



## Signals exchanged between legumes and *Rhizobium*: agricultural uses and perspectives

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### Abstract

Legumes and rhizobia exchange at least three different, but sometimes complementary sets of signals. Amongst the variety of substances normally and continuously secreted into the rhizosphere by plants are phenolic compounds. Flavonoid components of these mixtures are especially active in inducing rhizobial *nodulation* genes. Many *nod*-genes exist. Some (e.g., *nodD*) serve as regulators of transcription, but most code for enzymes involved in the synthesis of a family of lipo-chito-oligosaccharides (LCOs) called Nod-factors. Nod-factors possess hormone-like properties, are key determinants in nodulation, and allow rhizobia to enter the plant. As Nod-factors also stimulate the synthesis and release of flavonoids from legume roots, the response to inoculation is amplified. Once the bacteria enter the plant, other sets of signals are exchanged between the symbionts. These include extra-cellular polysaccharides (EPSs) as well as proteins externalised via type-three secretion systems. These carbohydrates/proteins may be active in invasion of the root. At the time of writing, only flavonoids and Nod-factors have been chemically synthesised and of these only the former are available in large quantities. Field trials in North America show that seed application of flavonoids stimulates nodulation and nitrogen fixation in soybeans grown at low soil temperatures. The biological basis to these responses is discussed.

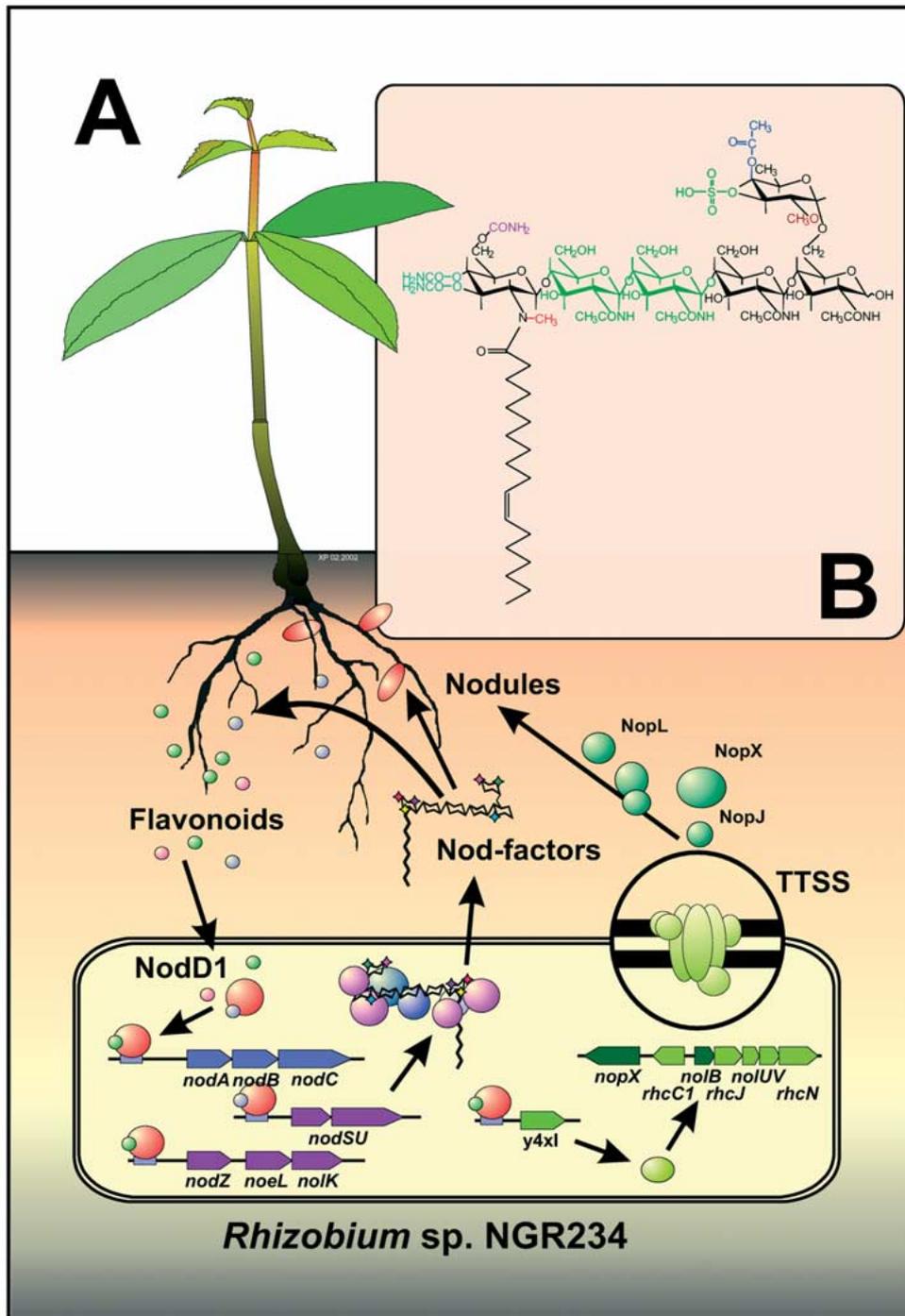
### Introduction

Under conditions of nitrogen limitation, rhizobia may induce formation of highly specialised organs on the roots or stems of their leguminous hosts. Within these nodules, rhizobia convert to an endosymbiotic form, the bacteroids, in which dinitrogen (N<sub>2</sub>) is reduced to ammonia. Specificity in symbiotic associations varies greatly amongst the symbionts. (*Azo*)*rhizobium caul-inodans* nodulates *Sesbania rostrata*, *Sinorhizobium meliloti* can initiate nodule formation on a few host plants (*Medicago*, *Melilotus* and *Trigonella*), whereas *Rhizobium* sp. NGR234, nodulates more than 112 genera of legumes, as well as the non-legume *Parasponia andersonii* (see Pueppke and Broughton, 1999).

Symbiotic interactions are controlled by signal exchange between the two partners (see Figure 1). Plants secrete flavonoids, phenolic compounds that, in conjunction with the bacterial activator protein NodD, induce the expression of rhizobial nodulation (*nod*, *nol*

and *noe*) genes. As a result, rhizobia produce Nod-factors. Nod-factors induce various plant responses, including root-hair deformation, cortical cell-division, 'pseudo-nodule' and nodule-formation (see Cullimore et al., 2001; Irving et al., 2000; Miklashevichs et al., 2001; Perret et al., 2000; Schultze and Kondorosi et al., 1998). Nodulation occurs via a cascade of developmental steps, that begin with bacterial colonisation of the rhizosphere and attachment to root-hairs. Rhizobia are then entrapped in the folds of curled root hairs where they penetrate the cell wall and form infection threads. Concomitantly, certain cortical cells divide to form nodule primordia and it is towards these primordia that the infection-thread grows. Further development gives rise to nodules that differ from tumours in having defined anatomical structures. Bacteria multiply within the infection threads that grow centripetally towards the root. Rhizobia begin to enlarge within the infection threads before being released into the cortical cells where they differentiate into bacteroids and begin to fix nitrogen.

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**Figure 1.** Flavonoid-inducible determinants of nodulation in *Rhizobium sp. NGR234*. (A) Flavonoids secreted from the roots trigger the expression of the rhizobial nodulation genes (*nod*, *nol* and *noe*) required for nodulation. Regulation of these genes is mediated by the transcriptional regulator NodD1. Most nodulation genes are involved in the synthesis of a family of nodulation signals called Nod-factors. In NGR234, NodD1 also controls *y4xl* that regulates the expression of genes encoding components of a bacterial type III secretion system (TTSS). Secretion of nodulation outer proteins (Nop) by the TTSS affects the ability of NGR234 to nodulate various host plants. (B) Nod-factors are modified lipo-chito-oligosaccharides i.e.,  $\beta$ -1,4-linked oligomers of *N*-acetyl-D-glucosamine, with a fatty acid replacing the *N*-acetyl group on their non-reducing terminus. The Nod-factor core is synthesised by NodC (a *N*-acetyl-glucosaminyltransferase required for chain elongation), NodB (a deacetylase that removes the *N*-acetyl group at the non-reducing terminus), and NodA (an acyltransferase that links the acyl chain to the deacetylated oligosaccharide). Synthesis of NGR234 Nod-factors requires a number of additional nodulation genes (e.g., *nodS* is involved in *N*-methylation, *nodU* in carbamoylation, and *nodZ* in fucosylation).

At least three different sets of symbiotic signals are exchanged between legumes and rhizobia during nodule development. Flavonoids, the first of these, emanate from the plant and interact with rhizobial NodD proteins that serve both as environmental sensors and activators of transcription of rhizobial nodulation genes. A second set of signals is synthesised when NodD-flavonoid complexes activate transcription from conserved 'nod-box' promoters. Most of the genes immediately downstream of these promoters are involved in the synthesis of Nod-factors that provoke deformation of root-hairs and allow rhizobia to enter the root through infection threads. Fine-tuning of *nod*-gene transcription is probably related to sequence variations in individual *nod*-boxes (there are 19 on the symbiotic plasmid of the broad host-range *Rhizobium* sp. NGR234; Freiberg et al., 1997). Other rhizobial products are necessary for continued infection thread development, and these represent a third set of signals. Amongst them are EPS and related compounds, as well as proteins exported by the type three-secretion system (TTSS) (see Broughton et al., 2000; Marie et al., 2001). Protein export is also dependent on NodD1 and flavonoids in NGR234 (Viprey et al., 1998). Flavonoids, Nod-factors, EPS and extra-cellular proteins (NOPs = nodulation outer proteins) are thus elements of the molecular dialogue that bacteria and legumes exchange during nodulation. Some of these compounds also have effects on non-legumes. Flavonoids applied to the root promote mycorrhizal colonisation of various plants (Vierheilig et al., 1998). Nod-factors mimic chitin oligomers in eliciting pH changes in tomato cell cultures (Staehelin et al., 1994a). Nod-factors are able to rescue a temperature-sensitive carrot mutant that is deficient in somatic embryo-genesis (De Jong et al., 1993). Rice plants transformed with the promoter of the early nodulin-gene *Mtenod12* (from the legume *Medicago truncatula*) fused to the  $\beta$ -glucuronidase reporter gene are sensitive to treatment with Nod-factors (Reddy et al., 1998). LCOs of *R. etli* induce systemic resistance against nematodes in potato roots (Reitz et al., 2000). EPS of *Rhizobium* sp. YAS34 may function in plant growth promotion and aggregation of sunflower rhizospheres (Alami et al., 2000). Our question is whether any of these signals could be used in agriculture to accelerate nodulation of legumes under specific environmental conditions in soils that already contain adequate populations of *Rhizobium*?

### Environmental effects on nodulation and nitrogen fixation

Nodule formation is strongly affected by sub-optimal soil conditions, such as temperature extremes, salt stress, high or low soil pH, low water content, pesticide application and nutrient deficiency (reviewed by Hungria and Vargas, 2000; Zahran, 1999). The availability of biotin and other water-soluble vitamins in the rhizosphere may also limit rhizobial growth (Streit et al., 1996). Nitrogen fertiliser application and high nitrate contents in the soil severely restrict *Rhizobium* infection, nodule development and nitrogen fixation in legumes. Breeding programmes have yielded legume genotypes with improved nitrogen fixation when grown at elevated nitrogen concentrations however (Caetano-Anollés and Gresshoff, 1991; Herridge and Rose, 2000; Zahran, 1999).

Introduction of *Rhizobium* strains with high tolerance to stress has been used to improve symbiotic efficiency and crop productivity in agricultural systems. In the central region of Argentina for example, tolerance of rhizobial strains to acidic soils (which have become progressively more acidic over the last two decades) is a prerequisite for nodulation of alfalfa (*Medicago sativa* L.). Consequently, research has focused on the isolation and characterisation of *Rhizobium* strains exhibiting acid tolerance and effective symbiosis with alfalfa (del Papa et al., 1999). It has been widely observed however, that the response to inoculation with specific rhizobia is strongly influenced by native soil rhizobia that compete with the introduced strain (see Dowling and Broughton, 1986; McDermott and Graham, 1990; Toro, 1996; Triplett and Sadowsky, 1992). Soil temperature is another variable that has important effects on nodulation of legumes (Lynch and Smith, 1993a,b, 1994; Walsh and Layzell, 1986). As an example, although soybeans (*Glycine max* (L.) Merr.) originate from sub-tropical Asia, attempts are constantly being made to adapt the plant to less favourable climates, especially in Europe and Northern America. Un-adapted soybean varieties require temperatures of 25–30 °C for optimal symbiotic activity. Soil temperatures below this range restrict nodulation and nitrogen fixation. For each degree of decrease in temperature within the range 25–17 °C, the lag between inoculation and the onset of nitrogen fixation is 2 to 3 days. At soil temperatures below 17 °C this restriction is even more pronounced with each decrease of 1 °C delaying the onset of nitrogen fixation by about one week (Zhang and Smith,

1994; Zhang et al., 1995a). Temperatures below 10°C are completely inhibitory to nodulation (Matthews and Hayes, 1982).

Microscopic observations show that rhizobial attachment to root-hairs, initiation of infection threads and nodule development in soybeans are progressively delayed at low root temperatures (Zhang and Smith 1994). Low temperatures also decrease biosynthesis and secretion of genistein (a flavonoid) from plant roots (Zhang and Smith, 1996a). In turn, it is possible that low temperatures affect nodule initiation by decreasing Nod-factor production. Of course, temperature has direct effects on the rates of chemical reactions, on molecular diffusion, the permeability of nodules to gases (particularly oxygen), on solubility, etc., that are difficult to separate from biological effects.

One of the few published molecular analyses of low temperature effects on nodulation concerns certain pea (*Pisum sativum* L.) cultivars. Nodulation of cv. Afghanistan by *R. leguminosarum* bv. *viciae* is at least partly controlled by Nod-factors that carry a modification on the reducing terminus (an acetyl group encoded by the acetyl transferase NodX; Firmin et al., 1993; Ovtzyana et al., 1999) as well as by a single genetic locus *sym2<sup>A</sup>* in the host-plant. *R. leguminosarum* bv. *viciae* strains producing Nod-factors lacking this modification cannot nodulate these pea plants at low temperatures. Somehow, blockage of nodulation is overcome at higher temperatures suggesting a temperature-sensitive gene-for-gene relationship between *nodX* and *sym2<sup>A</sup>* (Kozik et al., 1995; Lie, 1984; Olsthoorn et al., 2000).

#### *Effects of flavonoids on nodulation*

Flavonoids produced via the phenylpropanoid biosynthetic pathway (see Figure 2) are the strongest inducers of rhizobial nodulation genes (e.g., Firmin et al., 1986; Peters et al., 1986; Redmond et al., 1986). Many flavonoids are stored and released as glycosides or related conjugates. These conjugates, which are more soluble in water than aglycones, are usually less active in inducing rhizobial nodulation genes (and therefore Nod-factor production), but they can be hydrolysed to more active substances (Hartwig and Phillips, 1991).

Genistein and daidzein are important inducers of rhizobial nodulation genes in the early stages of symbiosis between soybean and *Bradyrhizobium japonicum*. Paau et al. (1990) reported that adding

flavonoid-containing soybean meal to the fermentation medium could increase the readiness of rhizobia to nodulate. Later observations showed that pre-incubation of *B. japonicum* cells with genistein increased nodulation and nitrogen fixation of soybeans growing at lower root temperatures (Zhang and Smith, 1995). Furthermore, genistein applied to seeds in the furrow at the time of planting, accelerated nodulation and increased N<sub>2</sub> fixation at low soil temperatures, which would normally delay or inhibit nodulation (Zhang and Smith, 1996b, 1997). Pre-incubation with genistein also encouraged nodule occupancy by the inoculant (Pan and Smith, 2000).

How do flavonoids stimulate nodulation? In soybean, as well as a number of other legumes, secretion of flavonoids into the rhizosphere is enhanced by rhizobia and purified Nod-factors (Dakora et al., 1993; Recourt et al., 1992; Schmidt et al., 1994). In turn, elevated flavonoid concentrations, through their effect on the induction of *nod*-genes, stimulate Nod-factor secretion by rhizobia. In other words, positive auto-regulation of flavonoid secretion occurs. As Nod-factors themselves play crucial roles in nodule development (Perret et al., 2000; Relić et al., 1994), it is thus likely that flavonoid concentrations remain below threshold levels under certain environmental conditions, such as low soil temperature.

It is also possible that exogenously applied flavonoids have direct effects on plants. Flavonoids act as auxin transport inhibitors (Jacobs and Rubery, 1988; Mathesius et al., 1998), thus inducing changes in the auxin/cytokinin balance of the root cortex (Boot et al., 1999; de Billy et al., 2001; Hirsch et al., 1997). Flavonoids applied to plants in the field may also promote colonisation by widespread vesicular arbuscular mycorrhizal fungi (AMF). AMF colonise plant roots and improve plant nutrition, mainly by acquiring and transferring phosphate from the soil to the host plant (Harrison, 1999). Under greenhouse conditions, various flavonoids applied to the root strongly stimulate mycorrhizal colonisation of many plants, including soybean (Vierheilig et al., 1998; Xie et al., 1995). In turn, colonisation by AMF promotes nodulation of soybeans (Kawai and Yamamoto, 1986; Zhang et al., 1995b). It is thus possible that the stimulatory effects of applied flavonoids on nodulation observed in field experiments result from accelerated mycorrhizal colonisation. Further research is needed to distinguish the relative importance of these different effects.

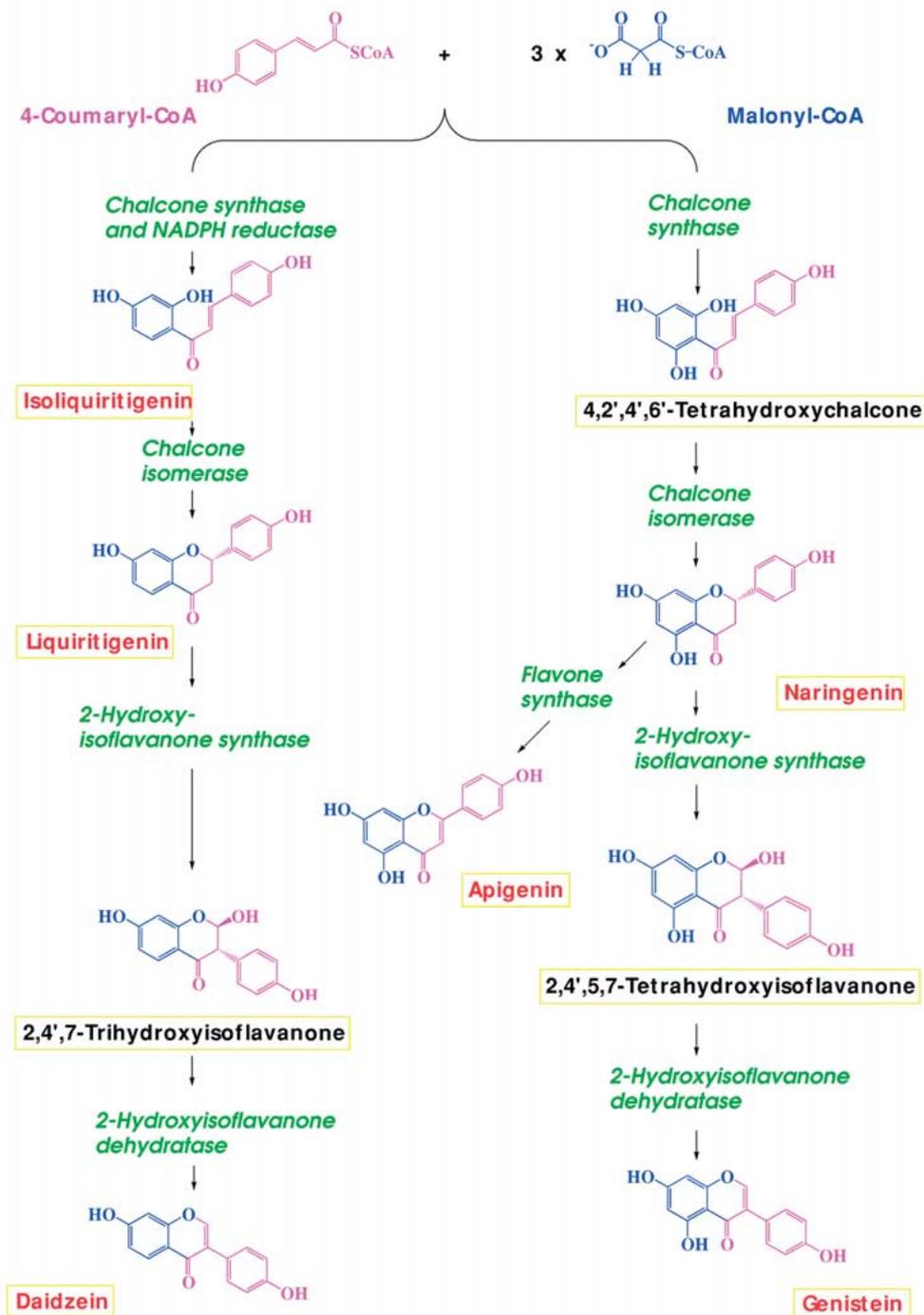


Figure 2. Biosynthesis of flavonoids and related compounds in plants. Two pathways exist. In most plants, the first committed step is catalysed by chalcone synthase (CHS). In certain species, the concerted action of CHS and an NADPH-dependent reductase generates isoliquiritigenin. In both cases, the succeeding reaction, which is shared by both biosynthetic pathways, is catalysed by chalcone isomerase. Those parts of the molecules that originate from 4-coumaryl-CoA are marked in purple while those derived from malonyl-CoA are shown in blue. Enzymes are given in green, while the names of the compounds are boxed in yellow. Names in red denote that the particular compound is found in legume rhizospheres (see Broughton et al., 2000), while the presence of those marked in black has not been demonstrated. The information was collated from O'Hagan (1991) and Buchanan et al. (2000).

Table 1. 'SoyaSignal' field tests in Canada and USA over a 6-year period (1994–1999). The data were collected from field trials in Ontario, Québec, and Prince Edward Island in Canada as well as North Dakota, Ohio, Illinois, Indiana, Iowa, Minnesota, and Wisconsin in the USA. The numbers represent the increase of grain yield after 'SoyaSignal' treatment (percentages over the non-treated controls)

Countries / Means	1994 (%)	1995 (%)	1996 (%)	1997 (%)	1998 (%)	1999 (%)	Average (%)
Canada	21.2	1.9	4.8	12.9	5.7	9.7	8.5
USA			5.6	7.9	5.4	5.5	5.5
Total	21.2	1.9	4.9	11.8	5.5	7.2	7.0
Number of trials	2	1	5	13	62	44	

### 'SoyaSignal' in North America Soybean Production

Of the molecular signals exchanged between legumes and rhizobia, only the flavonoids have so far been produced on an industrial scale. It is thus not surprising that they were the first to be exploited agronomically. A commercial product 'SoyaSignal', which consists mainly of genistein and daidzein, has been marketed in Northern America for about four years (Smith and Zhang, 1999). 'SoyaSignal' can be applied either directly to the seed or in furrows in soils that contain adequate populations of *Bradyrhizobium*. Results of more than 100 field trials in Northern America over a 6-year period show that 'SoyaSignal' significantly improves nodulation and nitrogen fixation, resulting in an average increase in grain yield of 7% (Table 1) (S. Leibovitch, P. Migner, F. Zhang and D. L. Smith, unpublished results). As expected, the results of 'SoyaSignal' application are temperature dependent. Strongest effects on yield were seen after cool springs, when planting temperatures were lower than 17°C.

### Agricultural uses of Nod-factors?

At micro- to nano-molar concentrations, Nod-factors trigger a series of early root responses, such as root-hair deformation, expression of early nodulin genes, cortical cell division and the induction of bacteria-free 'pseudo-nodules' on certain legumes (Cullimore et al., 2001; Irving et al., 2000; Miklashevichs et al., 2001; Perret et al., 2000; Schultze and Kondorosi et al., 1998). Nod-factors are also the signal required for bacterial entry into the root-hair. Rhizobial mutants unable to produce Nod-factors cannot infect legumes, but addition of purified Nod-factors enables these

mutants to enter (D'Haeze et al., 1998; Relić et al., 1993, 1994). Moreover, legumes treated with purified Nod-factors exhibit accelerated mycorrhizal colonisation, indicating a general 'symbiosis-promoting effect' by these molecules (Xie et al., 1995, 1998).

The fact that flavonoids enhance nodulation under field conditions raises the question of whether Nod-factors supplied directly with seeds would have similar stimulatory effects? Field trials of Nod-factor effects on nodulation (and AMF colonisation) are expensive to perform given the low yields obtained both chemically and via fermentation. Stability of Nod-factors in the soil presents another problem. Nod-factors are rapidly hydrolysed in the rhizosphere by chitinases and other plant enzymes of the host plant (Heidstra et al., 1994; Minic et al., 1998; Ovtsyna et al., 2000; Staehelin et al., 1994b, 1995). Compared to intact Nod-factors, the re-purified, hydrolysed derivatives are at least 1000-fold less active in inducing symbiotic responses, indicating that hydrolases of the host plant inactivate Nod-factors (Heidstra et al., 1994; Staehelin et al., 1994b). Non-*N*-acetylated Nod-factor derivatives are resistant to hydrolytic enzymes in the rhizosphere of alfalfa, but these molecules exhibit only low biological activity (Staehelin et al., 2000). Perhaps other modifications may confer increased stability while retaining biological activity. A promising approach would be to stabilise those glycosidic linkages in Nod-factors that are sensitive to hydrolytic degradation. In chemically synthesised Nod-factors for example, a  $\beta$ -1,4-glucosidic bond could be replaced with a  $\beta$ -1,3 linkage.

Elevated concentrations of Nod-factors however, desensitise root-hairs so rendering rhizobial entry impossible. Indeed, addition of Nod-factors to pea (cv. Afghanistan) roots inhibits nodule formation upon inoculation with *R. leguminosarum* by *viciae* strain TOM (Hogg et al., 2002). Hence, Nod-factor inac-

tivation by enzymes of the host plant could be an important feed-back mechanism, which avoids overstimulation of root-hair responses (Staelin et al., 1995). Finally, pre-existing nodules generally suppress further nodule development ('auto-regulation of nodule development', Caetano-Anollés and Gresshoff, 1991), and Nod-factors can reasonably be expected to have similar effects. Nevertheless, finding a way to stabilise Nod-factors and supply them to seeds could be a challenge to applied research.

Further research is also required to improve Nod-factor signalling in other ways. As an example, the effect of *Rhizobium* strains carrying a flavonoid independent transcription activator (FITA) *nodD* gene (Spaink et al., 1989), which produce Nod-factors in the absence of flavonoids (i.e., Nod-factor synthesis is constitutive), should be tested under various environmental conditions. Current research that focuses on host genes involved in the perception and transduction of Nod-factor signals (Cullimore et al., 2001; Perret et al., 2000) opens up another avenue for improving symbiosis. A plant gene (the early nodulin gene *enod40*) has been identified, whose levels of expression modify the sensitivity to Nod-factors and the early stages of *Rhizobium* infection. *M. truncatula* plants that over-express *Mtenod40* nodulate more rapidly than non-modified plants (Charon et al., 1999).

### Concluding remarks

Populations are dependent on stable food supplies. Continuous improvements marked by occasional revolutions in agriculture have permitted vastly more people to be fed. Eventually, advances in molecular genetics will also lead to increased food production. Progress will be come in a number of ways — transformed plants are being tailored to resist insects, or pathogens. Cereals containing higher quality proteins are being developed. A less obvious avenue of plant improvement involves knowledge of how beneficial micro-organisms interact with plants. Bacterial symbioses with legumes are amongst the best studied of these associations, and it is becoming apparent that the molecular signals that they exchange can be manipulated in agronomically significant ways. As the rhizosphere is a relatively hostile environment, signals emanating from or going to the plant must be relatively stable. For this reason, it is not surprising that flavonoids, which regulate bacterial gene expression and are secreted by the plant, were the first to be used in commercial agriculture. Applied to soybeans

growing at low soil temperatures, they significantly improve nodulation and nitrogen fixation, resulting in increased grain yields. Other applications of flavonoids with different legumes in various environments will undoubtedly be found.

Equally, Nod-factors could have important agricultural benefits. Limited stability in the rhizosphere and overstimulation of root-hair responses may restrict their use however. Ways of making them resistant to degradation by hydrolytic enzymes while maintaining their biological activity will have to be found. Finally, the other signals exchanged between legumes and their symbionts that include various forms of polysaccharides and proteins (Broughton et al., 2000) seem at the moment too expensive to prepare, and too difficult to deliver to the target. As they are probably even less stable than Nod-factors, they will most likely be the last such signals to find agricultural applications.

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