3]			
Bean	79.23 ab	118.96 B	2.02 a	1.71 A	1.45 a	2.70 A
Maize	61.00 b	91.59 C	1.15 b	0.82 B	0.82 b	2.18 B
Bean/maize	90.77 a	136.28 A	1.80 a	1.73 A	1.48 a	2.69 A

[1]All data represent the mean of four replicates and, when followed by the same letter, did not show statistical differences for each parameter in each experiment ($P \leq 0.05$, Tukey's test).
[2]Experiment performed during 17 y, without inoculation for the last 7 y and data represent the mean of seven harvests performed during 18 months, with three replicates per harvest and two crops per year.
[3]Experiment performed during 2 y, without inoculation and plants were harvested at the end of the second year.
[4]Soil extracts obtained by shaking 100 g of soil with 100 ml of 40.5 mM $Ca(OH)_2$ for 12 h and phenolic content calculated by log ϵ for genistein (4.50 at 263 nm).
[5]Using the *Rhizobium* strain RBL 1283, as described in Table 3.
[6]Cells counted in soybean plants.
[7]Cells able to nodulate bean (probably *R. etli*), to nodulate bean and leucaena (probably *R. tropici*) and cells able to nodulate exclusively leucaena (*Rhizobium* spp.).

area to better define the factors that control and mediate these interactions.

Increasing competitive nodulation by inoculant strains

A few studies have shown that the nodulation of economically important legume crops can be limited by availability of *nod* gene-inducers. For example, the exogenous addition of luteolin to the roots of certain alfalfa cultivars was shown to significantly increase nodulation (Kapulnik *et al.*, 1987). Likewise, bean nodulation by *R. leguminosarum* bv. *phaseoli* or *R. tropici* was increased by the addition of quercetin- and malvidin-3-*O*-glucoside (Hungria and Phillips, 1993). Pre-treatment of *Rhizobium* with an ether extract of seed exudate, ethanol extract of seed, genistein or biochanin A (two flavonoids that comigrated with the bioactive seed *nod* gene-inducing peaks) increased nodulation and yield of field grown peanut (*Arachis hypogea*). These benefits were measurable even with inoculants kept for up to 12 months (Hopper *et al.*, 1995). In Brazil, in an oxisol (dark red latosol) with an established population of 10^4 *Bradyrhizobium* cells g^{-1} of soil and 10^6 *Rhizobium* cells g^{-1} of soil, bean or soybean seeds inoculated and treated with with 40 μM of the isoflavone genistein produced plants with significantly higher nodule numbers (i.e. 15 and 20% for soybean and bean, respectively; M. Hungria, unpubl.). Abdalla (1994) reported that irrigation of soybean plants with phenolic compounds also increased nodulation, as well as NADH-GOGAT and NADH-GDH activities. These results suggest that it may be possible to routinely increase nodulation of some legume species by the addition of exogenous *nod* gene-inducers. Such inducers could likely be obtained cheaply from seeds or chemically synthesized and, since such inducers are active in low concentrations, their addition to inoculants should be possible at a low cost. Another approach is to select legume cultivars that produce more of

Table 5. Effects of the addition *B. elkanii* parental strain SEMIA 566 or its natural variant CPAC 15, or of the addition of sterile supernatants of these bacteria induced for *nod* gene expression, to host plant roots [i.e. thick-short-root (Tsr), root hair deformation (Had) and hair induction (Hai)]. Plants were grown in a growth chamber in jars containing N-free nutrient agar medium, and harvested at 15 d after transplanting. Competitiveness was evaluated by the percentage of nodule occupancy, at 30 d after inoculation, with a mixture of 1:1 with *B. elkanii* strain 29w (M. Hungria, C. Y. M. Nishi, J. Cohn and G. Stacey, unpubl.)

Strain	Cells Tsr[2] Length (cm)	Tsr[2] Thickness (mm)	Had[2] Length (μm)	Had[2] Thickness (μm)	Supernatant Hai[2] (no. $field^{-1}$)	Supernatant Hai[2] (no. $field^{-1}$)	Nodule occupancy (%)
566	19.95 a[1]	2.38 A	62.50 b	23.75 A	50.25 b	72.75 B	55.70 b
CPAC 15	19.75 a	2.50 A	50.00 b	18.75 AB	> 100.00 a	> 100.00 A	89.40 a
Control[3]	22.73 a	1.88 B	205.00 a	10.00 B	23.75 c	23.00 C	—
CV (%)	14.71	22.83	17,18	26.08	12.77	18.64	28.15

[1]Means of four replicates and, when followed by the same letter, did not show statistical differences within each column (Tukey's test, $P \leq 0.05$).
[2]Thick and short root, evaluated by the length and thickness of main root; Had, hair deformation, by length and thickness of each root hair (100 root hairs evaluated); Hai, hair induction, by the number of root hairs per field of Neubauer chamber.
[3]For the treatment cells, control consisted of plants grown in the absence of bacteria and, for the treatment supernatant, control consisted of bacteria supernatant produced in the absence of seed exudates.

the *nod* gene-inducers. For example, bean (Hungria and Phillips, 1993) and soybean (M. Hungria, unpubl.) cultivars with higher content of seed *nod* gene-inducers were found to exhibit higher nodulation. However, such breeding efforts need to take into account undesirable traits associated with higher flavonoid content, e.g. an increase in isoflavonoid content in soybean seeds is not nutritionally desirable, since it causes bitterness and astringency.

Competitiveness is another important trait that can be affected by the exchange of molecular signals. Comparison of *B. elkanii* strain SEMIA 566 with its natural variant CPAC 15 (isolated from a Brazilian savanna soil, "Cerrado", several years after the last inoculation with this strain) has shown that CPAC 15 is more competitive (Nishi *et al.*, 1996). This correlates with a greater ability of sterile supernatants of cultures induced for *nod* gene expression to induce root hair deformation when applied to soybean roots (Table 5). These results suggest that the LCO Nod signals could be associated with greater competitiveness and we are currently exploring this possibility, since for another pair of parental and variant strains (CB 1809 X CPAC 7) we have recently confirmed differences in LCO Nod signals (M. Hungria, C. Y. M. Nishi, J. Cohn and G. Stacey, unpubl.). Likewise, the use of specific genotypes that restrict nodulation of indigenous strains but allow nodulation of inoculant strains could be used to control nodulation competition. The molecular basis of such genotype specific nodulation is unknown, but may involve the recognition of LCO Nod signals.

Environmental effects

High temperature, low soil moisture, pH, P content and toxic amounts of Al are usually the main factors that limit N_2 fixation in tropics and there is evidence that they can also affect the exchange of molecular signals. High temperature was found to increase the release of *nod* gene-inducers from seeds during the first 24 h, but decreased *nod* gene-inducing activity from bean and soybean roots, and bean plants were found to be more sensitive to temperature stress than soybean (Hungria, 1995) (Table 6). These results are consistent with current biosynthesis in roots, but not in seeds (Maxwell and Phillips, 1990). As previously reported (Richardson *et al.*, 1988), similar decreases in nodulation were observed when plants were grown at pH 4.5 in comparison with pH 5.8 (Table 6). The decrease in nodulation due to either temperature or pH stress could be largely alleviated by the addition of the *nod* gene-inducer genistein (M. Hungria, unpubl.). Consequently, the decrease on the release of *nod* gene-inducers can explain, at least partially, the deleterious effects of high temperatures and acidity on early infection of bean and soybean roots.

Table 6. Effects of high temperature and acidity on *nod–lacZ* expression [β-galactosidase activity (U seed^{-1} or root^{-1} h^{-1})] of bean (cv. Carioca) and soybean (cv. BR-16) seed and root exudates

Plant	Treatment	Seed exudates[2] (hours of incubation)						Root exudates[2] (hours of incubation)					
		2	4	6	8	12	18	24	36	48	72	96	120
						Temperature							
Soybean	28°C[3]	618[1]	2146	1408	1259	416	380	360	254	191	115	155	122
	39°C	137[1]	1210	1355	1228	411	372	352	164	112	57	44	31
P[1]		*	*		*				*	*	*	*	*
Bean	28°C	546	889	993	1012	824	762	683	385	189	182	162	130
	39°C	899	1115	1282	1118	845	742	630	339	100	120	106	99
P		*	*	*						*		*	
						Acidity							
Soybean	pH 4.5	606	1206	1300	1504	805	550	406	280	220	182	190	160
	pH 5.8	617	1302	1480	1190	990	460	380	115	116	40	45	28
P					*				*		*	*	*
Bean	pH 4.5	880	1005	1126	1270	995	700	730	420	380	298	250	170
	pH 5.8	910	1110	1281	1314	940	862	800	380	225	150	120	100
P										*	*	*	*

[1]Mean of four replicates and * means statistical difference for each sampling time ($P \leq 0.05$, Tukey's test).
[2]Exudates produced as described before (Hungria *et al.*, 1991a,b); bean inducers analyzed with *Rhizobium* RBL1283 and soybean with *B. japonicum* strain LB100.
[3]Temperature during 8 h per day, with 23°C at night.

CONCLUSIONS

The establishment of the symbiotic relationship between rhizobia and their legume hosts is complex and involves the mutual exchange of diffusable signal molecules. Among these molecules are plant host flavonoids that serve to induce bacterial *nod* gene expression and bacterially produced LCO Nod signals. Together, these signaling networks largely determine host range specificity. Basic research on these systems has yielded a great deal of exciting new information on the mechanisms of legume infection. Future work will likely uncover new complexities and may lead to the discovery of new signal molecules. At present, this new information has not found its way to field application. Nevertheless, some promising results have been obtained through the application of the knowledge gained concerning rhizobia–legume communication. Most notably, the exogenous addition of *nod* gene-inducing flavonoids to rhizobial inoculants appears to appreciably enhance nodulation of some crop species. This may be a promising and cost-effective method to enhance the efficacy of commercial inoculants. Likewise, knowledge of rhizobia–legume signaling pathways suggests new ways in which to attack the problem of competition with inferior, indigenous soil rhizobia. Selection through plant breeding of lines that specifically suppress nodulation by soil rhizobia, while allowing nodulation by inoculant strains, may yield varieties with improved agronomic performance. However, the complexity of rhizosphere interactions important to rhizobial soil ecology and competitive nodulation ability may hamper efforts to rapidly improve inoculant performance. Further research is needed in order to more fully understand rhizobial soil ecology and the full extent of bacterial–host plant interactions.

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