

Nodulation competitiveness in the *Rhizobium*-legume symbiosis

N. Toro

Successful nodulation of legumes by rhizobia is a complex process that, in the open field, depends on many different environmental factors. Generally, legume productivity in an agricultural field may be improved by inoculation with selected highly effective N₂-fixing root nodule bacteria. However, field legume inoculation with *Rhizobium* and *Bradyrhizobium* spp. has often been unsuccessful because of the presence in the soil of native strains that compete with the introduced strain in nodule formation on the host plants. This ability to dominate nodulation is termed 'competitiveness' and is critical for the successful use of inoculants.

Key words: Competitiveness, legume, *Rhizobium*, symbiosis.

Inoculation of legumes with *Rhizobium* and *Bradyrhizobium* spp. is a common worldwide agricultural practice and is used to increase the yield of leguminous crops without adding nitrogenous fertilizers. *Rhizobium* and *Bradyrhizobium* have been released into the soil since the end of the last century and is one of the best examples of deliberate microbial release into the environment. The success of rhizobial inoculants requires that the strains be highly efficient in N₂-fixation and also highly competitive for nodulation. With the current knowledge about the *Rhizobium*-legume symbiosis it is possible to achieve the first requirement but the second is still a significant barrier to the development of good performance rhizobial inoculants.

Nodulation of legumes by rhizobia is a complex process that requires the production of specific plant flavonoids that induce bacterial genes involved in the biosynthesis of specific signal molecules, lipooligosaccharides (LOS), that trigger the first steps of nodule formation. Additionally, in the open field, nodulation is dependent on different environmental factors: soil pH; the presence of ions such as aluminium, manganese or nitrate; the deficiency of essential elements such as calcium or phosphorous, and the temperature. Furthermore, the inoculant strains have to compete for nutrients with a resident microbial community living in the

rhizosphere, and which is well adapted to that environment. Generally, it is considered that inoculation with rhizobia should be performed under two different situations: 1) soils which are depleted or contain a low indigenous rhizobial population and 2) when there is an established but inefficient rhizobial population. Very often the use of rhizobial inoculants to resolve the first problem has led to the second, due to the low effectiveness of the introduced strains. To solve the competition problem two main approaches are being studied in addition to the improvement of the strains and inoculation methods: 1) the use of rhizobial strains with an altered host range or legume genotypes with restricted *Rhizobium* susceptibility and 2) the construction of strains highly competitive for nodulation. In this review, the progress in our understanding of nodulation competitiveness, the environmental factors involved and the approaches used to address the problem of the success of rhizobial inoculants is summarised. The 'nodulation competitiveness' problem has been recently reviewed by Triplett 1990a, Triplett & Sadowsky 1992, Olivares *et al.* 1993 and Streeter 1994.

Environmental Factors Affecting Nodulation Competitiveness

Rhizobial inoculants used to be prepared by culturing the bacteria and adding small samples to a carrier, usually non-sterile peat, which was then used to inoculate seeds. The methods of growing the cells, seed-inoculation and storing

The author is with the Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, C/Professor Albareda 1, 18008 Granada, Spain; fax: 34-58-129600.

procedures are important steps for the viability, competitiveness and survival of the inoculant strains once released in the soil. These aspects of the management of rhizobial inoculants have been reviewed elsewhere (Date & Roughley 1977; Smith 1987; Roughley 1988; Catroux & Amarger 1992) and therefore will not be considered here.

Rhizobia in the soil exist as saprophytes and are able to use a diverse range of energy sources from sugars to phenolic compounds. The presence of the host plant stimulates the rhizobia population in the rhizosphere, and it is in this environment that the inoculant strain has to compete for nutrients with the existing microbial community, including the indigenous rhizobia population. Plants and microorganisms use the soil not only as a support matrix but also as a source of energy and nutrients. Therefore it is not surprising that the soil's characteristics will influence the survival and competition not only of the indigenous population but also of the introduced strain. Furthermore, legumes are also affected by the soil conditions which in turn will influence the symbiotic process.

Neutral and slight alkaline soil pH favour legume nodulation, but at low pH many legumes are not nodulated. Carbon exudated by the plant root decreases in acidic soils thereby reducing the supply of substrate to the microorganisms living in the rhizosphere. In acidic soils the low pH leads to an increased availability of aluminium and manganese which become toxic, as well as to a low concentration of phosphate, calcium and molybdenum. Rhizobia are sensitive to the available soil manganese and aluminium, fast-growing strains appear to be less tolerant to aluminium than slow-growing rhizobia. Nevertheless, rhizobial strains tolerant to low pH and high aluminium concentration can be selected and in fact they have been successfully introduced as inoculants in Western Australian soils for growing Medics and pasture legumes (Glenn & Dilworth 1994; Howieson 1995). Studies on bean nodulation showed that the soil pH can influence which symbiotype of *R. leguminosarum* bv. *phaseoli* will competitively nodulate *P. vulgaris* (Frey & Blum 1994).

Soil temperature is also an important environmental variable that affects general biological activity. Nodulation and N₂-fixation are observed under a wide range of temperatures with an optimum between 20 and 30°C. Elevated temperatures affect nodule initiation and development in temperate legumes, whereas in tropical legumes it is mainly N₂ fixation efficiency that is affected. Low temperatures reduce nodule formation and N₂ fixation in temperate legumes. Temperature changes affect the competitive ability of *Rhizobium* strains, and there are also specific temperature-sensitive legume-*Rhizobium* combinations. This is the case in *R. leguminosarum* bv. *trifolii* TA1 that forms nodules with *Trifolium subterraneum* cv. Woogenellup in the laboratory above 25°C but not below 22°C, although it nodulates other cultivars at low temperatures.

In the field this strain does not nodulate this cultivar (Gibson 1968; Lewis-Henderson & Djordjevic 1991a,b).

Other factors such as salinity, presence of nitrate, low concentrations of calcium or phosphate and the presence of pesticides affect the early events of the nodulation process, but as in the case of soil pH it should be possible to select strains adapted to different soil conditions that could be further improved in their N₂-fixation effectiveness and competition abilities. It is worth noting that the soil environment has specific traits that are difficult to reproduce in the laboratory. Thus, in relation to soil pH, a chemical characteristic easy to simulate *in vitro*, strains of both *R. meliloti* and *R. trifolii* that grew well at low pH in the laboratory did not necessarily have superior persistence traits in the field at low pH (Howieson *et al.* 1988).

Finally, biotic factors other than the indigenous rhizobia population affect the survival and competition of the inoculant strain. For example, the presence of protozoa also stimulated by the carbon flow in the rhizosphere will affect the performance of the inoculant microorganisms according to a predator/prey relationship. The presence in the soil of epiphytic bacteria such as *Erwinia herbicola* may suppress nodulation by the production of a toxin that will block rhizobial attachment to the root hairs. Bacteriophages and bacteriocines will also affect the performance of the rhizobial inoculants.

Further information about the effect of environmental factors on nodulation and N₂-fixation can be found in reviews by Thies *et al.* (1992), Catroux & Amarger (1992), Glenn & Dilworth (1994) and Bordelau & Prevost (1994).

Rhizobial Strains and Legumes with Altered Host Range

To overcome the problem of low nodule occupancy by inoculant strains in the field it would be important to select plant genotypes that limit nodulation by indigenous rhizobia, whilst allowing nodulation by the inoculant strain. Several genotype-specific nodulation genes (GSN) have been found that allow nodulation of specific plant genotypes within a given legume species. These genes act either positively or negatively to control nodulation specificity. The *nolA* gene of *B. japonicum* strain USDA 110 encodes a transcriptional regulator that exhibits homology to MerR (Sadowsky *et al.* 1991) and when transferred by conjugation into serocluster 123 strains, confers on them the ability to nodulate serogroup 123-restricted plant genotypes. It has been suggested that No1A exerts a repressive effect on isoflavone-induced expression of the *nodD(1)* and *nodYABC-SUIJ* operons (Dockendorff *et al.* 1995). In *R. fredii* a chromosomal locus *nolC* has been identified (Heron *et al.* 1989; Krishnan & Pueppke 1991) that influences the cultivar specificity of strain USDA 257. The parental strain is able to nodulate 'Peking' soybean and certain agronomically

advanced cultivars, while retaining the ability to form N₂-fixing nodules on 'Peking'. The *nolC* gene has homology with *dnaJ* of *E. coli* and is apparently able to modify the level of EPS produced (Krishnan & Pueppke 1992). Mutants of *B. japonicum* serogroup 123 have also been isolated which overcome nodulation-restriction mediated by PI 377578 and soybeans containing the *RJ4* allele (Judd *et al.* 1993). Other soybean plant introduction genotypes (PI) that restrict nodulation by *B. japonicum* strains have been identified, e.g. genotype PI 417566 that restricts nodulation by *B. japonicum* MN1-1c (USDA 430) strains in serogroup 129 and USDA 110. Host-controlled restriction of nodulation is strain-dependent and mutants that overcome nodulation restriction have been selected (Lohrke *et al.* 1995).

Recent studies suggest that restriction of nodulation by *B. japonicum* is determined by the genotype of the root and is dependent on plant growth temperature. Microscopic analyses have indicated that the soybean introduction genotypes restrict nodulation of *B. japonicum* at symbiotic stages which occur both before and after the formation of nodule primordia (Sadowsky *et al.* 1995).

Strains of both *R. tropici* and *R. etli* have been selected that showed a limited ability to nodulate and fix N₂ with wild type *Phaseolus vulgaris* genotypes G21117 and G10002, but that were effective in symbiosis with the bean cultivated genotypes Jamapa and Amarillo Gigante. Competition experiments also showed that the restricted strains could compete successfully with the highly efficient strain CIAT632 eliciting over 40% of the nodules on Jamapa (Montealegre & Kipenolt 1994).

Dominant serogroups have been also identified in *R. meliloti* and *R. leguminosarum* bv. *trifolii*. Interestingly, in a recent study it was shown that the distribution of *R. meliloti* genotypes isolated from the soil differed markedly from those sampled from nodules (Bromfield *et al.* 1995). *R. leguminosarum* bv. *viciae* is able to nodulate *Pisum*, *Vicia*, *Lens* and *Lathyrus* plants. It was shown that both pea plants and faba bean plants have a distinct preference for particular strains of *Rhizobium* from a naturally occurring population. Competitiveness for pea nodulation correlated with the ability to catabolize homoserine (Hynes & O'Connell 1990).

The loci *nodM* and *csn1* have been shown to control nodulation of subterranean clover by *R. leguminosarum* bv. *trifolii* ANU794, this phenotype is controlled by a single recessive plant gene (*rw1-1*). Introduction of either NodT or NodL into ANU794 conferred tap root nodulation ability on the recipients. The increase in the proportion of LOSs possessing acetyl residues positively affects the nodulation ability which may have important implications for inter-strain competition (Lewis-Henderson & Djordjevic 1991b; de Boer *et al.* 1994; de Boer & Djordjevic 1995).

One of the best examples of genotype specific nodulation is the TOM strain of *R. leguminosarum* bv. *viciae* which is

able to nodulate the Afghanistan cultivar of peas (Brewin *et al.* 1980). The European strains are not able to elicit nodules in this cultivar but strain PF₂ is capable of blocking nodulation of Afghanistan peas by TOM. The *nodX* gene (Davis *et al.* 1988) located in the symbiotic plasmid near *nodJ*, is responsible for the ability of TOM to nodulate Afghanistan cv; *nodX* mediates an acetylation of pentameric Nod factors on the C-6 of the terminal reducing glucosamine residue (Firmin *et al.* 1993). In strain PF₂, genes *nodABC* and *nodFE* (Dowling *et al.* 1989) are involved in the competitive blocking phenotype (Cnb), this phenotype (Winarno & Lie 1979) is related to the amount of Nod factors made, strain TOM forms relatively low amounts of the Nod factors which are crucial for the nodulation of cv. Afghanistan peas (Downie *et al.* 1994). The symbiotic process is dependent on a gene-for-gene interaction, *nodX* in the bacteria and *sym-2* in the plant, the latter may function as a receptor or as a processor of the specific information carried by the nod factor.

Bacterial Genes Involved in Nodulation Competitiveness

The ability of a particular rhizobial strain to compete for nodule formation may be indirectly altered by changes in phenotypes such as motility or polysaccharide production or by any other alteration in the symbiotic process. Thus, mutations in the *nod*, *exo*, *lps*, *ndo* and *nif* loci that do not block nodule formation have been found to decrease the nodulation competitiveness of the rhizobial strains. In addition to the loci described above, there are rhizobial genes which are not required to establish the symbiotic process but that affect nodulation competitiveness. Some of these genes have known functions, for example the rhizopine and bacteriocin genes, whereas for others the functions are unknown, e.g. the *nfe* (nodule formation efficiency) genes. The possible role of proline catabolism in competition will also be discussed.

The Nodulation Formation Efficiency Genes (*nfe*)

The *nfe* genes are defined as genes involved in nodulation efficiency and competitiveness, which are not required to establish the symbiotic process (Sanjuan & Olivares 1989). Increased nodulation efficiency results in both earlier nodulation and a larger amount of elicited nodules (Toro & Olivares 1986). In inter-strain competition, the presence of *nfe* genes determines a higher nodulation occupancy in a 1 to 1 ratio of strains within a coinoculated mixture. The *nfe* genes were found initially in *R. meliloti* (Sanjuan & Olivares 1989; Soto *et al.* 1993) whereas *nfeC* was defined in *B. japonicum* (Chun & Stacey 1994). With the exception *nfeD*, the *nfe* genes are preceded by *RpoN*-regulated promoters and *nfeA* and *nfeC* can also be transcribed from a second promoter different to the *RpoN* promoter (Soto *et al.* 1993;

Chun & Stacey 1994). The function of the *nfe* genes is so far unknown, only the *nfeD* (Soto *et al.* 1994b) encoded protein showed any homology with established data banks. *NfeD* exhibits a 26% identity with ornithine cyclodeaminases (OCDs) from *Agrobacterium tumefaciens*, an enzyme that transforms ornithine directly into proline with the release of ammonia. These results suggest that at least some of the *nfe* genes may be related to secondary catabolic pathways; perhaps the selective use of some carbon sources available during infection would result in a higher nodule occupancy.

Rhizopine Production and Competition

In some *Rhizobium*-legume associations novel symbiotic compounds known as rhizopines are synthesized in nodules by bacteroids and are used by the free-living rhizobia present in the infection threads or the rhizosphere, but by few other bacteria (Murphy & Saint 1992). Genes for the synthesis (*mos*) and for the catabolism (*moc*) of the corresponding rhizopines are located in the Sym plasmids. Production of inositol rhizopines has been detected in *R. meliloti* and *R. leguminosarum* bv. *viciae* at a frequency of 10 and 14%, respectively. The *mos* but not the *moc* loci are regulated by NifA/NtrA regulatory system. Rhizopines influence competition for nodulation but the mechanism by which rhizopine production and catabolism lead to higher nodule occupancy is unclear. It has been suggested that rhizopines may influence the number of rhizobia by providing a selective growth substrate in the rhizosphere and infection threads. Alternatively, the rhizopine may have a direct effect on the nodulation process by preventing nodulation by strains which cannot catabolize the rhizopine. The rhizopine concept opens different possibilities in the manipulation of rhizobial inoculants. It should be possible to improve the competitiveness and even the persistence in the soil of beneficial rhizobial strains by transferring rhizopine genes to these bacteria or by selecting rhizobia producing interesting rhizopines. Rhizopines and their role in symbiosis and competition have been recently reviewed by Murphy *et al.* (1995).

Bacteriocin Production and Competition

Production of bacteriocin provides to the producer-bacteria a selective advantage in nodule occupancy. The role of bacteriocin in nodulation competitiveness has been studied in *R. leguminosarum* bv. *trifolii*. The best characterized is the anti-rhizobial bacteriocin known as trifolitoxin which is produced by the ineffective strain T24 allowing it to be highly competitive for nodulation when coinoculated with a sensitive strain (Triplett & Barta 1987). The *tfx* genes have been isolated and sequenced (Triplett 1988; Breil *et al.* 1993) and also transferred to effective strains such as TA1 (Triplett 1990b) which conserved the competitive ability when co-inoculated with a sensitive strain. Recently, it has

been found that sensitivity to trifolitoxin is extended to distinct groups of the α -proteobacteria (Triplett *et al.* 1994). However, it is unclear whether the production of this rhizobial peptide antibiotic trifolitoxin can be used for improving the performance of inoculant strains under field conditions.

Proline Dehydrogenase and Competition

In the *Rhizobium*-legume symbiosis, the C₄-dicarboxylic acids have been generally considered the major carbon source exported from plant cells to the bacteroids that support the N₂-fixation process (Finan *et al.* 1983). However, it has not been established that this is the exclusive or even the most efficient energy source under different environmental conditions and little is known about the carbon sources used by the microsymbiont during nodule formation and invasion. Proline is usually catabolized in prokaryotic cells *via* pyrroline-5-carboxylate by means of proline dehydrogenase (ProDH) yielding NADPH, or by proline oxidase. In *B. japonicum* it has been suggested that ProDH activity plays a role in N₂-fixation (Kohl *et al.* 1994). However, in other rhizobial species ProDH could not be related to N₂-fixation efficiency. Recently ornithine cyclodeaminase activity was found in *R. meliloti* (Soto *et al.* 1994b) and it was shown that the ornithine catabolic pathway in this bacteria proceeds *via* proline formation. An *R. meliloti* mutant affected in ornithine and proline utilization and which lacks ProDH activity has been also isolated. Apparently this enzymatic activity is required for nodulation efficiency and competitiveness (Jiménez-Zurdo *et al.* 1995). Whether the manipulation of ProDH activity in *R. meliloti* can improve the competitive ability of inoculant strains remains to be elucidated.

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