

## Competition for Nodulation

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### 1. Introduction

Nitrogen (N) is the nutrient that most often becomes limiting for plant growth. Soybean may obtain this nutrient from the air, thanks to its ability to perform a symbiosis with bacteria of the genera *Bradyrhizobium* (*B. japonicum*, *B. elkanii*, and *B. liaoningense*), *Sinorhizobium* (*S. fredii* and *S. xinjiangense*) and *Mesorhizobium* (*M. tianshanense*). These bacterial species are collectively known as soybean-nodulating rhizobia, but only *B. japonicum*, *B. elkanii*, and *S. fredii* were used as commercial inoculants for soybean crops, with *B. japonicum* being the most widely employed. In this symbiosis the rhizobial partner reduces the atmospheric  $N_2$  to  $NH_3$  in a reaction catalyzed by the nitrogenase enzymatic complex, while the plant partner supplies the C sources that provide the energy required for the  $N_2$  reduction reaction. Since atmospheric  $N_2$  is an unlimited source of N, the process of  $N_2$  fixation is of great potential for sustainable agriculture, and in the special case of legumes, the symbiosis is so efficient that in hydroponic culture the plant may satisfy all its N needs without resorting to any other N source. In addition, this symbiosis is a biological process that does not require fossil energy consumption, and does not leak any contaminant byproduct to the biosphere. Therefore, the inoculation of legume crops with selected rhizobial strains of high  $N_2$  fixation performance is an extended practice in agriculture since decades ago. In parallel, the industry of inoculants is very active, commercializing a variety of formulations with different strains and combinations with other plant-promoting rhizobacterial species such as *Azospirillum brasilense* or *Pseudomonas fluorescens*. For the farmers, inoculating a legume crop with active rhizobia is a simple procedure, and its economic cost is much lower than applying chemical fertilizers. All these advantages are, however, obscured by the fact that in field crops the symbiotic  $N_2$  fixation seldom provides the expected results, and the plants may consume the N from the soil.

Several factors account for this low performance of  $N_2$  fixation in field crops. In energetic terms,  $N_2$  fixation is more costly for the plant than soil N uptake and therefore soil N is preferred when this source is not limiting, or when  $N_2$  fixation is inefficient (Salon et al., 2009). This may be appreciated if one takes into account that the symbiosis only occurs in a specialized organ known as root nodule. It is there where the rhizobia differentiate into the state able to reduce  $N_2$  –the bacteroid– and where the  $O_2$  concentration is lowered at levels compatible with nitrogenase activity (Patriarca et al., 2004). Therefore, rhizobia must infect the roots and trigger the development of nodules, which finally will be occupied by the rhizobia. During the earliest steps of nodule development and root infection (Ferguson et al., 2010), the plant-rhizobia relationship is more similar to a pathogenesis than to a mutualistic symbiosis: rhizobia invade plant tissues, consume plant energy resources,

induce a tumor-like development, and proliferate inside plant cells, without any benefit for the plant until nodules are completely developed and  $N_2$  fixation starts. Indeed, rhizobial strains with low  $N_2$ -fixing efficiency or unable to fix  $N_2$  trigger typical plant defense reactions and lead to a weakening of the plant. In such a scenario, N removal from soil may be quite significant. It has been estimated that in soils with low N content, a good  $N_2$ -fixing symbiosis may provide 70 % of N that plant needs (i.e. 70 % of all plant N coming from the air) while an inefficient symbiosis only provides 20-30 % of N (Unkovich & Pate, 2000). Therefore, the difference between a good and a bad symbiosis would be around 40 % plant N being obtained from the air or the soil, respectively. N contents of soybean grains are around 2.5 % w/w, depending on the cultivar, all this N being removed from the ecosystem at grain harvest. Hence, considering a mean yield of 2,500 kg ha<sup>-1</sup> the difference between a good and a bad  $N_2$ -fixing symbiosis equals 25 kg N ha<sup>-1</sup> that are respectively conserved or removed from the soil each year.

In soybean-producing countries like Argentina, these crops are grown in soils with low N content, either because the soils were previously cropped with species of high soil N demand, such as wheat or corn, or because they are in marginal areas. Therefore,  $N_2$  fixation becomes a key input of sustainable soybean cropping, because this species has also a high N demand, and thus, the N that cannot be provided by  $N_2$  fixation must be supplied as chemical fertilizer, which involves many environmental problems and has a higher cost.

The efficiency of the  $N_2$ -fixing symbiosis depends on many factors. Of primary importance are the total number of nodules formed in each plant, and the  $N_2$ -fixing activity of each nodule. As mentioned before, nodulation has an energetic cost to the plant and therefore the number of nodules cannot be too high. Instead, an optimal number of nodules able to provide the necessary amount of fixed  $N_2$ , with a reasonable energetic cost involved in its maintenance, is regulated by the plant. In this way, once a given number of active nodules are established, the plant progressively inhibits the formation of new nodules (see below). This indicates that both the plant genotype (by its ability to assimilate fixed  $N_2$  and its ability to control the number of nodules) and the rhizobial genotype (by its  $N_2$ -fixing activity) are key determinants of the symbiosis performance. However, being a biological process, the environment also plays a fundamental role, not only by its influence on the activity of each partner, but also by its interaction with both genotypes.

Competition for nodulation between inoculated rhizobia and different rhizobial strains resident in the soil is a striking example of this complexity. Normally, the soils are populated with rhizobia either from the indigenous bacterial population or introduced by the inoculants used in previous crop seasons. Since rhizobia are soil bacteria, they readily adapt to a new soil and exchange genetic material with the local soil microbiota. However, in the soil there is not a high selective pressure for high  $N_2$  fixation performance and therefore, genetic drift leads to dispersion of the  $N_2$ -fixing potential among different genotypes with diverse efficiencies. Therefore, the soil rhizobial population is often of high efficiency to nodulate the plants, but of medium to low efficiency to fix  $N_2$ . Hence, the competition of this population for plant nodulation may prevent the newly inoculated strains to occupy a significant proportion of the nodules, leading to lack of  $N_2$  fixation response to inoculation (Toro, 1996). To get an approximation to the problem, we can consider that each nodule contains a clone of bacteria derived from a single precursor bacterium that initiated the root infection. Occasionally, two bacterial clones may share a nodule, but it is extremely unfrequent to find nodules with more than two clones. Given that a single soybean plant might possess, at most, in the order of  $10^2$  nodules at maturity,

this is the order of magnitude of the bacterial individuals that can survive the root penetration. However, in soils with several years of soybean cropping there may be in the order of  $10^5$  to  $10^7$  soybean-nodulating rhizobia colonizing the proximity of the root –the rhizosphere– and therefore, for each bacterial cell that succeeds in penetrating the root, there are 100 that remain outside. Considering that nodules are protected environments for rhizobia, a harsh competition is established among rhizospheric rhizobia to gain access to the root nodules. In this process, the bacterial genotypes will have a prominent role in defining their competitiveness, but also the plant genotype will dictate how and when the bacteria will be allowed to penetrate the roots, the interaction between the bacterial and plant genotypes will determine which strains will be favored, and the interaction of the environment with both genotypes will determine the relative advantages for each bacterial strain.

Soybean seeds are inoculated with around  $10^5$ - $10^7$  rhizobia seed<sup>-1</sup>, but more than 80 % of the rhizobia die within the first 2 h after inoculation (Streeter, 2003). From the survivors, only a small percentage reaches the rhizosphere after sowing (López-García et al., 2002). Thus, given the above figures, obtaining around 10 % of all the nodules occupied by the inoculated strain, as is currently accomplished in soybean crops, may be considered quite successful, but still it is completely insufficient to get a significant increase in plant N<sub>2</sub> fixation above the levels obtainable without inoculation. Therefore, to get a significant proportion of plant N coming from the air it is imperative to improve inoculant's competitiveness to obtain significantly higher percentages of nodules occupation, and active research about this problem is being done since decades. In this chapter we will provide a look on the methods employed to study the problem of competition for nodulation, the bacterial and plant traits that may influence the competition, the ecological aspects that modulate the plant and rhizobia interaction, and how these factors may be managed in order to profit the symbiosis to increase soybean crop yields.

## 2. Methods employed to study competition for nodulation

In any method, at least one of the competing strains has to be selectively labelled in order to discriminate the nodules occupied by it. Labels employed are not different than those used for strain selection or tracking, including antibiotic resistances, fluorescent proteins, antibodies, DNA probes or reporter proteins such as  $\beta$ -galactosidase or GUS. Special properties of certain rhizobial strains, like melanin production, were also employed (Castro et al., 2000). However, there are some caveats to be kept in mind for labelling. In the first place, it has to be demonstrated to which extent the label may alter the nodulating ability or the competitiveness of the strain. Ideally, labelling should not alter any of them, but provided the effect is accurately measured in comparison to the unlabeled wild type, strains altered after labelling may be confidently used (Thomas-Oates et al., 2003). The introduction of labels in replicative plasmids has the advantages of avoiding undesired modifications in the rhizobial genomic background, and the possibility of introducing the same plasmid in different host strains. However, the plasmid replication as well as the expression of genes carried by it may consume energy from the bacterial cell, and therefore may affect competitiveness. This may explain why strains labelled in this way tend to have diminished their competitiveness (Mongiardini et al., 2009). By difference, the introduction of labels in the genomic DNA of the bacterial cell may cause undesired loss or alteration of a given function. Several rhizobial genomes were completely sequenced

(<http://genome.kazusa.or.jp/rhizobase>) and therefore now it is possible to choose the exact location where a label may be introduced without affecting any coding region (Pistorio et al., 2002). However, both replicative plasmids and reporter genes recombined in the genomic DNA render gene-manipulated strains that cannot be safely released into the environment. An alternative may be the choice of a natural selection, such as for antibiotic resistance; however, enrichment in a strain tolerant to high antibiotic doses is also not desirable for the environment, because this trait may be dispersed by horizontal gene transfer, and in addition, several reports also indicate that selection for resistance to high antibiotic concentration may yield strains with diminished competitiveness (Lochner et al., 1989; Spriggs & Dakora, 2009; Thomas-Oates et al., 2003). Therefore, if this method is chosen, a careful assessment of symbiotic and competitive abilities of the antibiotic-resistant derivatives must be carried out before employing them in competition studies (López-García et al., 2002; Spriggs & Dakora, 2009). Intrinsic antibiotic resistance to low doses (without enrichment by selection) may be used but this method is not so efficient to distinguish an indicator strain in a population (Spriggs & Dakora, 2009). Other methods, which do not involve any manipulation of the strains, are antibody or DNA labelling. Antibody labelling of different strains recovered from nodules may be efficiently carried out by ELISA (Spriggs & Dakora, 2009); however, antibodies cannot distinguish among genotypically related strains. DNA labelling may be carried out either with probes or by PCR. In this last methodology, DNA fingerprints may be obtained, which have better discriminative potential than antibodies. The disadvantage of these methods is that they involve more manipulation and equipment, and are more expensive than the other methods mentioned.

Once the labeled indicator strain is available, the next step is plant culture and inoculation. Here there also exist a wide variety of methods, which can be divided in field assays and laboratory assemblies. In the last case, the rooting substrate may be sterile or nonsterile soil, or an artificial substrate such as sand, vermiculite, perlite or a mixture of them. Even more artificial root environments were employed, including liquid plant nutrient solution, agarized media, and plastic growth pouches. Depending on how close to the natural environment are the experimental conditions used, more accurate predictions on rhizobial competitiveness in field crops may be obtained, but more variables need to be considered. In many cases, an unnecessarily high number of variables may obscure the conclusions about a given factor under study, such as the effect of a bacterial single mutation. The influence of some important environmental variables will be considered in another section.

Field trials are normally done in plots arranged in complete random blocks. Factorial analysis also is employed and is useful when more than one variable is under study (López-García et al., 2009). As mentioned before, field trials are the most close to the real crop, but at the same time are subjected to the whole ensemble of natural variables. For this reason, field trials should not be restricted to a single location or crop season, and an analysis of weather, soil, and previous land use should accompany the nodulation experiment. Generalizations require repeating the experiment in more than one season and ideally, at several locations. Otherwise, it must be clearly stated that the conclusions are restricted to the site and season where the experience took place.

The most popular laboratory assemblies used to contain the rooting substrate are the pots and the Leonard jars (Vincent, 1970). They differ in the watering method, which in turn lead to differences in the water content of the substrate. Pots are watered from the top at given intervals and therefore, it is possible to maintain the field capacity within reasonable limits,

provided that drainage is incorporated to the pots (for instance, by means of holes at the bottom). The disadvantage of this method is that, since periodic watering is required, pots are quite exposed to cross-contamination. To avoid it, layers of inert material are commonly placed on top to prevent penetration of undesired rhizobia to the pots. In addition, it is always necessary to distribute uninoculated pots among the test pots to serve as negative controls and for assessing the absence of cross-contamination. By contrast to pots, Leonard jars are not irrigated from top. They are assemblies consisting in an upper container filled with the desired rooting substrate and a bottom reservoir that contains the irrigation solution. The rooting substrate is moistened by a wick running the length of the upper container and extending out into the solution reservoir. In this way, the irrigating solution rises by capillary from the reservoir avoiding the need of irrigation by hand (the only requirement is to maintain the liquid in the reservoir). Hence, Leonard jars are less prompt to cross-contamination than pots but provide higher moisture around roots. Later we will see how this variable may affect competitiveness.

When soil is used as rooting substrate it may be sterile or nonsterile, depending on whether the influence of biotic factors is to be analyzed. Since this experimental technique is commonly performed in a greenhouse, variables associated to climatic factors are eliminated. However, it is often overlooked that if several soil samples are pooled and sieved, soil variables like soil structure and patching are also eliminated. Various methods were also used for soil sterilization. When available, gamma radiation is preferable to heat (dry or wet), since that method is less likely to provoke changes in organic matter. The other rooting substrates mentioned above are chemically inert and possesses water retention capacity, normally in the order vermiculite > perlite > sand. Although these substrates do not substitute for soil, they share some common properties like water retention and porosity. Thus, they may be used in combination with defined plant nutrient solutions to provide a controlled environment where some of the most important soil variables (e.g. moisture, porosity, pH, and nutrients level) may be manipulated. Moreover, plant nutrient solutions are used in many experiments as liquid or agarized media, or to wet plant stands, like a paper towel, without employing any other rooting substrate. In these cases the influence of both water potential and porosity is lost, which could lead to misinterpretations when phenomena like bacterial motility or chemical diffusion may play a role on the results. The essence of measuring competition for nodulation rests on the count of the proportion of nodules occupied by a given strain on a plant. Therefore, these approaches need also an appropriate statistical analysis. The methods more widely employed are the analysis of variance (ANOVA) and the  $\chi^2$  test. Whatever the method applied, two important aspects are the number of replicas of each treatment and the proper data input in the statistical analysis. The number of replicas depends on the variability of the experimental material but it is not recommended to pool all the nodules from different plants. Instead, nodules from each plant should be treated separately in order to express the results in a per plant basis, thus considering each plant as an experimental unit. Regarding data input, it has to be kept in mind that the above mentioned statistical methods suppose that certain conditions are obeyed by the data. One important condition is the homogeneity of the variance, which means that all the experimental groups have the same variance. Although the number of nodules in different samples obeys this condition, the proportion of nodules occupied by a given strain does not. In this case, the variance is maximal for a proportion of 0.5, and tends to zero as the proportion approaches 0.0 or 1.0 (Lison, 1968). To obtain a dataset obeying the homogeneity of variance, these proportions must be transformed to the arc sin root square before applying a test such as

ANOVA to them. Then, the whole analysis is carried out with the transformed data, but if averages are to be compared (for instance, with the Tukey test) the data need to be used with the original values, i.e. are not used with the transformed values. A rather frequent error in the literature is the use of the proportions (or percentages) of nodule occupation without transformation in ANOVA tests, which in this case lose sensitivity.

A special method was developed in the 80s by Amarger and Lobreau (1982). Since its proposal, the method was widely employed and allows the determination of strains competitiveness quite accurately. It is based on the use of two competitor strains at a range of concentration ratios. For instance, strain A is competed against strain B at 100:1; 10:1; 1:1; 1:10, and 1:100 A:B initial concentrations ratios in the inocula, which are termed  $I_A : I_B$ . Then, nodules are harvested and the proportions of nodules occupied by strain A and strain B,  $N_A$  and  $N_B$  respectively, are registered for each  $I_A : I_B$  ratio. With these data a plot of  $\log(N_A/N_B)$  against  $\log(I_A/I_B)$  is constructed, which gives a straight line that cuts the ordinate  $\log(N_A/N_B)$  axis when  $I_A/I_B$  equals 1.0, thus giving the  $C_{A:B}$  value that represents the competitiveness of strain A on strain B when both are inoculated at exactly the same concentration. Although this method is laborious because several inoculum rates must be tested for each pair of competing strains, the result of  $C_{A:B}$  is more exact than the obtained in a single competition at approximately equal concentrations. This accuracy is especially valuable when differences in competitiveness are not wide (as may be the case when soil isolates are evaluated against a collection strain).

### 3. Bacterial and plant traits that affect competition for nodulation

Both the rhizobia and the plant genotypes influence the competition for nodulation. In particular, genes determinant for symbiosis, like those for production, transduction and binding of symbiotic soluble signals, development of infections and nodules, and  $N_2$  fixation, will have obvious effects on competitiveness, since they affect rhizobial and plant activities that are prerequisite for competition. Therefore, these genes will not be reviewed here and the reader is forwarded to excellent recent reviews on the subject of plant infection and nodulation (Ferguson et al., 2010; Patriarca et al., 2004). Furthermore, rhizosphere colonization is a previous and necessary step for nodulation and therefore, any gene or set of genes that favor the adaptation of rhizobia to the environment may have a positive effect on competition for nodulation, although as we will see later, the relation between efficiency in rhizosphere colonization and competitiveness for nodulation is not so straightforward. Anyway, tolerance against environmental stress, metabolic efficiency in the use of nutrients from the rhizosphere, growth speed and persistence in the environment, and resistance against predation are also related with competitiveness. After rhizosphere colonization, adhesion to root surfaces is also required for nodulation and therefore, surface components of both symbionts have a role in competitiveness. However, other genes unrelated to these activities may also influence competitiveness in unexpected ways. In the following, some important traits for which specific effects on competitiveness for nodulation were observed, are reviewed.

#### 3.1 Bacterial traits

Some approaches were developed to identify genes associated to competitiveness, which required solving two problems: 1) testing a high number of candidate genes in plant assays and 2) screen the competitiveness of all these candidates. These problems were addressed in two ways, although none of them in soybean-nodulating rhizobia.

The earliest attempt was to employ a highly competitive strain of *Rhizobium etli* to extract from it large DNA fragments (in the order of 25 kb) that were introduced into cosmids, which were used to transform the less competitive reference strain. These transconjugants were inoculated in mass on common bean plants, the nodules were obtained, and rhizobia occupying these nodules were extracted and used in a second round of inoculation, nodules extraction and rhizobia recovering (Beattie & Handelsman, 1993). According to the authors, if competitiveness conferred by the cosmids was as high as that of the donor strain, these two rounds of enrichment by nodules passage should allow a high chance of recovery of clones with enhanced competitiveness. The authors recovered nine such clones, but surprisingly, when these clones were cured from the cosmids their high competitiveness remained intact. In addition, the introduction of the cosmids in the reference strain did not render this strain more competitive, whereby the enhanced competitiveness of these nine clones seems to be the consequence of the enrichment procedure and not a trait conferred by the DNA fragments obtained from the more competitive strain. Unfortunately this strategy was not continued and the causes for the higher competitiveness obtained are unknown. More recent studies indicated that bacterial strains cultured continually under laboratory conditions tend to lost some traits related with their adaptation to the natural environment (Marks et al., 2010). Therefore, the enrichment procedure by nodules passage might have reversed a laboratory adaptation of the reference strain, which could include heritable genetic changes. If this is the case, such an enrichment procedure may be considered for strains improvement without genetic manipulation.

The second approach was to employ signature-tagged mutants (Pobigaylo et al., 2008). Briefly, different short DNA fragments are introduced in transposons in such a way that a collection of transposons is obtained, where each transposon can be identified by the DNA sequence tag that it carries. Then, these transposons are used to mutagenize a given bacterial strain and as a consequence, a set of mutants where each member can be distinguished from the others is obtained. By combining this technique with microarray screening, each tag can be mapped into a corresponding insertion sequence. Pobigaylo et al. (2008) employed this technique to inoculate two sets of 378 tagged *S. meliloti* mutants on alfalfa plants and were able to recover 67 mutants attenuated in symbiosis among which 23 were altered in genes that affected competitiveness but are not obviously related to nodulation or N<sub>2</sub> fixation. Many of these mutants were affected in metabolic or transport functions and two encode hypothetical proteins. The question as to whether modification of the expression of any of these genes could improve rhizobia competitiveness remains to be elucidated.

The above-mentioned work was done in aeroponic plant cultures, which allowed inoculation and homogeneous distribution of a high number of mutant strains as well as the recovery of many nodules for screening. However, as mentioned earlier, such an artificial assembly does not allow evaluation of other important features, like rhizosphere colonization. Rhizosphere is a nutrient enriched zone in comparison to the rest of the soil, due to the many compounds released in plant root exudates, among which various sugars, organic acids, aminoacids and vitamins serve as sources for microbial growth, and flavonoids and related compounds may act as signal molecules. Therefore, rhizobia colonize this soil compartment and an important question is if this colonization involves an active mobilization of the rhizobia towards rhizosphere. Rhizobia are motile bacteria, expressing active flagella and able to move by swimming and swarming (Bahlawane et al., 2008; Braeken et al., 2007; Daniels et al., 2006; Nogales et al., 2010; Soto et al., 2002; Tambalo et al., 2010). For many years, various studies informed that rhizobia can move from soil to legume

roots to initiate root colonization and infection (Brencic & Winans, 2005; Fujishige et al., 2006; González & Marketon, 2003; Yost et al., 2003). Evidence includes measurements of rhizobial dispersal in soil (Lowther & Patrick, 1993), the *in vivo* observation of *S. meliloti* motility towards infection sites on legume roots (Gulash et al., 1984), the characterization of rhizobial attraction by root exuded molecules and specific flavonoids (collectively referred to as chemoattractants) in *R. leguminosarum*, *S. meliloti*, and *B. japonicum* (Barbour et al., 1991; Caetano-Anollés et al., 1988a; Chuiko et al., 2002; Gaworzewska & Carlile, 1982; Pandya et al., 1999), and the observation of diminished root adsorption, colonization, and nodulation rates in motility defective mutants of *S. meliloti* and *R. leguminosarum* (Ames & Bergman, 1981; Caetano-Anollés et al., 1988b; Hunter & Fahring, 1980; Mellor et al., 1987; Parco et al., 1994).

This notion of rhizobial movement in soil towards root exudates and surfaces underlies also the agronomic practice of seed inoculation for soybean crops. Accordingly, it is expected that rhizobia will move in some way from the inoculation site on the seed surface to the infectable root cells that lie near the root tip to produce a nodule, even when these infection sites continuously migrate away from the inoculation site as the roots grow (Bhuvanewari et al., 1980). However, most of the above evidence was obtained in laboratory experiments performed in saturated aqueous media. When porous media more similar to soil were employed, some conflicting results were obtained. It was reported long ago that vertical motility of rhizobia in the soil profile is restricted to a few millimeters unless other factors, like percolating water, earthworms, or tillage, aid in moving rhizobia to a greater depth (Madsen & Alexander, 1982). This correlates with a poor root apex colonization of seed-inoculated *B. japonicum* when these root apical regions –the infectable zone– penetrate a few centimeters into the rooting substrate, and with the observation that *B. japonicum* inoculated on soybean seeds sowed in vermiculite where a rhizobial population, isogenic with the inoculant, was previously established, occupied less than 20 % of the nodules regardless of its intrinsic infectivity (López-García et al., 2002). More recently, the motility of *S. meliloti* towards root exudates in a peat substrate was again observed as being very restricted, unless nematodes able to be attracted by specific volatile compounds produced by *Medicago truncatula* are also present. In these experiments, rhizobia were observed to be transported both on the nematodes surface and into the nematodes gut (Horiuchi et al., 2005). In agreement with these results, Liu et al. (1989) found that lack of motility barely affected nodulation competitiveness of *B. japonicum* in unsaturated, non-sterile soil, and suggested that the encounter between roots and rhizobia depends not on rhizobial movement, but on soil exploration by growing roots. In agreement with these observations, *B. japonicum* non-motile flagella-defective mutants were similarly competitive as the wild type in vermiculite at field capacity but were totally displaced from nodules occupation by the wild type when the vermiculite was flooded, indicating that bacterial swimming may be a factor of competition for nodulation only in this last situation (Althabegoiti et al., 2011). Therefore, in soils at field capacity rhizobial motility may be retarded by many factors that are absent both in flooded rooting substrates or liquid media (Horiuchi et al., 2005; Liu et al., 1989; López-García et al., 2002; Madsen and M. Alexander, 1982; McDermott & Graham, 1989). Among these factors we can mention chemoattractant diffusion, which at field capacity is slower due to the lower water potential, paths impairment due to the tortuosity and size of the soil pores, and retardation of bacterial displacement due to attachment/detachment to and from soil particles (Tufenkji, 2007; Watt et al., 2006). Hence, it was suggested that the limited motility of rhizobia in soils at field capacity might be a primary factor in the problem of competition for nodulation (López-García et al., 2002). To solve this problem with the



inoculants, two measures were proposed: the use of in-furrow inoculation instead of seed inoculation, and the selection of inoculant strains with higher motility (Althabegoiti et al., 2008; Bogino et al., 2008; López-García et al., 2009). Both techniques yielded promising results in soybean field assays (López-García et al., 2009), but the in-furrow inoculation method still needs technical improvement for its application to soybean crops at a similar cost-benefit relationship as seed inoculation.

Since plants roots often release protons, rhizosphere is usually an acidic compartment (Hinsinger et al., 2005). In addition, the interior of root hairs where rhizobia will penetrate is also acidic. Therefore, acid tolerance was raised as a trait related with efficiency of rhizosphere colonization and root infection. However, few studies were carried out in *B. japonicum* since it was early recognized that slow-growing *Bradyrhizobium* species are more tolerant to acid stress than fast-growing rhizobial species, being able to tolerate pH below 5.0 (Graham, 1992). Moreover, regarding acid stress, soybean plants seem more sensitive than *B. japonicum* and hence, most of the efforts directed towards improvement of acid tolerance were directed to the plant partner of this symbiosis. Despite this, acid tolerance may be involved in competitiveness even in acid tolerant species. Studies in *R. tropici* indicated that the substitution of Ala for Ser in a domain of AtvA, a protein homologous to the virulence protein AcvB from *Agrobacterium tumefaciens*, caused a significant drop in competitiveness. The authors observed that mutation of the membrane protein LpiA, whose gene lies in the same operon as *atvA*, caused a similar effect; however, these competitiveness defects took place also under non-acidic conditions, and none of these changes altered nodulation or N<sub>2</sub> fixation (Vinueza et al., 2003). Therefore, the requirements of these genes seems to authentically affect competitiveness, and might be related to coping with the acidic environment of the root surfaces or the interior of plant cells rather than a general environmental adaptation.

Rhizosphere may be also a dry environment because of the continuous root water suction activity. Therefore, drought tolerance is also a trait that might be relevant for rhizosphere colonization and competitiveness for nodulation. Among the strategies employed by microbes to cope with desiccation, the accumulation of solutes such as trehalose seems widespread. *B. japonicum* cannot use trehalose as C source, and therefore, trehalose incorporation to growth media leads to its accumulation in the cytoplasm. This treatment yielded *B. japonicum* cells more tolerant to desiccation and led to increased survival of rhizobia inoculated on soybean seeds (Streeter, 2003). Moreover, trehalose spontaneously accumulates in *B. japonicum* during desiccation. Three pathways of trehalose biosynthesis were found in this bacterial species: trehalose synthase, trehalose-6-phosphate synthetase, and maltooligosyltrehalose synthase. A transcriptomics study of *B. japonicum* during desiccation showed that the expression of the genes encoding trehalose-6-phosphate synthetase (*otsA*), trehalose-6-phosphate phosphatase (*otsB*), and trehalose synthase (*treS*) was significantly induced and in parallel, the activity of trehalose-6-phosphate synthetase and the trehalose intracellular concentration were increased thus indicating that trehalose accumulation is a regulatory response of desiccation tolerance in *B. japonicum* (Cytryn et al., 2007). However, no studies are available about the role of trehalose accumulation on competition for nodulation in soybean. In *R. leguminosarum* by *trifolii* a mutant unable to accumulate trehalose was less competitive for nodulation than the parental strain but capable of nodulation and N<sub>2</sub> fixation (McIntyre et al., 2007). In agreement with this result, an *S. meliloti* triple mutant in *treS*, *treY*, and *otsA* was impaired in competitiveness although its intrinsic nodulation and N<sub>2</sub>-fixation abilities were not altered (Domínguez-Ferreras et al.,

2009). By difference, an *otsA* mutant of *R. etli*, although still able to accumulate trehalose, was defective in nodulation and nitrogenase activity in common bean (Suárez et al., 2008). Therefore, the requirements of these genes for nodulation in the absence of competitors might be more stringent for determinate nodules formation. An interesting result was obtained by Ampomah et al. (2008), who observed that both *S. meliloti* and *S. medicae thuB* mutants, unable to catabolyze trehalose (and therefore, accumulating this solute in the absence of stress conditions) were improved in competition for nodulation in two cultivars of *M. sativa* and one of *M. truncatula* but were equally competitive as the wild type for rhizosphere colonization. Moreover, the authors observed that *thuB* expression is induced during *S. meliloti* penetration of root hairs and suggested that osmotic stress and trehalose accumulation occur in this environment. Therefore, desiccation tolerance appears to be necessary for root infection, independently of the drought conditions in the rhizosphere.

In addition to physicochemical factors such as barriers to motility, acidity, and dryness, the ability of rhizobia to use rhizospheric nutrients for growth is also an important factor for rhizosphere colonization, root infection and competitiveness (Toro, 1996). Moreover, several rhizobial species may induce the production of specific nutrients by the plant, in an analogous manner as *A. tumefaciens* induces the production of opines, which only this bacterial species may catabolyze. Therefore, these substances, derived from *myo*-inositol, were termed rhizopines (Murphy et al., 1987). Rhizopines are produced by bacteroids into the nodules, exported to the rhizosphere, and consumed there by free-living rhizobia. Only a limited range of strains of *S. meliloti* and *R. leguminosarum* were found to produce and consume rhizopines, so that this ability is considered a selective advantage for rhizosphere colonization and competition for nodulation. In both species, rhizopine catabolism requires a functional *myo*-inositol catabolic pathway (Bahar et al., 1998; Galbraith et al., 1998). Although rhizopine production/consumption was not observed in soybean-nodulating rhizobia, *myo*-inositol catabolism was found as related with nodulation competitiveness in this symbiosis. An *S. fredii* mutant in *idhA*, which encodes *myo*-inositol dehydrogenase, nodulates normally but is severely impaired in competition for nodulation. In addition, this mutant is defective in N<sub>2</sub> fixation and bacteroid morphology (Jiang et al., 2001). In an *R. leguminosarum* *viceae* strain that does not produce rhizopines, catabolism of *myo*-inositol was also found as required for competition for nodulation, although this requirement was not observed for rhizosphere colonization, nodulation or N<sub>2</sub> fixation, whereby the authors concluded that *myo*-inositol catabolism is required during early plant root infection (Fry et al., 2001). Other genes related with catabolism of specific rhizosphere substances were found as determinant for competition for nodulation. Rosenblueth et al. (1998) searched for genes induced by bean root exudates in a library of *R. tropici* and found the *teu* genes, which are induced only by *Phaseolus vulgaris* and *Macroptilium atropurpureum* root exudates (both plants are symbionts of *R. tropici*). However, the compound responsible for *teu* operon induction was not identified. To search whether a similar pathway existed in other rhizobial species, the authors incubated the bean root exudate with bacteria from different rhizobial species to sequester the inducer and found that only *R. etli*, *R. leguminosarum* *phaseoli* and *R. giardinii*, all symbionts of *P. vulgaris*, had this capacity. Therefore, the system of *teu* induction by root exudates seems a specific trait of this group of rhizobia and plant species. An *R. tropici* CIAT 899 mutant in *teuB* was not affected in nodulation when inoculated alone, but was less competitive than the wild type for nodulation at various inoculum rates.

In addition to the ability of metabolizing specific substrates, the general metabolic activity of rhizobia also influences their competitiveness. Normally, soybean is cultivated in N-limited soils, and in addition, N limitation is a prerequisite for nodulation and N<sub>2</sub> fixation. It is well known that legumes are able to inhibit nodulation in the presence of abundant soil N-sources, but research about the influence of N-limitation on the rhizobial side is scarcer. López-García et al. (2001) found that *B. japonicum* can grow with minute amounts of NH<sub>4</sub><sup>+</sup> in the culture medium, and N-limitation leads to derepression of glutamine synthetase I and II, change in C-sinks with accumulation of exopolysaccharides (EPS) at the expense of polyhydroxybutirate (PHB), and higher sensitivity to genistein for *nodC* expression. Correlating these physiological changes, the rhizobia are more infective and competitive for nodulation. These changes can be exploited in the formulation of improved inoculants. Likewise, *Burkholderia mimosarum* competitiveness to nodulate three *Mimosa* species was increased in low-N incubation media (Elliott et al., 2009). Nitrogen assimilation is regulated by the *ntt* system, which includes *nttB-nttC-nttY-nttX*. Two genes downstream this system is encoded the small RNA binding protein Hfq. Transcriptomic and proteomic analyses of *S. meliloti* mutants in *hfq* indicated that alteration of this gene leads to imbalance in C and N metabolism, suggesting that the mutant strain tends to use aminoacids instead of primary C substrates as energy sources (Torres-Quesada et al., 2010). Furthermore, the authors found that *hfq* mutation did not affect nodulation but severely diminished N<sub>2</sub>-fixation and when the mutants were coinoculated with the wild type in 1:1 relationship on alfalfa plants, no nodules were found occupied only by the mutant. However, this diminished competitiveness might be due to the repression of *iolC*, *iolD*, *iolE* and *iolB*, encoding *myo*-inositol catabolic activities, which as we saw, are required for competition at an early step of nodulation (Fry et al., 2001). The adaptations mentioned before, i.e. acid tolerance, drought/osmotic tolerance, and ability to metabolize specific organic nutrients from root exudates, are important examples of improvement in cell fitness to the root environment. However, competence may be exerted not only by doing things better than competitors, but also by precluding competitor's activity. Soybean-nodulating rhizobia are known to have natural resistance to several antibiotics, among them chloramphenicol, neomycin, and penicillin (Cole et al., 1979). In addition, some strains of other rhizobial species are able to produce an anti-rhizobial substance known as trifolitoxin. This is a ribosomally synthesized, posttranslationally modified peptide, which was found in *R. leguminosarum* bv *trifolii* T24, against which various  $\alpha$ -proteobacterial species are sensitive (Triplett, 1994). Among the soybean-nodulating rhizobia, *S. fredii* strains are sensitive, while *B. japonicum* seems resistant. Trifolitoxin production and resistance was considered as an interesting trait to enhance competitiveness for nodulation. Therefore, the ability to produce trifolitoxin was introduced with a replicative plasmid in *R. etli*. Then, the competitiveness of trifolitoxin-producer or non-producer *R. etli* strains against a sensitive strain for nodulation of common beans was assayed in field trials, in soils with a low bean-nodulating rhizobial population (in the order of 10<sup>2</sup> rhizobia g of soil<sup>-1</sup>). As a result, the trifolitoxin-producer strain occupied significantly more nodules than the sensitive strain, while the non-producer strain did not differ from the sensitive strain. In turn, grain yield was not modified in the inoculated or in uninoculated beans, indicating that the indigenous soil population was proficient for N<sub>2</sub> fixation (Robledo et al., 1998). However, the release of high numbers of rhizobia genetically modified to produce antimicrobial substances involves a number of serious environmental concerns, whereby this technology needs more studies before its commercial implementation.

After rhizosphere colonization, rhizobial adhesion to root surfaces is also a key aspect in competitiveness. Therefore, cell-surface characteristics conferred by surface polysaccharides are important for competitiveness (Bhagwat et al., 1991; Parniske et al., 1993; Quelas et al., 2010; Zdor et al., 1991). Rhizobial adhesion depends on several factors such as the medium composition where rhizobia and plants are put in contact, the composition of the culture medium where rhizobia were grown previously, and rhizobial growth state at the moment of their contact with the roots (Vesper & Bauer 1985; Smit et al., 1992). In addition, Vesper & Bauer (1985) observed that in a batch culture of *B. japonicum* only a subpopulation of bacterial cells is proficient for adhesion. This is consistent with more recent findings indicating that bacterial culture populations are not homogeneous even under controlled growth conditions (Ito et al., 2009). Moreover, bacterial cells seem to recognize specific adhesion sites in the plant roots, as mediated by plant and rhizobial agglutinins (Laus et al., 2006; Lodeiro & Favelukes, 1999; Loh et al., 1993; Mongiardini et al., 2008). These agglutinin-mediated modes of rhizobial adhesion are related to infectivity and competitiveness. In *B. japonicum* a lectin called BJ 38 was described, which mediates both polar adhesion among rhizobial cells that form special structures known as stars or rosettes, and polar adhesion of rhizobial cells to the soybean cells (Ho et al., 1990; 1994). A *B. japonicum* mutant defective in BJ38 activity was less infective on soybean plants (Ho et al., 1994). This bacterial lectin is located at one cell pole of the rhizobia (Loh et al., 1993) but it is unknown whether it is part of a larger cell appendage. Vesper & Bauer (1986) found that *B. japonicum* pili are required for adhesion to soybean roots, but it is uncertain whether BJ 38 is part of these pili. Similarly to BJ38, a bacterial agglutinin called RapA1 was found in the cell poles of *R. leguminosarum* and *R. etli* (Ausmees et al., 2001). Overproduction of this agglutinin in *R. leguminosarum* by *trifolii* led to higher rhizobial adhesion to different plant roots, but had no effect on the speed of nodulation in clover (Mongiardinini et al., 2008). However, the overproducing rhizobia were more competitive than a control strain for clover nodulation (Mongiardinini et al., 2009). In addition to bacterial agglutinins, the plant agglutinins also exert an influence on rhizobial adhesion and competitiveness. Pretreatment of *B. japonicum* in low concentrations of soybean seed lectin before plants inoculation improves rhizobial adhesiveness, infectivity, and competition for nodulation (Halverson & Stacey, 1986; Lodeiro et al., 2000). This lectin is bound by the bacterial EPS at the opposite cell pole of BJ38, in a growth state-dependent manner (Bhuvanewari et al., 1977). It was found that the sugar receptor in the EPS is galactose (Bhuvanewari et al., 1977), and mutants unable to incorporate galactose in their EPS are severely impaired in lectin binding, adhesion to soybean roots, infectivity, and N<sub>2</sub> fixation (Pérez-Giménez et al., 2009; Quelas et al., 2006; 2010). This sugar moiety is modified according to the physiological state of the bacteria: rhizobia in exponential growth have in their EPS acetylated galactose, while rhizobia in stationary phase have methylated galactose. Likewise, acetylated galactose has higher affinity for lectin than methylated galactose, and rhizobia in exponential phase bind more lectin, adhere better to the roots and are more infective and competitive than rhizobia in stationary phase (Bhuvanewari et al., 1977; Lodeiro & Favelukes, 1999; López-García et al., 2001; Vesper & Bauer, 1983). Soybean lectin binding to rhizobia may be enhanced by culture conditions that increase the amount of EPS: as we mentioned before, *B. japonicum* cultured under N-limiting conditions produces more EPS at the expense of PHB, and this EPS overproduction leads to higher soybean lectin binding activity of the bacterial cells, which become more infective and competitive to nodulate soybean against isogenic bacteria grown in normal N media (López-García et al., 2001). Likewise, in *R. leguminosarum* by *trifolii* and *R. etli* the overexpression of the regulatory genes *psaA* and *rosR* led to increased

EPS production and competitiveness for nodulation (Bittinger et al., 1997; Janczarek et al., 2009). Therefore, the possibilities of manipulating the expression of agglutinins in the rhizobia (Ho et al., 1994; Mongiardini et al., 2009) or increasing rhizobial sensitivity to plant agglutinins by culture conditions (López-García et al., 2001) or gene manipulation are interesting ways to increase rhizobial competitiveness.

Adhesion of bacteria to diverse surfaces leads to development of biofilms, which are complex structures, where bacteria differentiate from the single-cell planktonic state (Stoodley et al., 2002). Therefore, many determinants of bacterial adhesion to plant roots also play a role in biofilm formation on inert surfaces (Danhorn & Fuqua, 2007). However, it is controversial whether biofilm formation is related to nodulation. Although biofilms may be formed on legume roots, the time required for the development of a mature biofilm is larger than the time required for root infection and nodule initiation. In addition, factors affecting both processes like soybean seed lectin or the basic core of lipochitooligosaccharide Nod factors seem to be required in different manners for legume root infection or biofilm formation (Fujishige et al., 2008; Pérez-Giménez et al., 2009). Hence, biofilm formation and nodulation were regarded as alternative strategies for rhizobial survival in the soil rather than sequential steps of the symbiosis (Pérez-Giménez et al., 2009). In the soil, free-living rhizobia may tend to form biofilms on biotic or abiotic surfaces (Seneviratne & Jayasinghearachchi, 2003) and this may also explain their low motility at field capacity (see above).

### 3.2 Plant traits

The importance of competition for nodulation is also highlighted by the fact that plants exert a control on the number of nodules formed in the roots. The earliest nodules are often the most active in N<sub>2</sub> fixation. Therefore, the occupation of the earliest nodules by the inoculated strain is of prime importance to determine the global N<sub>2</sub> fixing activity of the plant. Control of nodulation involves a systemic signaling mechanism that was described thanks to the availability of hypernodulating mutants, which lose the autoinhibition of the formation of new nodules once sufficient nodules were formed. This autoregulation is systemic and the signals responsible for this pathway were not yet found. The mutations are related to the ability to nodulate in the presence of high concentrations of combined N compounds, and the insensitivity to ethylene or light (Oka-Kira & Kawaguchi, 2006). In soybean, the process of autoregulation of nodulation involves the production of a cue signal in the nodulated root, called "Q", which travels to the shoot where it is perceived by a LRR RLK with a serine/threonine kinase domain called *GmNARK*. As a response to the perception of "Q" in the leaves, a shoot-derived inhibitor (SDI) is produced and released to the roots, where it inhibits further nodulation (Ferguson et al., 2010). Current work is in progress to elucidate the chemical nature of "Q" and SDI. The "Q" signals may be CLAVATA3/ESR-related (CLE) peptides, highly modified by proline hydroxylation and glycosylation, and recent work identified three such CLE peptides in soybean, two of which may be related to nodulation inhibition by *B. japonicum* and the other, by nitrogen (Reid et al., 2011). In turn, the SDI seems a low molecular weight (< 1,000 Da) molecule, which is heat-stable and seems not RNA or protein (Lin et al., 2010).

The plant growth regulator ethylene is also an inhibitor of nodulation, although it is not clear whether ethylene takes part in the autoregulation response (Ding et al., 2009; Oka-Kira & Kawaguchi, 2006). As mentioned before, nodulation is an energy-consuming process, and the role of ethylene might be related to prevent this process if environmental conditions are not adequate (or alternative N-sources are available in the soil) in order to save

photosynthates in stressful situations. Nevertheless, strains able to locally counteract the nodulation inhibition by ethylene seem more competitive for nodulation. Some strains of *B. elkanii* are able to produce an ethylene synthesis inhibitor called rhizobitoxine [2-amino-4-(2-amino-3-hydropropoxy)-transbut-3-enoic acid]. Rhizobitoxine-producing strains were more competitive for nodulation of *M. atropurpureum* (Siratro) than non-producing strains (Okazaki et al., 2003). Likewise, the expression of 1-cyclopropane-1-carboxylate (ACC) deaminase in free-living cells of *M. loti* enhanced their competitiveness to nodulate *Lotus japonicus* and *L. tenuis*. This enzyme degrades the ethylene precursor ACC and therefore, lowering ACC levels in the rhizosphere or during initial infection might have been avoided inhibition of nodulation in a local manner (Conforte et al., 2010). However, this strategy may be not useful in the soybean symbiosis, since nodulation of this species seems not sensitive to ethylene (Schmidt et al., 1999).

A very interesting phenomenon that seems not related with autoregulation is restriction of nodulation of certain soybean genotypes by some serogroups of *B. japonicum* and *S. fredii* (Cregan & Keyser, 1986; 1988). Despite the initial interpretation of this phenomenon, discrimination of serogroups is not absolute, since restriction is not necessarily involving all the members of a given serogroup (Scott et al., 1995). Genetic studies lead to the identification of plant loci controlling restriction of nodulation, in particular, the dominant genes Rj2, Rj3, and Rj4, which restrict nodulation by strains in the USDA 122 and c1 serogroups, and the recessive genes Rj1, Rj5, and Rj6 that restrict nodulation by virtually all bradyrhizobia strains (Pracht et al., 1993; Weiser et al., 1990; Williams & Lynch, 1954). Therefore, the use of such genes in cultivated soybeans may help in selecting a set of soybean-nodulating rhizobial strains for nodulation in detriment of an undesired soil rhizobial population (Devine & Kuykendall, 1996). However, discrimination in host-controlled restriction of nodulation seems not so specific and more studies are required before this strategy can be transferred to farmers. Some advances were done in the molecular characterization of the soybean genes involved in host restriction of nodulation. The Rj1 gene was mapped to the soybean molecular linkage group D1b of chromosome 2 and was identified as the Nod factor receptor *Gm NFR1a*. Its recessive allele has a 1-bp deletion that introduces a premature stop codon eliminating the protein kinase domain of *Gm NFR1a* (Lee et al., 2011). The dominant Rj2 gene as well as its allele Rfg1, which restricts *S. fredii* serogroups related with USDA 257, were mapped to the soybean linkage group J in chromosome 16 and encodes Toll interleukin receptor/nucleotide-binding site/leucine-rich repeat (TIR-NBS-LRR) class of plant resistance (R) proteins (Yang et al., 2010). Therefore, this genetic system of host restriction of nodulation has similarity with the gene-for-gene resistance system against plant pathogens. Counterpart genes in the bacterial side were also identified. The PI (plant introduction) 417566 genotype restricts the USDA 110 serogroup (Lohrke et al., 1995). Interestingly, host-restriction of nodulation requires high inoculum doses of *B. japonicum* USDA 110, while low-inocula are not restricted (Lohrke et al., 2000). It was found that USDA 110 mutants in *nodD2* are not restricted at high cell-densities, and are more competitive for nodulation than the non-restricted USDA 123 serogroup (Jitackson & Sadowsky, 2008). *nodD2* is part of the complex circuit of nodulation genes expression in *B. japonicum* (Loh & Stacey, 2003). This gene is activated by *nolA*, which in turn is activated by a special quorum signal of *B. japonicum* called bradyoxetin. Furthermore, *nodD2* is an inhibitor of *nodD1*, the activator of nodulation genes in *B. japonicum*. Thus, at high cell densities, *nodD2* indirectly responds to quorum sensing and inhibits the expression of the other *nod* genes. It still remains to be elucidated whether *nodD2* fits also to the gene-for-gene model.

Similarly as the case of soybean, in other legumes such as pea and *M. truncatula*, the plant cultivar also exerts some selection on the rhizobial genotypes that will have preference for nodulation, although a genetic system of host-restriction of nodulation was not described with the same detail as in soybean (Depret & Laguerre, 2008; Rangin et al., 2008). Of particular interest is the case of pea, where Depret & Laguerre (2008) observed that not only the plant genotype exerts a strain selection for nodulation, but also the set of strains that preferentially occupy the nodules changes across the different phenological stages of the plants. The authors employed three pea cultivars, two of which (Austin and Athos) share a common ancestor, and the other (Frisson) is more ancient. In addition, two hypernodulating mutants of Frisson were included. The *R. leguminosarum* bv *viceae* strains came from two different soils and were genotypically classified according to the neutral 16S intergenic space and the symbiotically functional *nodD* gene into 68 genotypes, 5 of which predominated in one of the soils and 6 in the other. In each soil, the cv. Austin and Athos tended to be nodulated by a similar rhizobial population, which had a different structure in the nodules from cv. Frisson. In addition, there was a difference in nodule population structure between cv. Frisson and its hypernodulating mutant P118, indicating that the single gene change in the latter was enough to induce a change in the nodule bacterial population. Moreover, the *nodD* genotypes were diverse in the nodules produced before the beginning of flowering, but tended to be dominated by a single genotype in all three cultivars in the nodules produced between the beginning of flowering and the beginning of seed filling. The authors attributed these differences at least in part to the differences in rhizosphere composition at the different phenological states, which might lead to differences in the rhizobial population structuring. In addition, the metabolic state and structure of the roots may also influence the plant preference for certain rhizobial genotypes.

#### 4. Ecological aspects of the plant-rhizobia interaction

Rhizobia are world-wide distributed bacteria and therefore traits for adaptation to almost every environment where agriculture is carried out may be found. In addition, local populations of rhizobia are not restricted to nodulate the indigenous legume species. Horizontal gene transfer of rhizobial symbiotic genes was documented not only for those rhizobial species that carry this information in transmissible symbiotic plasmids (Torres-Tejerizo et al., 2011) but also for those species that carry this information in the bacterial chromosome (Gomes-Barcellos et al., 2007; Sullivan & Ronson, 1998), and therefore the ability to nodulate a newly introduced legume species is rapidly acquired by the local population. A mathematical model was developed to simulate the propagation of horizontally transferred symbiotic genes to a local non-symbiotic population and the prediction is that such genes can be fixed in the local population in a few generations (Provorov & Vorobyov, 2000). Nevertheless, the major richness in rhizobial genotypes for a given legume species is often found in the centers of origin of that species. Soybean is originated in Asia and therefore, it may be presumed that most of the soybean-nodulating rhizobia are originated in this same region. Therefore, searches for new soybean-nodulating strains with special adaptations were conducted there. A recent survey was performed in soils from four different regions in China (Thomas-Oates et al., 2003). Many fast-growing rhizobia were isolated and classified according to various physiological, biochemical and genetic characteristics, to build a catalogue of fast-growing soybean-nodulating strains with different adaptations that can be used according to local requirements.

Competitiveness of inoculant strains for nodulation of certain plant cultivars in special areas may benefit from such collections. Nevertheless, it is important to understand how the peculiarities of a region may influence competitiveness. It is not a simple task to predict the most limiting factors in a given environment. Sometimes these factors are climate and soil characteristics that can be readily noted, but in many instances there are environmental influences that are hard to identify. These influences may take place at the onset of root infection or during rhizosphere colonization. Rhizosphere is a complex and dynamic habitat (Hinsinger et al., 2005; Watt et al., 2006) that requires a particular approach for its analysis. New cell labeling and microscopy tools are expanding our knowledge on rhizosphere events, since now it is possible to follow a single living cell in real time in the rhizosphere. The knowledge that we are gaining thanks to these methodologies will bring new ideas and applications in the near future.

Although rhizosphere colonization is of prime importance in competition for nodulation, there are reports indicating that the relationship is not so straightforward. In two pea fields (named sites I and II) inoculated with *R. leguminosarum* bv *viceae* it was found that, although the rhizobial indigenous populations were similar, the inoculant resulted more competitive at site I but less competitive at site II, even when at this site the inoculum was applied at very high concentration; however, in laboratory tests of competition of the inoculant against the dominant strain from site II, both resulted equally competitive (Meade et al., 1985). Strains of *R. leguminosarum* bv *trifolii* that were the most abundant nodule occupiers in field-grown subclover were not the most competitive in laboratory experiments carried out in Leonard jars with perlite-vermiculite as substrate (Leung et al., 1994a). In particular, there were four isolates, classified according to their electrophoretic types (ET), which were the most abundant in the field crops. Two of them, named ET 2 and 3, were studied in more detail. Although the ET 3 isolate was in general more competitive than the others, the authors recovered some isolates that, although were rarely present in the field nodules, were more competitive than ET 3 in the laboratory. Regarding the isolate ET 2, although it was found in a large proportion of field nodules, it had a low competitiveness in laboratory. The authors attributed these behaviors to a series of factors, among them the obvious possibility that the environment exerted a decisive influence in the field, but also considered more subtle alternatives, such as a possible non-random distribution in the soil of the more competitive, yet rare isolates. Similarly, a dominant serotype of *R. leguminosarum* bv *trifolii* was found in field nodules of subclover, although there were 13 distinct serotypes in that site. However, the rhizosphere effect, i.e. the ratio between rhizosphere and non rhizosphere population densities increased much more among the rare serotypes than in the dominant one in spring, while this effect was similar in other seasons (Leung et al., 1994b). Moreover, in a study carried out by Laguerre et al. (2003) in France, a difference between fava bean and pea was observed for the relationship of dominance in soil or nodules in the *R. leguminosarum* bv *viceae* population. While the success in nodule occupancy of rhizobial genotypes in fava bean was mainly determined by the rhizobial symbiotic genotype independently of the soil conditions, in pea there was a stronger influence of the rhizosphere colonization ability (linked to the genomic background but not necessarily to the symbiotic phenotype) on the competition for nodulation. These results underscore the complexity of the environmental effect on genotypic expression, which is differentially exerted on a given rhizobial population according to the plant genotype, the season, and the soil structure. It has been argued that rhizobia from soil or rhizosphere are more competitive than rhizobia from rich broths due to a physiological state induced by nutrients limitation, which



predispose the rhizobia to seek for plant root infection. Such physiological conditioning was observed for processes that may be related with rhizosphere colonization and nodulation, such as motility and roots infectivity (Lodeiro et al., 2000). However, nutrients limitation achieved by suspending in poor media rhizobia previously grown in rich broths is different than nutrients limitation achieved by rhizobia themselves at stationary growth phase, when nutrients from the broth are exhausted. In the last case, infectivity is diminished (López-García et al., 2001). Several reports indicated that strains isolated from highly competitive soil rhizobial populations frequently lack their superior competitiveness in laboratory tests or when reintroduced into soil containing an established population. In turn, in several instances an apparently poor competitor for nodulation in laboratory experiments resulted very competitive in soil (Lochner et al., 1989 and references therein). Hence, López-García et al. (2002) tested directly this concept on the basis of competition between two nearly isogenic *B. japonicum* strains (Fig. 1). They established a population of strain LP 3004 (USDA 110 Sm-resistant) in vermiculite pots by inoculating the pots with the bacteria in N-free plant nutrient solution and leaving the pots without plants for at least 1 month in the greenhouse. After an initial period of cell divisions, the rhizobial population stabilized in around  $10^6$  rhizobia  $\text{ml}^{-1}$  without decaying along this period. After this incubation, when the nutrient-limited physiological state seemed to be acquired by this rhizobial population,

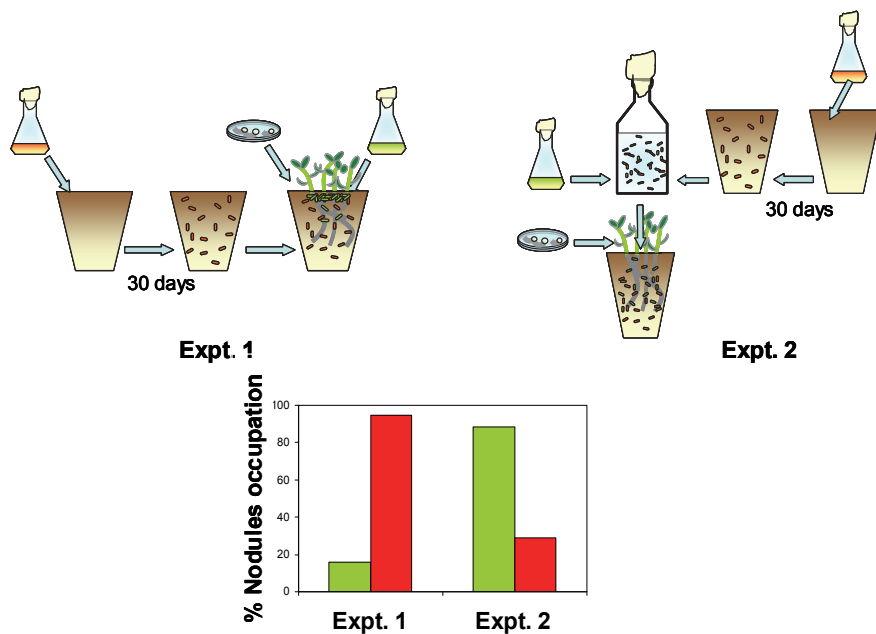


Fig. 1. Demonstration that the rhizobial position in the rooting substrate is determinant for competition for nodulation. Two nearly isogenic strains (indicated in red and green) were inoculated either on the seeds and the rooting substrate or mixed homogeneously before added to the pots. As result, the strain inoculated on the seeds occupied few nodules, despite being intrinsically more competitive. For further details see text.

the pots were divided in two groups. To one of them soybean plantlets were planted and inoculated on the seedlings with around  $10^8$  rhizobia plant<sup>-1</sup> of the strain LP 3001 (USDA 110 Sp-resistant) freshly obtained from a rich broth. From the other half of the pots the rhizobia were removed, suspended in N-free plant nutrient solution, and mixed there with another aliquot of rich broth-grown LP 3001 in 1:1 relationship ( $10^6$  rhizobia ml<sup>-1</sup> of each strain). This mixture was added to fresh vermiculite pots, and soybean plantlets were planted. After 20 days in the greenhouse the nodules were recovered and their contents were identified according to their antibiotic resistances. As a result, it was observed that in the pots where the rich broth rhizobia were inoculated on the seedlings, these rhizobia formed around 20 % of the nodules, but in the pots where both strains were homogeneously mixed before pouring them into the vermiculite, the rhizobia from the rich broth formed more than 80 % of the nodules. This experiment demonstrated that the superior competitiveness of the established population is not caused by a nutrient-limited physiological state, but simply by the better position that they had in the vermiculite with respect to the growing roots: the authors also observed that at field capacity the movement of the rhizobia in the vermiculite is very scarce, which was recently corroborated with non-flagellated mutants (Althabegoiti et al., 2011), and supported the idea that the initial cells that colonize the rhizosphere do not arrive swimming but are "scavenged" by the displacement of the growing roots.

Life in the soil is nevertheless very important for the rhizobia. They may persist even in the absence of legume crops, and it was observed that *Bradyrhizobium sp. (Lotus)* retains its nodulation, competition, and N<sub>2</sub> fixing characteristics even after 10 years in the soil (Lochner et al., 1989). If we consider a soybean crop season, and take into account that nodules start to senesce at grain filling, we can estimate that the rhizobia are into the nodules for less than 40 % of a year; the other 60 % of the time they have to survive in the free-living state in the soil. During this period the rhizobia have to face diverse threats, including UV irradiation, temperature changes, predation, drought, flooding, etc. Since these microorganisms are unable to sporulate, a preferred state of endurance is the biofilm (Danhorn & Fuqua, 2007). To this end, the cell surface components play a major role, but flagella are lost and biofilm rhizobia are not motile, which may in part explain the lack of effects of motility on competition for nodulation in non-flooded soils. In addition, plant lectins may help in developing the biofilms. It was observed that soybean lectin enhances the biofilm formation by *B. japonicum* in a way that is dependent on the presence of the receptor EPS molecule in the bacteria (Pérez-Giménez et al., 2009). Since this process seems not related with plant infection, it was argued that lectin-assisted biofilm formation may favor *B. japonicum* biofilms in the vicinity of decaying soybean roots or even on dead roots, where soybean lectin may have been released. This is supported by the observation that soybean lectin is remarkable stable, being unaltered even after a week of incubation at 70 °C. Thus, this enhancement of biofilms formation where soybeans were recently cultivated may keep a localized high rhizobial population for the next nodulation cycle, thus explaining the heterogeneities of rhizobial distribution in the soil previously postulated by Leung et al. (1994).

## 5. Conclusion

Competition for nodulation still remains a very complex and largely unknown phenomenon, yet a very important issue for N<sub>2</sub> fixation technology. Nevertheless, understanding of this phenomenon has advanced in the last years, and several measures to improve competitiveness of rhizobial inoculated strains may be proposed. Among that are

the manipulation of host-controlled restriction of nodulation, the genetic manipulation of the plant and bacterial partners, selection of superior strains, improvement of inoculant formulations by manipulating the culture media and the physiological and metabolic state of the bacteria, and the improvement of inoculant application technologies, particularly with in-furrow inoculation. These methods, as well as the new developments that are in progress, are necessary for the sustainable agriculture of the future.

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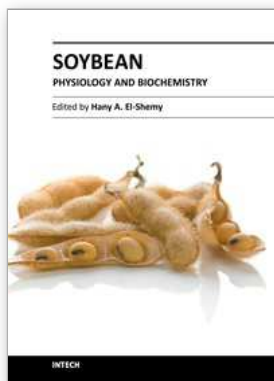


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Worldwide, soybean seed proteins represent a major source of amino acids for human and animal nutrition. Soybean seeds are an important and economical source of protein in the diet of many developed and developing countries. Soy is a complete protein and soyfoods are rich in vitamins and minerals. Soybean protein provides all the essential amino acids in the amounts needed for human health. Recent research suggests that soy may also lower risk of prostate, colon and breast cancers as well as osteoporosis and other bone health problems and alleviate hot flashes associated with menopause. This volume is expected to be useful for student, researchers and public who are interested in soybean.

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