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Contribution of biological nitrogen fixation to cowpea: a strategy for improving grain yield in the semi-arid region of Brazil

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Abstract Nodulating bacteria from the family Rhizobiaceae are common in the semi-arid tropics around the world. The Brazilian semi-arid region extends over 95 million hectares of which only 3% is suitable for irrigation, therefore leaving an immense dryland area to be exploited by peasant farmers, who often lack appropriate technologies for sustainable management. Cowpea is an important crop in this area, representing the staple protein source for human nutrition. This work aimed to identify rhizobial strains capable of guaranteeing sufficient nitrogen derived from biological fixation for cowpea cultivated in dryland areas, evaluating not just efficiency but also the ecological parameters of competitiveness and survival in the soil. Grain yield and nodulation parameters showed that strain BR 3267 is capable of establishing efficient nodulation, improving both yield and total N accumulated in grain. Cowpea inoculated with strain BR 3267 showed grain productivity similar to plants receiving 50 kg of N per hectare, which is the amount of fertilizer commonly used in the north-east region. These characteristics associated with previously determined ecological properties makes strain BR 3267 an important resource for the optimization of biological nitrogen fixation in cowpea in the dryland areas of the semi-arid tropics. Data on the dynamics of rhizobial populations in such areas have shown that (1) the naturalized rhizobium population is very small and, by themselves, do not promote proper nodulation and, (2) the inoculant rhizobia do not persist between crops. Such characteristics represent an opportunity for the introduction of superior rhizobia strains, such as BR 3267, during the cowpea crop.

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Introduction

Nodulating bacteria from the family Rhizobiaceae are common in the semi-arid tropics around the world. In Brazil, the semi-arid region comprises 95 million hectares of which only 3% are suitable for irrigation, leaving an immense dryland area to be exploited by sustainable social and economic practices. Under these conditions, grain production may represent an important economic alternative.

Cowpea (*Vigna unguiculata* (L.) Walp) is a leguminous plant high in protein cultivated over 1.6 million hectares in Brazil, mainly in the semi-arid region. Cowpea agribusiness generates resources of above US$ 350 million per year exploited by large and medium scale farmers, but also by a significant number of peasant farmers, representing a cash crop and an important protein source for more than 27 million Brazilians (Freire Filho et al. 1999). Amongst other advantages such as drought tolerance, cowpea may benefit from an efficient N_2-fixing symbiosis when associated with effective rhizobia.

The symbiotic specificity and host range of indigenous tropical rhizobia which nodulate cowpea and other tropical legumes has not yet been well characterized, but the rhizobia are often described as promiscuous, capable of nodulating a wide range of legumes but with poor effectiveness (Singleton et al. 1992). These characteristics may have a negative impact on the establishment of more efficient inoculant strains, acting as a barrier to the optimization of nodule formation and biological nitrogen fixation, representing an important agronomic limitation (Mpepereki et al. 1996). In order to address these problems, studies of tropical rhizobia/leguminous associations are focusing more on ecological parameters such as competitiveness and soil survival of isolates as well as dynamics of rhizobial populations (Martins et al. 1997).

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Competitiveness in the context of the *Rhizobium* spp. means the ability of a strain to form nodules through successful competition with various strains present in the seed or root environment (Simon et al. 1996). Although, the effect of the rhizosphere is not clearly understood, it may contribute to the selection of competitive but ineffective rhizobial strains (Athar and Johnson 1996).

More studies on aspects related to rhizobia ecology, capacity of colonizing nodulation sites and competitiveness determinants, as well as identification of plant traits related with specificity are therefore needed to overcome the difficulties in selecting competitive, persistent and effective rhizobia strains, allowing the maximization of biological nitrogen fixation contribution to this crop. The interaction of plant, rhizobia and edaphic/climatic factors should also be given special attention. Several factors have been pointed out as being capable of influencing rhizobial populations: soil fertility, physical properties such as pH and clay content, biotic factors such as distribution of host plant and prevalence of predators and, climatic effects including temperature and rainfall (Hirsch 1996). Nodulation is also dependent on an adequate supply of calcium and phosphorous and is adversely affected by aluminium, manganese and NO_3^- (Toro 1996). Xavier et al. (1998) demonstrated a strong correlation between competitiveness determinants and aluminium concentration in soil.

Biological nitrogen fixation associated with cowpea is able to improve plant growth and grain production avoiding the increase in production costs associated with the application of nitrogen fertilizers. This study aimed to select rhizobial strains capable of guaranteeing sufficient N derived from biological fixation for cowpea cultivated in dryland areas of the north-east semi-arid region of Brazil, evaluating not just efficiency but also ecological parameters such as competitiveness and soil survival.

Materials and methods

Rhizobial isolates

The strains of rhizobium tested were selected from amongst isolates obtained from cowpea nodules cultivated in soil samples from the north-east region of Brazil: Atlantic Forest, transition zone and semi-arid areas, located in the states of Pernambuco and Sergipe. The isolates had been previously characterized according to nodulation host range, biological nitrogen fixation efficiency, tolerance to temperature and salt stress and competitiveness determinants (Martins 1996). The strains used belong to the Germoplasm Bank of Embrapa Agrobiologia, Seropédica, RJ, Brazil.

Quantification of the native rhizobial population

The rhizobia population in the experimental area was quantified using the most probable number (MPN) technique with *Macroptilium atropurpureum* as host plant. Three soil samples (0–10 cm depth) comprising 20 sub-samples each, were collected four times in a period starting 1 month before the first crop and extending until the end of the second crop. Soil samples were suspended in sterile water (10^{-1} to 10^{-6}) and 1 ml each dilution was used as inoculation for plants growing in sterile test tubes. Four repetitions were used. Thirty days after inoculation, the number of nodulated plants was determined and rhizobia population was estimated using Mc-Crady's MPN table (Vincent 1970).

Field experiment

The selected isolates were tested as inoculant for cowpea cv. IPA 206, in small plots during two consecutive crops from 1998 to 1999. The experimental field was located in a dryland area (non-irrigated plot) located near Petrolina, Pernambuco State, north-east region of Brazil. A complete randomized block design with three repetitions was used, with two factors: factor 1, 10 inoculants, a control receiving N (+ N) fertilizer and an absolute control (uninoculated and – N); and, factor 2, three types of inoculation. The inoculation schedules were: (I) seeds were inoculated for the first crop, (II) seeds were inoculated for the second crop and, (III) seeds were inoculated for both first and second crops. Three harvests were taken: onset of nodulation at 30 days after emergence (DAE), during flowering (45 DAE) and during grain production (60 DAE). Plant growth parameters and nodulation were determined for all plants collected. Fresh nodules were dried over silica gel and used later as substrate for the immunoassay.

Isolates identification by enzyme linked immunosorbent assay

Rhizobia-forming nodules were determined by ELISA. Ten anti-sera were produced in young rabbits inoculated subcutaneously with selected strains and purified in Hitrap column (Amersham Bioscience cat. no. 17040201) following the producer's instructions, which enriches the anti-sera IgG concentration. Ten nodules from each treatment were selected at random and used as antigens. Each nodule was placed in a microtube with 500 μl water and then macerated. ELISA plates (Hemobag 96 wells, Hemobag) were prefilled with 50 μl poly-L-lysine, 0.01% (Sigma cat. no. P8920) for 20 min at room temperature and then washed with 200 μl Tris-saline buffer (TBS, g l^{-1}: Tris-HCl 1.37 g; Tris-base 0.16 g; NaCl 8.767 g). Nodule macerate was mixed and 50 μl was added to a well. The plate was incubated (30 min; 37°C) and washed with TBS (200 μl). The wells were blocked with bovine serum albumin, BSA (3%) in TBS (30 min; 37°C). The anti-serum was diluted with TBS according to the titration curve performed and 50 μl was added to the well and then incubated (30 min; 37C). The wells were washed 3 times with BSA (0.5%) in TBS (200 μl). Then, 50 μl the secondary antibody (anti-rabbit IgG alkaline phosphatase conjugate; CalBiochem cat. no. 401392) were added and incubated (1 h; 37°C). The plate was washed 5 times with BSA (0.5%) in TBS (200 μl). Finally, the substrate solution (p-nitrophenylphosphate substrate; CalBiochem cat. nos. 487663 and 487664) was added to the wells (100 μl) and incubated protected from light (30 min; room temperature). The color formed was determined at 405 nm in a Labsystem Multyscan Plus (Ribeiro 1999).

Positive and negative reactions to the ELISA represented, respectively, nodules formed by strains reacting or not to the anti-serum used. Crossed reactions were found not to interfere significantly with the results, therefore, positive reactions were assumed as nodules formed by inoculant strains, and negative reactions by naturalized strains.

Results

Rhizobia population in the experimental area

Quantification of rhizobia showed that the population is small before the first crop, where values were around 10 cells per gram of soil (Fig. 1). An increase in the rhizobia population to 10^2 cells per gram of soil occurred

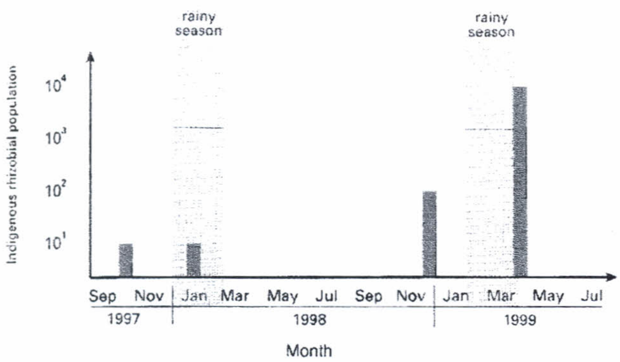

Fig. 1 Indigenous rhizobial population of the soil in the experimental area during the study period

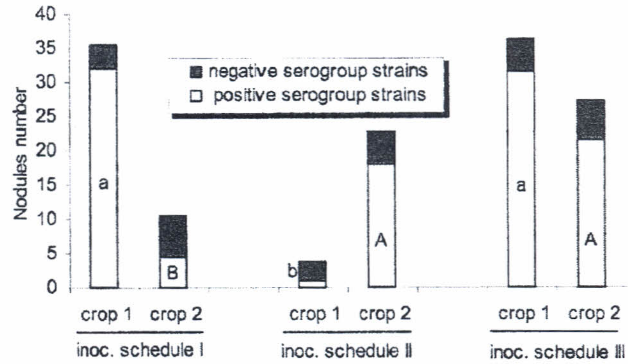

Fig. 2 Effect of inoculation schedules (*I* inoculation during the first crop, *II* inoculation during the second crop, *III* inoculation during first and second crops) on nodule numbers per plant formed by inoculant and naturalized strains observed during first and second crops (*crop 1* and *2*). Data refers to first harvest and it is a mean of data obtained for all the inoculants. *Same letters* mean no significant difference at 5% (Tukey's test) for nodule numbers formed by inoculant strains for each crop separately, while naturalized strains did not show any significant differences

after the first introduction of cowpea and another increase to around 10^4 cells per gram of soil after the second crop. Cowpea is generally cultivated in the semi-arid region without irrigation and planted just after the beginning of the rainy season when the rhizobia population is quite low. The rainy season often occurs from November to April, but during 1998 and 1999, the El Niño phenomenon, corresponding to an abnormal temperature increase in the Pacific ocean waters near the Equator which alters the climatic conditions of the semi-arid region, promoted an unusually long dry season.

Effect of the inoculation schedules on the establishment of the inoculant

Nodule formation on cowpea roots was strongly influenced by the inoculation practice. Three inoculation schedules were employed: (I) seeds were inoculated for the first crop to evaluate strain survival during the period between the two crops; (II) seeds were inoculated for the second crop only to evaluate the influence of the first crop of cowpea on the native rhizobial population; and, (III) seeds were inoculated for both crops to determine the effect of the second inoculation on the competitiveness of the inoculant strain. Figure 2 shows that both treatments inoculated during the first year (inoculation schedules I and III) led to significantly more nodules being formed by the inoculant strains compared to plants that did not receive inoculation (inoculation schedule II), where naturalized rhizobia were responsible for nodule formation. A similar pattern was observed during the second year.

Inoculation had a dramatic effect on the number of nodules formed while spontaneous nodulation due to naturalized strains was quite poor, as may be seen during the first crop year in schedule I. Inoculation in the first year was also not enough to ensure nodulation in the second year. This was interpreted as evidence of poor persistence by the inoculum strains.

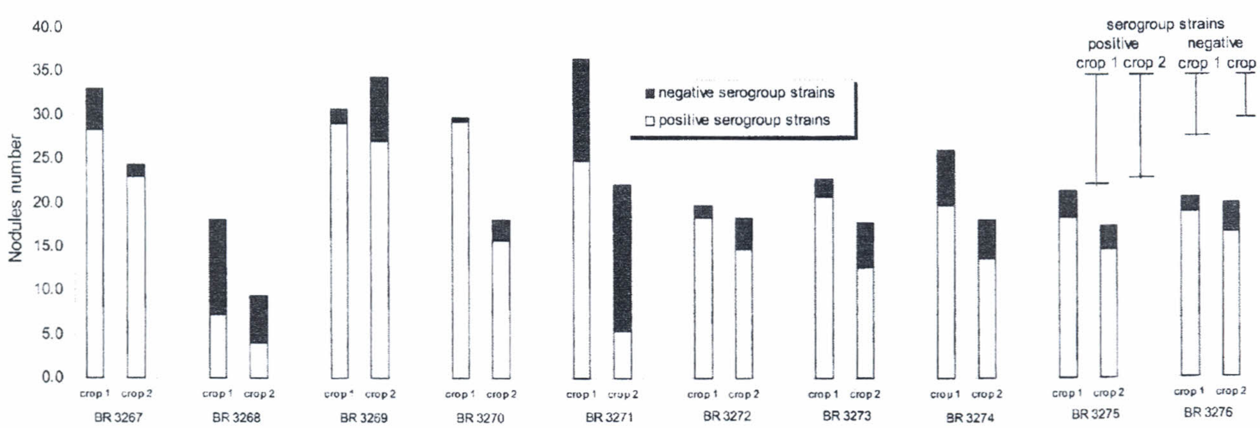

Fig. 3 Effect of inoculation on nodule numbers per plant formed by inoculant and naturalized strains observed during first and second crops (*crop 1* and *2*). Nodule numbers formed by inoculant and naturalized strains were compared by LSD test at 5% level for each crop separately. Data refers to first harvest and it is a mean of data obtained for the three inoculation schedules

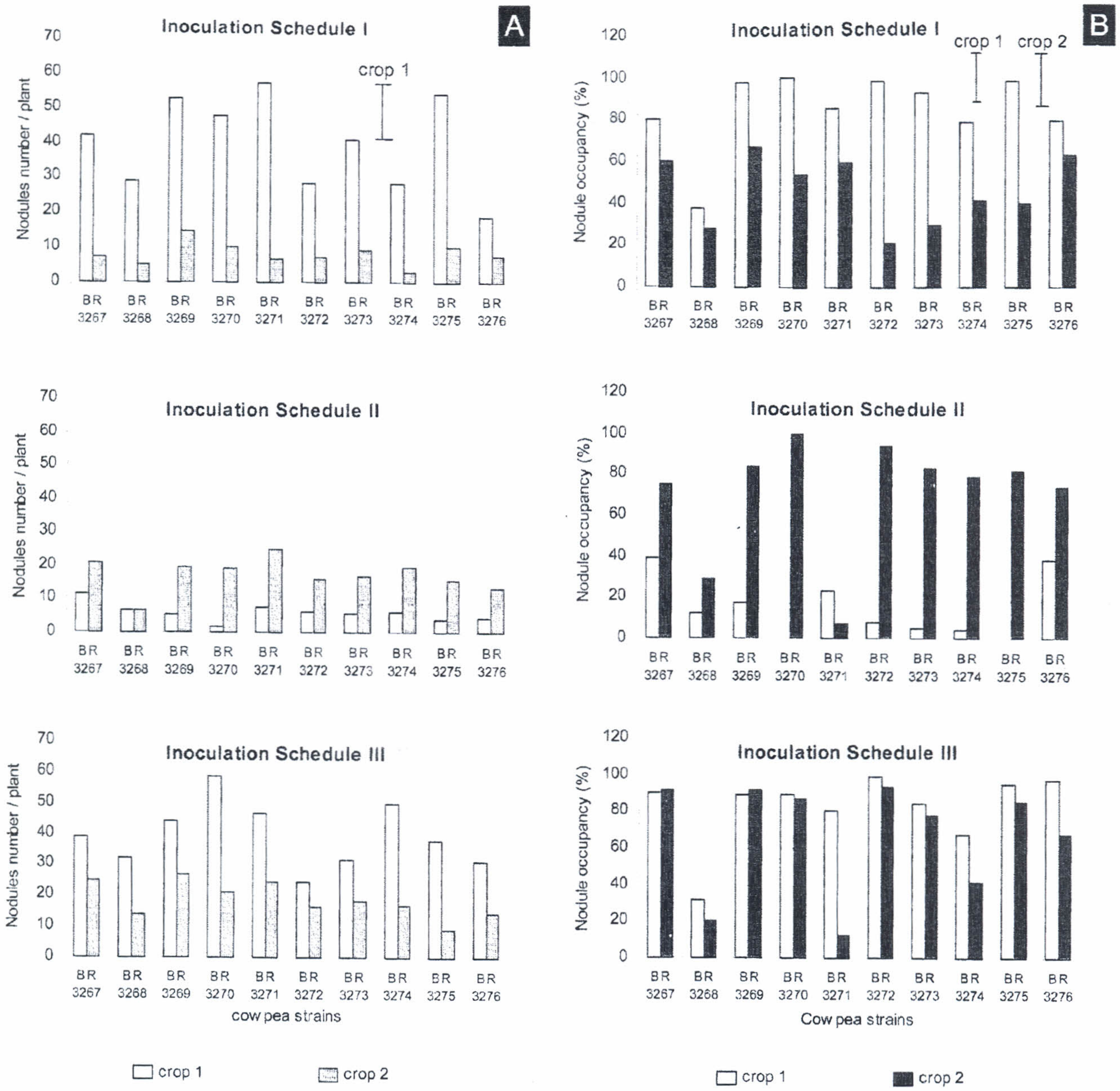

Fig. 4 Total nodule numbers per plant (**A**) and nodule occupancy (%) by inoculant strains (**B**) during first and second crops (*crop 1* and *2*), submitted to the inoculant schedules: *I* inoculation during the first crop, *II* inoculation during the second crop, *III* inoculation during first and second crops. Nodule numbers were compared by LSD test at the 5% level for the first crop, while nodule numbers obtained for the second crop did not show significant differences. Nodule occupancies were compared by LSD test at the 5% level for the first and second crop separately. Data is a mean of the three harvests

Effect of the inoculant strains on nodulation

The data in Fig. 3 refer to the number of nodules formed by the various inoculant strains. Total nodule numbers varied from 10 to 35 per plant depending on the inoculant used. Here also there was a reduction in nodule number during the second year for most of the treatments, indicative of poor persistence. However some strains, especially strains BR 3269, BR 3272 and BR 3276, were more persistent. In most treatments nodule formation by naturalized rhizobia was less than a quarter of the total. This showed that in this region, it is possible to select persistent rhizobia strains capable of inducing effective nodulation. On the other hand, strains BR 3268 and BR 2371showed poor persistence and poor competitiveness with naturalized strains.

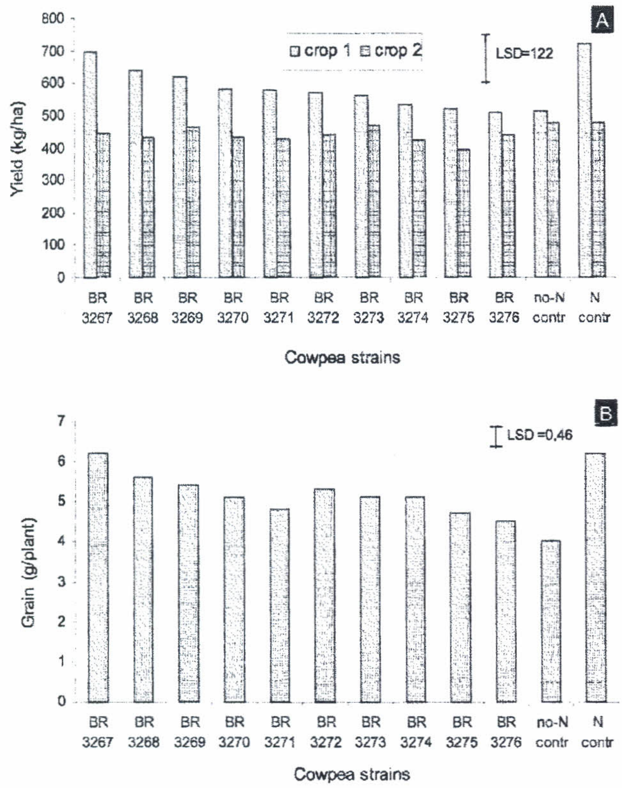

Fig. 5 Grain productivity (**A**) during first and second crops and N accumulated in grain during the first crop (**B**). For both parameters, data are means of the three inoculant schedules, compared by LSD at the 5% level for each crop

Inoculant behavior under the inoculant schedules applied

Inoculant survival in soil was evaluated at the end of the second year (inoculation schedule I). Nodule number data shown in Fig. 4A indicate that (1) the naturalized rhizobial population was responsible for little nodulation and (2) the inoculant strains were not capable of surviving during the period between the two crops. The naturalized population was never responsible for more than 5 nodules per plant (Fig. 4B, inoculation schedule II). Establishment of the inoculant was successful for eight of the ten strains tested. Strain BR 3271 was most capable of promoting nodule formation during the first crop but, like the other inoculants, its performance fell considerably in the second crop. Combining data from Fig. 4A and B (inoculation schedule III), it is possible to separate the other inoculant strains into two main groups: (1) strains capable of promoting good nodulation during the first and second crops: BR 3267, BR 3269, BR 3272 and BR 3273; and (2) strains capable of promoting good nodulation during the first crop only: BR 3270, BR 3274, BR 3275 and BR 3276.

Grain yield

Grain yield for the first crop showed significant differences between treatments. Addition of 50 kg N ha^{-1} promoted an increase of almost 30% on grain yield when compared to the uninoculated, non-N control plants. Grain yield of plants inoculated with strain BR 3267 was 693 kg grain ha^{-1}, similar to the yield observed in the control treatment receiving N fertilizer (Fig. 5A). Grain yield was closely related to grain N levels (Fig. 5B). On the other hand, there were no significant differences due to treatment in the second crop and plants displayed a mean value of 440 kg grain ha^{-1}.

Discussion

Cowpea is a plant generally recognized as being nodulated by a large range of soil rhizobia. This condition is thought to make the establishment of an effective inoculant strain very difficult. As a result, cowpea inoculation is rarely performed under field conditions.

In the semi-arid region, the data obtained showed that soil populations of cowpea rhizobia in dryland areas are very low and incapable of promoting proper root nodulation. A small native rhizobium population has also been identified in stressed areas by Woomer et al. (1992) and Athar and Johnson (1996).

The identification of rhizobium strains colonizing nodules was performed using ELISA, a technique based on immunological reaction and already described by other authors (Martensson and Gustafsson 1985; Evans et al. 1996). In this work, anti-sera were prepared against the inoculant strains and submitted to a purification step consisting of a column filled with *Staphylococcus aureus* protein A, which eliminates most of the non-specific immunoglobulins, IgMs, promoting a reduction of 30% in the level of crossed reactions, increasing specificity and, as a result, avoiding false positives (Ribeiro 1999).

ELISA results showed that antigens from strains of the rhizobium naturalized populations reacted with some of the antisera such as BR 3276 and BR 3267. However, this occurred only at a low frequency and had little impact on differentiation between naturalized and inoculant rhizobia. Strain BR 3276 has been isolated from a place near the experimental field so it is likely to occur all over the area, while the BR 3267 serogroup has been found in both the semi-arid region and Cerrado areas of north-east Brazil, suggesting that it is common in Brazil's tropical regions (Zilli 2001).

In this experiment, nodule number response to inoculation increased for most of the strains tested and results obtained by ELISA showed that the majority of nodules were colonized by inoculant strains.

As a general behavior, the inoculant strains do not persist between seasons and it is likely that, under the adverse conditions of this region, the presence of the host plant is essential to guarantee survival. The rhizosphere provides a rich nutrient environment where the existing microbial community, including the rhizobia population, is stimulated (Toro 1996). Therefore, not only edaphic-climatic characteristics but also plant presence are determinants for the survival and competition performance of the inoculant strain. The results indicate the need for yearly inoculation with effective rhizobia in the cowpea crop sown in dryland areas.

Cowpea is commonly recognized as being nodulated by slow-growing rhizobia and only recently have fast-growers been identified as capable of promoting efficient root nodulation (Zilli et al. 1999). Inoculant strain BR 3268, a fast-grower, although capable of increasing nodule number, did not colonize nodules efficiently. Nevertheless, during the first crop, it increased grain productivity. It is possible that this strain may somehow elicit molecular signals to the root cells making them more receptive to other soil rhizobia strains, which could explain the data obtained.

Besides its fast-growth capacity in culture media, strain BR 3268 showed tolerance to a high level of several antibiotics, salt and also tolerance to high temperature (Martins 1996). This set of characteristics have been considered important for competitiveness, establishment and soil survival and it is possible that co-inoculation of cowpea by fast-growing rhizobia may represent an important alternative for improving biological N fixation, which should be a subject for further studies.

This experiment was carried out from June 1998 to April 1999 and, during this period, the Brazilian semi-arid region was submitted to even more rigorous dry conditions than usual due to the El Niño effect. Average annual rainfall was as low as 200 mm during this period compared to 470 mm observed during 1996 and 1997. The presence of the El Niño phenomenon during two consecutive years in the region imposed a severe stress on the plants, resulting in a reduction of nodule number to 50% of the total observed during the first crop. As a consequence, grain yield was also reduced and no contribution of the inoculation could be observed during the second crop.

Grain yield and nodulation parameters showed that strain BR 3267 is capable of establishing an efficient symbiosis with cowpea. Grain yield of plants inoculated with this strain was similar to that of plants receiving 50 kg N ha^{-1}, which is the amount of fertilizer commonly used in the north-east region. These characteristics associated with ecological properties determined previously, such as tolerance to several antibiotics and high temperature stress, make strain BR 3267 an important component of a strategy for optimizing biological N fixation in cowpea in the dryland areas of the semi-arid tropics.

Cowpea inoculation in the dryland areas of the semi-arid region was shown to be a feasible practice due to the low rhizobium population present in the soils at the moment of crop sowing. The use of inoculation, despite not improving costs significantly, may increase grain yield, contributing to an increased food supply for the semi-arid Brazilian population.

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