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Ensifer (Sinorhizobium) fredii Interacted More Efficiently than *Bradyrhizobium japonicum* with Soybean

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Authors' contributions

This work was carried out in collaboration between all authors. Author GNP designed and evaluated the competitive ability in co inoculated plants and made the statistical analysis. Author LV works in the identification of organisms in the soil, designed and performed the study. Authors IM and JS design and performed the experiments aimed at analysing infection foci in inoculated plants and measured nodulation on a subset of these plants. Author VMA performed the evolution of nodulation efficiency by inoculating different cell concentrations. Author PAB collaborated in the design of the experiments, the statistical analysis of the results and wrote the paper. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The purpose of this work was to compare the efficiency of *Bradyrhizobium japonicum* and *Ensifer fredii* to infect and develop nodules on soybean. Furthermore we also evaluated the competitive ability of both species and how this was altered by the plant genotype and the soil pH.
Study Design: The design of the experiments was completely at random and the number of replicates was different on each of the different experiments tested.

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Place and Duration of Study: The place of the studies was the Facultad de Ciencias Agrarias y Forestales Universidad Nacional de La Plata and the duration of the study was a year and a half.
Methodology: Roots of inoculated soybean plants were fixed and the number of infection initiation sites was evaluated by means of microscopic observation. The number of nodules developed by inoculated plants was also evaluated.
Results: Bacteria were equally effective at developing infection initiation sites on soybean however, *E. fredii* induced more nodules than *B. japonicum*, probably due to the fact that *E. fredii* is more efficient than *B. japonicum* at nodulating soybean. However, *Bradyrhizobium* was more competitive than *E. fredii* which was unrelated to the soybean genotype but altered by the soil pH. Under the conditions described *E. fredii* was less competitive than *B. japonicum* probably due to the high cultivar-rhizobia specificity.
Conclusion: *E. fredii* was as efficient as *B. japonicum* at nodulating soybeans. However *Bradyrhizobium* was a better competitor though this is affected by the plant genotype and the soil pH. The selection and use of fast growing rhizobia in inoculant production seems to depend on broadening the genetic base of soybean or in selecting cultivars with specificity for fast growing rhizobia.

Keywords: Bradyrhizobium; Ensifer fredii; nodulation; competition.

1. INTRODUCTION

Legumes constitute the third most important family of angiosperms (*Fabaceae*) [1,2] and most of them establish a symbiotic association with Gram (-) bacteria commonly known as rhizobia [3].

Soybean (*Glycine max* (L.) Merr.) is the most important legume worldwide and nodulates with soil bacteria from the genus *Bradyrhizobium, Mesorhizobium, Ensifer (Sinorhizobium*) and *Rhizobium* [4,5,6,7,8]. *Bradyrhizobium* species that nodulate soybean include *B. japonicum* [9], *B. elkanii* [10], *B. liaonengense* [11] and *B. daqingense* [12]. In addition to this, soybean is also nodulated by fast growing rhizobia [13,14] that include *Ensifer (Sinorhizobium) fredii, E.* (*S.*) *xinjiangense* and other unclassified rhizobia as well [15,16,17]. Additionally, Hungría et al. [18] and Appunu [19] found that also *Mesorhizobium tianshanense* and *Rhizobium tropici* nodulate soybean.

While *B. japonicum* and *B. elkanii* nodulate and fix nitrogen efficiently in association with improved soybean cultivars [10], fast growing rhizobia have been reported to interact inefficiently with soybean though several reports argue about this [20,21,22,23]. Unlike *Bradyrhizobium* siblings, Pueppke [24] found that nodule development induced by *E. fredii* is highly affected by the environment. Furthermore, Buendía Claveria et al. [21] and Yang et al. [23] found that inoculation of soybeans with *E. fredii* in alkaline soils in Spain and China resulted in a higher number of nitrogen fixing nodules compared to *B. japonicum* inoculated plants. Furthermore, Videira et al. [22] found a strong genotype-rhizobia interaction and Yang et al. [25] demonstrated that plant R genes are also involved in the interaction. Considering this, the success of rhizobia to develop nodules on soybean might be dependent on the ability of the isolates to compete for infection sites, on their ability to evade the plant's autoregulatory mechanism and on the cultivar nodulation capacity and genotype.

Nodulation of soybean has been carefully studied and described by Calvert et al. [26]. Pierce and Bauer [27] and Takats [28] described an autoregulatory mechanism in soybean that arrests infections, whether plants were inoculated with fast or slow growing rhizobia [29,30]. However, it has been observed that under defined environmental conditions fast growing rhizobia developed more nodules than *B. japonicum* inoculated plants (Balatti, P., personal communication, Universidad Nacional de La Plata, 2012). This raised the question, is the higher number of infections a result of the ability of fast growers to avoid the plant's mechanism of autoregulation? Therefore, the purpose of this work was to study the number of infections and nodulation of soybean roots inoculated either with *B. japonicum* or *E. fredii*, what might lead us to see if any of these bacteria can avoid the plant autoregulatory mechanism. Also, we evaluated the effect of the soybean cultivar and soil pH on the competitive ability of *E. fredii* and *B. japonicum*.

2. MATERIALS AND METHODS

2.1 Growth and Numbers of Bacterial Strains Used

Stocks and cultures of the strains used in this study were done as described by Chatterjee et al. [31]. Bacteria were grown in yeast extract mannitol (YEM) medium [32] in an orbital shaker at 150 rpm at 28°C. *E. fredii* SMH12 [33] and S40 [34]) were kindly provided by Dr. J E. Ruiz Sainz, (Universidad de Sevilla) Seville, Spain. *B. japonicum* E109 (a derivative of USDA138) is a commercial strain used in Argentina. Mutant *E. fredii* S40-1 is Streptomycin (Str) resistant mutant (Martinez Alcántara, this work).

Bacterial cell concentration was adjusted by means of the Optical Density (O.D.) at 625 nm in a spectrophotometer Shimadzu UV160A [32].

2.2 Plant Growth Conditions

Seeds of soybean (*Glycine max* (L.) Merr.) cv Asgrow 4400 RG were surface sterilized [31], and were germinated in plates containing 1% water agar, which were incutabed at 28°C for two days. Then, seedlings were transplanted to modified Leonard Jars, filled with a mixture of 2 parts of vermiculite and one part of soil (sieved horizon A) [30]. The soil used in the experiments contained 23 ppm of P,53 ppm of NO_3, and 3.3% of organic matter. Plants were grown in the greenhouse under a 16 h photoperiod at 27°C±2 and 20±2°C during the night. Samples were taken 5 and 30 days after inoculation and roots were screened for the presence of nodules. Shoot dry weight was determined by drying the shoots in an oven at 60°C until constant weight.

2.3 Infection Initiation in Roots

Seeds were inoculated by immersing plantlets roots in bacterial suspensions of a) 10^3; b) 10^4; c) 10^5; d) 10^6; e) 10^7; and f) 10^8 cell.ml^{-1}. Plants were harvested five days after inoculation and the roots were stained by the Haematoxylin method [35]. The number of initiation sites was counted by means of a stereoscope Leitz SM Lux microscope. A replicate included 4 plants and the number of replicates per experiments was 6 (total number of plants assayed were 24). Three independent experiments were run. The experimental design was completely at random and the statistical analysis performed was the ANOVA (Statgraphics software). Means were compared by the Least Significant Difference (LSD). A remaining subset of plants (24) was cultivated in the greenhouse for 25 additional days, then 30 days old plants were harvested and the nodules were counted.

2.4 Competitive Ability of the Strain

We performed a different set of experiments to evaluate the competitive ability of two strains, *E. fredii* SMH12, a highly efficient nitrogen fixing bacteria, S40 an isolate pretty much diverse from SMH12 (molecular data) and *B. japonicum* E109 (the commercial strain used in inoculants in Argentina) to infect and colonize soybean roots, which was analyzed by identifying the rhizobia occupying the nodules of soybean grown in Leonard jars, as described before. We also studied the effect of the soil pH and plant genotype on competition. Three independent experiments were performed each included the following treatments: A) uninoculated plants; B) Plants inoculated with 10^8 cells of *B. japonicum*; C) Plants inoculated with 10^8 cells of *E. fredii*, D) Plants inoculated with a mixture of *B. japonicum* and *E. fredii* cells in a 2:1 ratio; E) Plants inoculated with a mixture of *B. japonicum* and *E. fredii* cells in a 1:1 ratio F) Plants inoculated with a mixture of *B. japonicum* and *E. fredii* cells in a 1:2 ratio. In the first experiment we evaluated the competitive ability of *E. fredii* strain S40 while in the second one that of strain *E. fredii* SMH12, both of them on soybean cultivar Asgrow 4400. In the third experiment, we evaluated the effect of the soybean cultivar on the competitive ability of SMH12 repeating the competitive experiments on three different soybean cultivars NA2018GR, NA4990GR and A7053GR. In addition to this, we also evaluated the effect of pH upon the competitive ability of SMH12 by growing cultivar NA4990GR in media buffered at two different pHs, 7 and 8. Plants were seeded and inoculated with a 2:1, 1:1 and 1:2 *E. fredii / B. japonicum* ratio. In this experiment, bacteria were inoculated by adding them to the soil substratum, before the seedlings were transplanted. Thirty days after inoculation nodules were collected, counted and surface sterilized by immersing in 50% ethyl alcohol for 5 min and in 50% sodium hypochlorite for 5 min. Then, they were rinsed with sterile distilled water and rounded in circles on media to check for the presence of contaminants on their outside. The experiments were performed in a randomized block design with three replicates and a one way ANOVA statistical test ($P \leq 0.05$) was run by means of the Statgraphics software.

2.6 Identification of Soil and Nodule Bacteria

Each nodule was crushed in 20 µl sterile water and two drops of the homogenate were plated on TY [36] or YEM containing 2 g of yeast extract, supplemented with the required antibiotics: Streptomycin (Str), (400 $\mu g.ml^{-1}$), and Tetracycline (Tet), (5 $\mu g.ml^{-1}$). While *E. fredii* S40 and SMH12, grew on TY and YEM media, *B. japonicum* E109 grew on YEM in the presence of Tet and did not grow on TY media. The identity of the bacteria recovered either from the nodules or the soil was confirmed by means of the amplification of a 900 bp or 750 bp bands that correspond to *B. japonicum* and *E. fredii*, respectively [37]. Briefly, the soil DNA was extracted based on a modified procedure described by Volossiuk et al. [38]. Loops picked up from the colonies were used as templates for the Multiplex PCR.

3. RESULTS AND DISCUSSION

Both experiments where soybeans were inoculated with increasing cell numbers of *E. fredii* or *B. japonicum* generated identical results, therefore only one of them is presented (Figs. 1A-D). The number of infections sites were independent of the number of rhizobia inoculated (R^2= 0.012) (Figs. 1A and 1B). *B. japonicum* and *E. fredii* were equally effective at inducing infections. A subset of plants cultured for 25 additional days developed nodules and their number was a function of the rhizobium cell concentration (Figs. 1C and 1D). However, nodule number was much lower than the number of infections observed 5 days after inoculation suggesting that as has been described by Calvert et al. [26] infections might have been arrested. However, *E. fredii* inoculated plants developed a higher number of nodules (17 nodules) than *B. japonicum* inoculated ones (11 nodules). Furthermore, the number of *E. fredii* cells required by soybeans to nodulate was lower than with *Bradyrhizobium*. This basically suggests that at least fast growing rhizobia seem to be more effective in evading autoregulation.

On a per plant basis or expressed as nitrogenase specific activity, the plants had similar levels of fixation, whether they were inoculated with fast or slow growing rhizobia (data not shown), another argument about the inefficiency of fast growing rhizobia.

As another index of efficiency, we analyzed the number of nodules developed by *E. fredii* and/or *B. japonicum* when soybeans were coinoculated in a 2:1, 1:1 and 1:2 ratios of rhizobia, which was confirmed by a Multiplex PCR reaction [37] (Fig. 2). *B. japonicum* seemed to be a better competitor than *E. fredii,* considering that independently of the ratio of cells inoculated, whenever they were coinoculated, most nodules contained *B. japonicum*, whether the *E. fredii* strain coinoculated was SMH12 or S40 (Table 1) (Fig. 2). When the assays were performed with *E. fredii* strain S40, coinoculation with a 2:1, 1:1 or 1:2 *B. japonicum / E. fredii* S40 ratio, 91.9, 78.2 and 92.2 % of the nodules contained *B. japonicum*, respectively and the rest either *E. fredii* alone or both *B. japonicum* and *E. fredii* (Fig. 3).

We evaluated further the effect of the plant genotype and soil pH upon the competitive ability of *E. fredii* and *B. japonicum* and found that whether the soybean cultivar belonged to the short, middle or late maturity group, nodules contained mostly bradyrhizobia (Fig. 3). In addition to this, we found that *B. japonicum* consistently formed more nodules than *E. fredii* (Fig. 3), which tended to decrease at pH8 (data not shown). This is in a way in agreement with the findings reported by Zhang et al. [39]; these authors stated that rhizobia distribution in the soils of China was a function of the soil pH and also the availability of nutrients such as N, P and K. This was such that they demonstrated biogeographic clustering of rhizobia, since the major soybean producing areas of North China Plain, together with a subtropical region in China and alkaline soils in India have a unique composition of rhizobia that were mostly fast growers.

Our results showed that both fast and slow growing bacteria developed a high number of infections in soybean, suggesting that both species were equally efficient in doing so, which was independent of the number of rhizobial cells inoculated. Heron and Pueppke [29] found that the slow growing strain 3G4B16 had an infection/nodulation ratio 39 times greater than fast growing strain USDA191. However, while these authors looked at infection threads in a defined portion of the root, we looked at nodule initiation sites along the whole root and by means of a different staining technique.

Fast growing rhizobia were more efficient than *B. japonicum* at inducing nodulation and two lines of evidences support this. One is that fast growing rhizobia induce the same number of nodules with

three order of magnitude less bacterial cells than *B. japonicum* (Figs. 1A y 1B), which might be related to a quorum sensing response along the establishment of a successful symbiosis [40]. These mechanisms seem to be mediating early to intermediate stages signaling events and probably in the case of *E. fredii*, quorum sensing influenced the events that occurred later in nodule development. The other line of evidence is that plants inoculated with *E. fredii* developed a higher number of nodules than with *Bradyrhizobium*, which was also reported by Heron and Pueppke [41] and Buendía Clavería et al. [21]. However, the number of infections developed 5 days after inoculation and the number of nodules were unrelated, which suggest that the autoregulatory mechanisms of the plant prevented nodule development [28]. Therefore, *E. fredii* was more effective than *B. japonicum* in infecting and nodulating soybean suggesting that infection foci induced by fast growers, somehow were less prone to the autoregulatory mechanism of soybean, at least with the cultivars assayed in these experiments.

Still, compared to *B. japonicum, E. fredii* had a low competitive ability. Whenever soybeans were coinoculated while most nodules were occupied only by *B. japonicum*, some contained both fast and slow growing rhizobia suggesting either that they shared the port of entry or complemented each other to successfully infect the plant, without affecting their viability. McLoughlin et al. [42] found that the nodules of soybeans inoculated with fast growing rhizobia and grown in soils containing native *Bradyrhizobium* contained almost exclusively bradyrhizobia. Manjanatha et al. [43] and de O. Chueire and Hungria [44] found that *Bradyrhizobium* strains outcompeted fast growing ones. These findings and ours allowed us to conclude that under the conditions described, basically neutral pH, fast growing strains are competitive deficient. Under natural conditions if soybean plants are nodulated mostly by bradyrhizobia, their cell number in soils will increase and will lead to their prevalence in the soil.

Our findings confirmed that *B. japonicum* was more efficient than *E. fredii* to nodulate soybean cultivars whether they belong to short or long life cycle groups. Regarding this, it should be pointed out that the cultivars assayed in these experiments were all closely related with improved American cultivars; therefore they might have low specificity for *E. fredii.* Yang et al. [23] found that the subpopulations of rhizobia in the soils of China showed strong cultivar specificity; therefore the competitive ability of fast and slow growing rhizobia might be a function of the soybean genotype genome. Still Han et al. [45] stated that the soil environment is critical for the prevalence of fast growing rhizobia such as pH and P availability.

The pH of the media often influences the rhizobia-plant interaction. Li et al. [8] and Zhang et al. [39] described that *E. fredii* is highly affected by the soil environmental conditions, such as pH and available P [5;45]. In our experiments, the pH of the media did not subvert the fitness of *B. japonicum* and *E. fredii*, to nodulate improved soybean cultivars, since even at high pH *B. japonicum* was the strain that occupied the highest percentage of nodules. However, it should be mentioned that as the pH of the media increased, a reduction in the number of nodules containing *B. japonicum* occurred even though no statistically significant difference was found (data not shown). Furthermore, at high pH we failed to isolate rhizobia from a considerable number of nodules, that PCR demonstrated had *B. japonicum*. Also in field experiments were soybeans coinoculated with fast and slow growing rhizobia presented empty nodules (PhD Balatti, P., personal communication, Universidad Nacional de La Plata, 2012). No bacteria were recovered from these surface sterilized nodules on YEM but the Multiplex PCR lead to the amplification of the 950 bp *Bradyrhizobium* specific fragment, suggesting that these nodules contained extremely low numbers of *B. japonicum*, which might be difficult to detect, unless a highly sensitive technique is used such as PCR. So even though the pH affected *Bradyrhizobium* it did not subvert the genomic determinants that made *B. japonicum* more competitive than *E. fredii*.

Our results showed that fast and slow growing rhizobia were equally efficient at developing the early events of nodulation as well as fixing atmospheric nitrogen in soybean. However, it appears that fast growing rhizobia nodulation events evaded the autoregulatory mechanisms of nodulation in plants more efficiently than those provoked by bradyrhizobia. Although the plant genome plays a critical role in the symbiosis it was unrelated to the competitive ability of rhizobia. Under the conditions described the soil pH had no effect on the competitive ability of both partners.

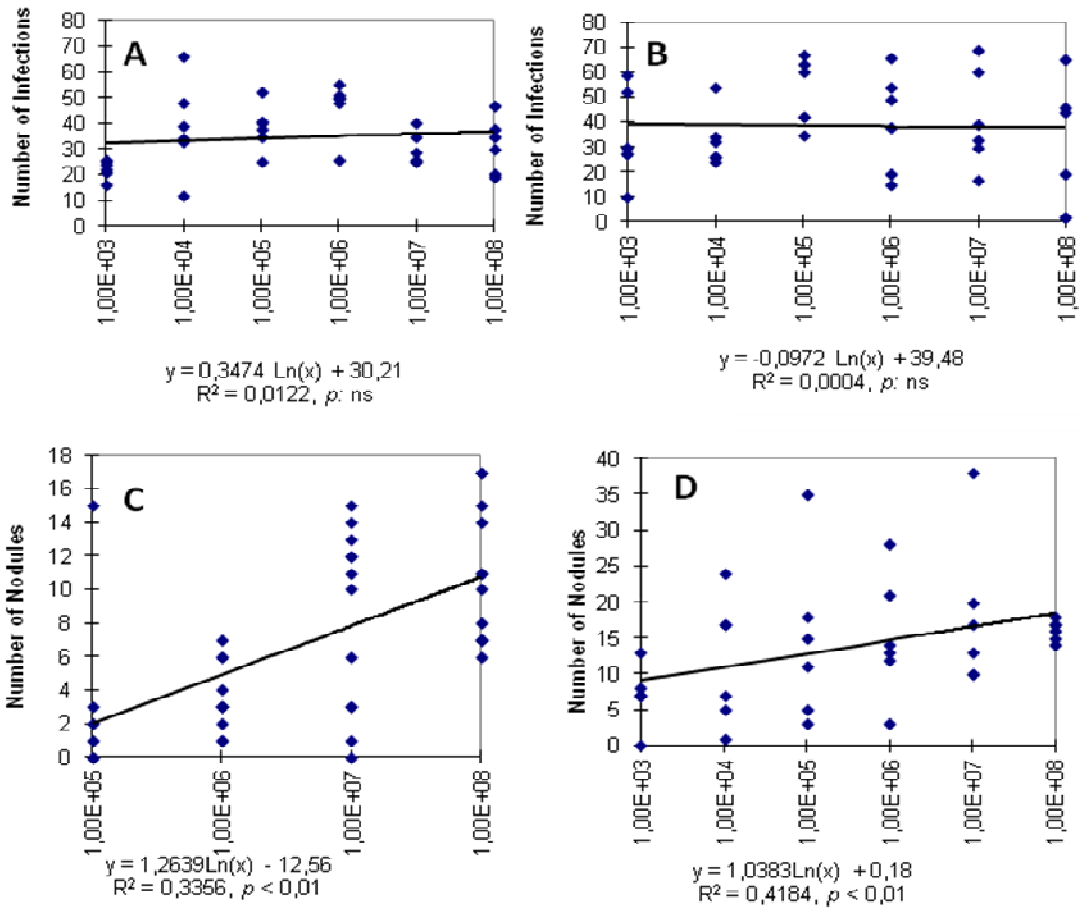

Fig. 1. Development of initiation sites and nodulation in roots of soybean plants inoculated with fast and slow growing rhizobia

Treatments were as follows a) 10^3; b) 10^4; c) 10^5; d) 10^6; e) 10^7; f) 10^8 bacterial cells.ml^{-1} of culture. A) Number of initiation sites developed by B. japonicum on soybean. B) Number of initiation sites developed by E. fredii on soybean. C) Number of nodules found on soybean plants inoculated with B. japonicum. D) Number of nodules found on soybean plants inoculated with E. fredii. Plants were inoculated and five days after infection, sites were counted as described. ns = non significant at P<0.05

Table 1. Competitive ability of *E. fredii* strain SMH12 and *B. japonicum* E109 o*n soybean* cultivar A4400GR in a 1:2 mixture of soil: vermiculite, respectively

Strains recovered	% Nodule occupancy			Nodules evaluated	% of evaluated nodules among total
	B. japonicum	*E. fredii*	*B.j. + E.f.*		
Uninoculated control	-	-	-	-	-
B. japonicum	100 a	0	0	16	14
B.j. 2:1 *E.f.*	100 a	0	0	30	20
B.j. 1:1 *E.f.*	100 a	0	0	26	20
B.j 1:2 *E.f*	100 a	0	0	26	20
E. fredii	0 b	100	0	16	43

The pH of the soil was 7.0. Nodules were collected from plants grown in modified Leonard jars containing each three plants. Bacteria were added to the soil substratum each at a concentration of 10^8 bacteria.g^{-1} of soil. The number of replicates was 15 plants

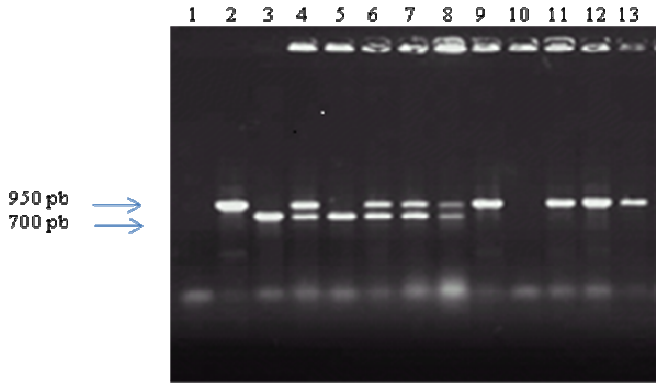

Fig. 2. *RSα* and *nolB* fragment amplification by means of the Multiplex PCR reaction performed with template DNA isolated from surface sterilized nodules

RSα is a 950 pb fragment that is specific of Bradyrhizobium japonicum and nolB is a 700 pb fragment specific of Ensifer fredii. Lanes 1 control reaction lacking template DNA; lane 2: B. japonicum template DNA; lane 3: corresponds to template DNA of E. fredii (S40); lanes 4-13: correspond each lane to the amplifications performed using template DNA from nodules extracts, lanes 4-7: corresponds from 1:2 (B.j.: E.f.) treatment; lanes 8-10 from 1:1 (B.j.: E.f.) treatment; lanes 11-13: from 2:1 (B.j.: E.f.) treatment. This is a 1% agarose gel run at 80 volts in TBE buffer

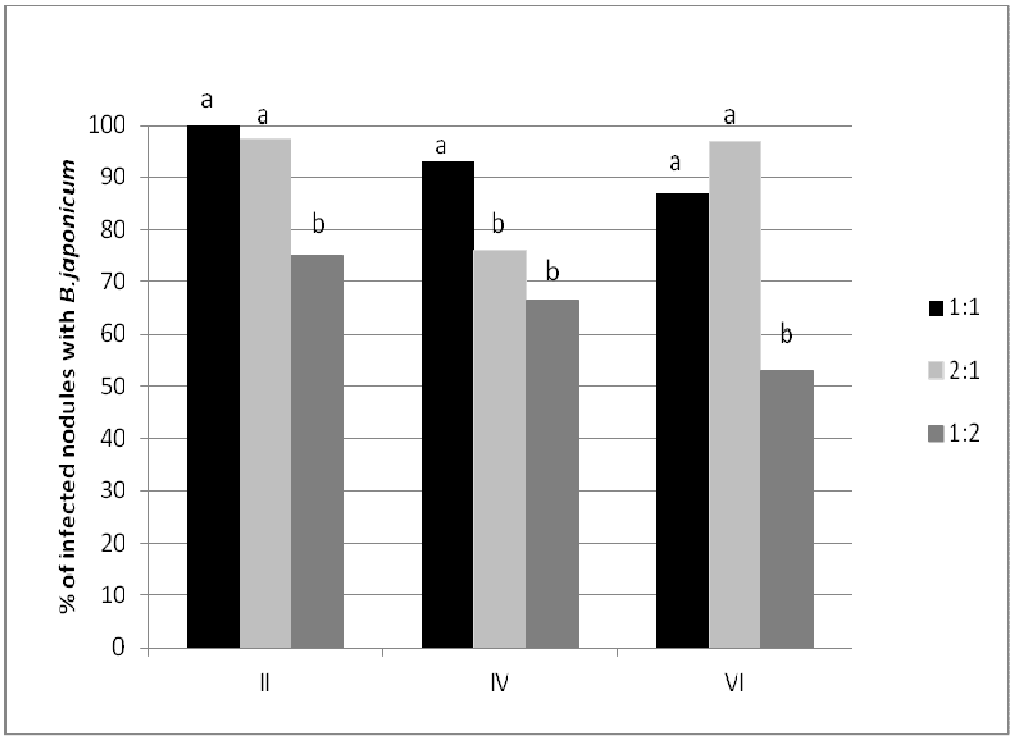

Fig. 3. Competitive ability of *E. fredii* SMH12 and *B. japonicum* E109 strains on soybean cultivar A4440GR cultivated in modified Leonard Jars

Percentage of nodules occupied either by fast and/or slow growing rhizobia in soils inoculated with different ratios of B. japonicum and E. fredii. Statistical differences are indicated by different letters (P<0.05). At the base of the figure roman numbers indicate the maturity group of the soybean genotype used in the experiments. On the right numbers indicate the ratio of B. japonicum to E. fredii cells inoculated. The number of nodules evaluated for each inoculation treatment was: 485 for B. japonicum (not included in the figure); 485 for 2:1. (B.j.: E.f.); 432 for 1:1 (B.j.: E.f.); 438 for 1: 2 (B.j.: E.f.); 497 for E. fredii (not included in the figure)

4. CONCLUSION

E. fredii was as efficient as *B. japonicum* at inducing infection initiation sites on soybean roots, however *E. fredii* developed more nodules suggesting that less infections are within the control of the autoregulatory mechanism of soybean. *Bradyrhizobium* proved to be more competitive than *E. fredii* though this might be alterd by the soybean genotype and the soil environment. If at seeding we modify the seed environment, we might enhance nodulation by fast growing strains, which should rise their number in the soil. So there is a potential to formulate inoculants with fast growing rhizobia though to do this the genetic base of soybean cultivars might be broaden.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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