Nodulation in peanut (Arachis hypogaea L.) roots in the presence of native and inoculated rhizobia strains

Stella Castro*, Marcela Permigiani, Marta Vinocur, Adriana Fabra
Facultad de Ciencias Exactas Físico-Químicas y Naturales. Universidad Nacional de Río Cuarto. Agencia Postal No. 3, 5800, Río Cuarto, Córdoba, Argentina

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Abstract

There is considerable interest in improving nitrogen fixation in legumes to increase soil fertility, particularly in the developing countries. The effect of inoculation of peanut (Arachis hypogaea L.) seed with Bradyrhizobium sp. USDA 3187 was examined under field conditions. In order to determine nodule occupancy, bacterial cells from surface-sterilized nodules of inoculated plants were isolated and characterized by their intrinsic antibiotic resistance and by agglutination test. Results obtained revealed that peanut roots were nodulated by native strain rather than the introduced one, indicating that strain USDA 3187 was not as competitive as naturalized peanut rhizobia. Data also showed that there were no significant differences in nodule dry weight, plant dry biomass, nitrogenase activity and seed yield between uninoculated and inoculated treatments. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Arachis hypogaea L.; Competition; Nitrogen fixation; Nodulation; Rhizobia

1. Introduction

Bacteria of the genera Rhizobium and Bradyrhizobium are the principal soil inhabitants. Several members of the family Leguminosae are of considerable ecological and economic significance due, in part, to their ability to form a nitrogen-fixing symbiosis with bacteria from these genera (Sangina, 1992). Thus, the nitrogen that is supplied by rhizobia to legumes replaces costly industrially fixed nitrogen.

Introduction of specific bacteria into soils may supply nutrients to crops, stimulate plant growth through the production of plant hormones, and control or inhibit the activity of plant pathogens and improve soil structure (van Veen et al., 1997). Inoculation is a practice of significant agronomic benefit in areas where the crop is planted for the first time (Brockwell et al., 1988; Hume and Blair, 1992) or in soils where the number of rhizobia is too low to allow efficient nodulation of the plant (Singleton et al., 1992). Simon et al. (1996) demonstrated that the number and species of these bacteria depend on the biotic and abiotic soil environment and on species of wildly grown or cultivated legumes. Thus, precise identification of strains introduced into the soil has great importance for ecological studies of competitiveness of rhizobia and their survival in the soil environment.

The term ‘competition’, when used for the Rhizobium spp., generally implies competition for nodule
formation between the various *Rhizobium* strains from the moment these strains are present in the same environment, until the moment of their presence inside the nodules (Simon et al., 1996). Triplett (1990) indicates that a high competitiveness of inoculum strains is as important as the effectiveness of symbiotic nitrogen fixation itself. The effect of interstrain rhizobial competition in the soil or rhizosphere is not clearly understood, and this sometimes results in the selection of competitive but ineffective rhizobial strains (Athar and Johnson, 1996).

Peanut (*Arachis hypogaea* L.) is an important legume grown in Argentina, with 98% of its production concentrated in the province of Córdoba. Argentina is ranked as the second largest peanut exporter in the world. Inoculation of peanut is not a common practice in the country, but in soils where the number of *Bradyrhizobium* sp. is too low to allow efficient nodulation of the plant, an increase in the number of bacteria may improve grain yield (Hume and Blair, 1992).

The study was undertaken to establish the competitiveness of the inoculated strain (*Bradyrhizobium* sp.) for nodule sites vs. indigenous soil microorganisms population, and the effect of the inoculation on peanut yield.

2. Materials and methods

2.1. Bacterial strains

The bacteria used in this study were *Bradyrhizobium* sp. USDA 3187 which was able to infect peanut plants, and a strain isolated from root nodules of peanut plants grown under field conditions.

2.2. Media and growth conditions

Strains were routinely maintained on yeast extract mannitol (YEM) agar slants (Vincent, 1970) and kept at 4°C. Purity was assured by routine plating on YEM agar supplemented with Congo red and selecting uniform colonies. The basal growth medium is that described by Vincent (1970). Primary cultures in 10 ml medium were started from agar slants and incubated at 28°C with shaking until the early logarithmic phase was reached. These cultures provided inocula for the experimental cultures that were also grown with shaking at 28°C in 25 ml in 100 ml Erlenmeyer flasks, with 1% inoculum.

2.3. Seed inoculation

The *Bradyrhizobium* strain was cultured in yeast extract mannitol broth until it reached the late logarithmic phase of growth. The inoculum was applied at an initial population level of $1.8 \times 10^6$ CFU seed$^{-1}$.

2.4. Field experiments

Field studies were conducted in 1994 on the Universidad Nacional de Río Cuarto (U.N.R.C.) Experimental Farm (33°07' latitude S; 64°14' longitude W; 421 m asl) on a sandy loam Typic Hapludoll. Soil properties are shown in Table 1. Plots which have never been cropped with peanut before were established inside a larger experiment described by Castro et al. (1997). A runner Virginia-type peanut cultivar (Florman INTA) was hand-planted on November 30, in rows 0.7 m apart and with a seed population of 12 plants m$^{-2}$. Half of the plots, randomly selected, were sowed with control seeds, and the other half with inoculated seeds. Weeds were controlled using the

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td>Soil profile properties</td>
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<tr>
<td>Horizon</td>
</tr>
<tr>
<td>A$_p$</td>
</tr>
<tr>
<td>A$_d$</td>
</tr>
<tr>
<td>B$_w1$</td>
</tr>
<tr>
<td>B$_w2$</td>
</tr>
<tr>
<td>BC</td>
</tr>
<tr>
<td>C$_k$</td>
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</table>
The herbicide Pivot (Imazethapyr ammonium) as a pre-emergent (1 l ha$^{-1}$), a compound that has no detrimental effects upon peanut microsymbiont survival (Fabra et al., 1997). At growth stage R1 (flowering, 30 days after planting) plants from a section of 1 m$^2$ in each plot were harvested. The roots with nodules were separated from the plant and used immediately to determine nitrogenase activity or nodule occupancy. The soil adhering to roots and other plant parts was removed under running tap water and the dry weight of the plants and nodules, after drying for 72 h at 70°C, was determined. At harvest, plants obtained from 1 m$^2$ were analyzed for yield determinations and the results expressed in kg ha$^{-1}$.

2.5. Nitrogenase assay

Nitrogenase activity was evaluated according to Hardy et al. (1973). Total root systems were transferred to culture vessels capped with serum stoppers. To start the assay, a 10% acetylene atmosphere was injected. After 1 h of incubation, the ethylene produced was measured by injecting 0.5 ml of the gas into a Model Shimadzu gas chromatograph GC-6A series. Conditions of analysis were: temperature detector (H$_2$FID): 100°C; oven temperature: 80°C; 1 m × 2 mm i.d. steel column packed with Porapak T 80–100 mesh.

2.6. Isolation of bacteria from nodules

Nodules were selected at random from plants at growth stage R1, surface-sterilized by standard methods (Vincent, 1970) and crushed aseptically in a small amount of sterile water. Bacteria were plated on a YEMA medium and occupancy evaluated.

2.7. Identification of nodule occupants

Nodule occupants were identified by their intrinsic antibiotic resistance characteristics and also by a serological technique.

2.7.1. Intrinsic antibiotic resistance

Cultures of strains USDA 3187, and those bacteria isolated from nodules, were tested against a broad spectrum of antibiotics to determine their intrinsic antibiotic resistance characteristics. The antibiotics used were as follows (μg per disk): ofloxacine, 5 μg; cefixime, 5 μg; chloramphenicol, 30 μg; cefuroxime, 30 μg; aztreonam, 30 μg; phosphomycin, 50 μg, ampicillin, 10 μg; netilmicin, 30 μg; and cephalothin, 30 μg. The diameters of the zone of rhizobial growth inhibition around each disk were measured after 48 h of incubation at 28°C.

2.7.2. Production of antiserum

In order to obtain antiserum of Bradyrhizobium sp USDA 3187 and the strain isolated from the nodules, the method described by Margni (1986) was followed. For the preparation of the antigen, Bradyrhizobium sp. USDA 3187 or native strain cultures were grown in yeast extract mannitol broth on a shaker until they reached an O.D.($_{620 \text{ nm}}$) of 0.3. Bacterial cells were harvested by centrifugation at 4000 × g for 10 min and the pellet resuspended in sterile saline (PBS) and washed thrice with acetone. Dry pellets were weighed and resuspended in physiological saline (0.85%) with the addition of formic acid (5% final concentration). The added volume was adjusted to reach $2\times10^9$ cells ml$^{-1}$. Antiserum was prepared using two adult rabbits per strain. Each rabbit was given intravenous injections of the appropriate antigen according to the following schedule: Day 1, 1.0 ml intramuscular injection, antigen concentration $2\times10^9$ cells ml$^{-1}$ [with the addition of Freund adjuvant containing liquid vaseline and glycerine (9 : 1 V/V)]; Day 15, 1.0 ml intravenous injection, antigen concentration $2\times10^9$ cells ml$^{-1}$; Day 16, 1.0 ml intravenous injection, antigen concentration $2\times10^8$ cells ml$^{-1}$; Day 17 and 18, 1.0 ml intravenous injection, antigen concentration $2\times10^8$ cells ml$^{-1}$; Day 25, bleed to check the antiserum production; Day 28: bleed by cardiac puncture. After bleeding, the collected blood was incubated for 1 h at 37°C to facilitate clotting, and held at 4°C in the refrigerator overnight to help extrusion of the serum. Serum was separated and centrifuged to remove residual cells and stored at –2°C in 0.5 ml samples without addition of any preservative.

2.7.3. Agglutination test to determine titer of antiserum

Bacterial cultures were grown under similar conditions to those described for antigen preparation. Antibody concentration used for agglutination test
was between 1 : 2.5 and 1 : 160. Saline solution was used for antiserum dilution. Antibody titer in serum was determined by the plate agglutination test (Margni, 1986).

2.8. Statistical analysis

The data were analyzed statistically using a Student’s t-test and a level of \( p < 0.05 \) being accepted as significant.

3. Results

3.1. Field experiments

Fig. 1 shows the results obtained from plants harvested at the R1 growth stage. Although there were some differences in the nodule dry weight, plant dry weight and acetylene reduction between uninoculated and inoculated plants, they were not statistically significant. No change was found in peanut seed yield due to the seed inoculation treatment (uninoculated seed = 2771 ± 643 kg ha\(^{-1}\); inoculated seed = 2884 ± 643 kg ha\(^{-1}\)).

3.2. Competitive ability of inoculated strain

Experimental results provided no evidence for differences in time of nodule appearance by either strain. Nodules could be clearly observed 30 days after inoculation, independent of the applied treatment. The strain recovered from the nodules shows a different intrinsic antibiotic resistance pattern when compared with *Bradyrhizobium* sp. (the inoculated strain) Table 2. Based on these results, we may assume that *Bradyrhizobium* sp. was not occupying the nodules. In order to confirm this hypothesis, agglutination test was done. It was consistently apparent that agglutination between the combination of homologous antiserum and antigen was stronger (>1 : 160) than the converse (1 : 40) (Table 3). Thus, when antiserum from USDA 3187 was combined with antigen from nodule isolated strain, a weak agglutination (1 : 40) was observed. These results indicated that the strain present in nodules from the inoculated treatment came from

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**Table 2**

Zones of rhizobial growth inhibition with different antibiotics

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Native strain(a)</th>
<th><em>Bradyrhizobium</em> sp. USDA 3187</th>
</tr>
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<tbody>
<tr>
<td>Ofloxacin</td>
<td>23.33 ± 2.31</td>
<td>30.33 ± 0.58(b/c)</td>
</tr>
<tr>
<td>Cefixime</td>
<td>15.67 ± 1.15</td>
<td>0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>28.00 ± 1.73</td>
<td>21.33 ± 1.15(b/c)</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>16.67 ± 2.89</td>
<td>0</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phosphomycin</td>
<td>10.67 ± 0.58</td>
<td>20.67 ± 1.15(d/c)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>14.33 ± 2.08</td>
<td>32.00 ± 3.41(e/c)</td>
</tr>
<tr>
<td>Netilmicin</td>
<td>16.67 ± 2.08</td>
<td>15.67 ± 1.15</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>19.67 ± 0.58</td>
<td>28.33 ± 2.89(b/c)</td>
</tr>
</tbody>
</table>

Data are the mean ± S.E. of three determinations.

\(a\) \( p < 0.0025\).

\(b\) \( p < 0.0001\).

\(c\) \( p < 0.0005\).

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**Table 3**

Maximum antisera dilution that gives positive agglutination test

<table>
<thead>
<tr>
<th>Antisera from</th>
<th>Antigen</th>
<th>Dilution</th>
</tr>
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<tbody>
<tr>
<td>USDA 3187</td>
<td>nodule isolated strain</td>
<td>1 : 40</td>
</tr>
<tr>
<td>Nodule isolated strain</td>
<td>USDA 3187</td>
<td>1 : 40</td>
</tr>
<tr>
<td>USDA 3187</td>
<td>USDA 3187</td>
<td>&gt;1 : 160</td>
</tr>
<tr>
<td>Nodule isolated strain</td>
<td>nodule isolated strain</td>
<td>&gt;1 : 160</td>
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</table>
the native population. The fact that heterologous agglutination gives positive results until a 1 : 40 dilution may be indicative of the fact that USDA 3187 and the native strains are sharing some antigens.

**4. Discussion**

The introduction of effective strains of rhizobia depends on the competition for nodules’ sites between the introduced strains and the native population of rhizobia. Thus, a key property of an inoculum strain must be the ability to outcompete the indigenous soil bacteria.

Triplett (1990) indicates that a high competitiveness of inoculum strains in comparison with native rhizobia strains is as important as the effectiveness of symbiotic nitrogen fixation itself. Nodulation competitiveness is the ability of a given strain to dominate nodulation in the presence of other strains of the same species. We were unable to detect or reisolate *Bradyrhizobium* USDA 3187 from the nodules obtained from inoculated plants, using the agglutination test or by the analysis of the bacteria intrinsic antibiotic resistance pattern. Since we observed that indigenous strains were occupying the nodules, they might have a competitive advantage.

Peanut is considered a highly promiscuous species because it is nodulated by rhizobia able to nodulate a diverse group of legumes (Alwi et al., 1989). However, not all peanut microsymbionts are equally effective in fixing nitrogen in symbiosis with legumes. At field site, well-adapted native strains, with low nitrogen-fixing ability and/or high nodulation ability, may dominate the highly efficient, laboratory-tested inoculated strains (Martensson and Gustafsson, 1985). McLoughlin et al. (1984) reported that inoculation attempts failed to improve legume productivity because the indigenous strains occupied the root nodules rather than the inoculum strains. This was related to a more competitive nature of the indigenous population or to concentration of the inoculum used. It was also observed that when the inoculation improved yield, the indigenous population was very small (Triplett and Sadowsky, 1992). From our results, we may assume that this is not the case for our field site, although peanut was not present in the cropping history of this soil. Other factors may influence the competition between the native rhizobia and the selected inoculum strain by the occupation of legume nodules, for instance, biological factors, such as bacteriophages (Evans et al., 1979) or epiphytic bacteria (Handelsmann et al., 1988), as well as environmental factors, such as temperature, pH, nitrate content, etc. (Beattie et al., 1989).

Many approaches have been followed to address the competition problem. One method was to massively inoculate legumes with superior inoculum strains many times over a period of several years (Triplett and Sadowsky, 1992). This method may not only allow a permanent establishment of the inoculated strain in the soil but also increased the competitiveness of the strain in consecutive years. Another method was to increase the level of the inoculum used. Since Coventry et al. (1985) observed that rhizobia number may be low where cultivation during cropping has disturbed the soil organic matter content, we are carrying out studies to determine if it is possible to establish inoculant strains in low fertility soils using a higher inoculum dose.

In summary, we demonstrated that under our field conditions, natural bacterial populations are not only able to infect peanut plants but they are also highly adapted, competitive and are capable of replacing an introduced rhizobia strain.

**Acknowledgements**

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