Identification of effective strains of *Bradyrhizobium* for *Arachis pintoi*

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***Arachis pintoi*, a perennial peanut native to Brazil, is well nodulated with native rhizobia although the symbiosis is often ineffective. Since this leguminous plant has great potential as a multipurpose forage and cover crop in soils with low fertility, a study was initiated to select and identify effective *Bradyrhizobium* strains for *A. pintoi*. Of 230 authenticated bradyrhizobia isolated mainly from centres of diversity of the genus *Arachis* in South America, only 48 were effective on *A. pintoi*, although all the isolates nodulated the two genotypes evaluated. A specific host × strain interaction demonstrated a potential for improving the symbiosis. Under greenhouse conditions, several *Bradyrhizobium* strains proved competitive with the indigenous microflora and produced more dry matter yield and higher N-fixation rates than the native rhizobia.**

Keywords: *Arachis pintoi*; *Bradyrhizobium*; Nodulation; Genotypes; Nitrogen fixation; Host × strain interaction

Biological N-fixation can provide an economic and environmentally safe source for efficiently-nodulated leguminous species. This can be especially advantageous in tropical soils which are generally low in available plant nutrients. Leguminous forage species adapted to these soils and capable of high N-fixation may, therefore, have an important impact on pasture-based animal production systems in these areas.

Arachis pintoi, a leguminous perennial plant native to Brazil, and locally known as tropical white clover, is considered a multiple purpose plant that can be utilised on low fertility soils as a forage and cover crop (Thomas, 1993; Hardy, 1995; Kiss, 1997). It is a promiscuous species and can nodulate well with native rhizobia strains, although this symbiosis may be ineffective (Oliveira *et al.*, 1996, 1997). In experiments carried out at Centro Internacional de Agriculture Tropical (Cali, Colombia), it was observed that plants inoculated with some selected strains had increased shoot N content when compared with uninoculated controls with abundant and apparently active nodules (Silvester-Bradley *et al.*, 1988). These results indicate that it is possible to enhance the biological N-fixation efficiency of *A. pintoi* by the selection of more effective strains of rhizobia to be used as the inoculant. Therefore, the objective of this work was to select and identify effective *Bradyrhizobium* strains for *A. pintoi* for improving its dry matter (DM) yield and forage quality.

Materials and Methods

Preliminary screening

In a preliminary screening test at North Carolina State University, U.S.A., 230 strains of *Bradyrhizobium* were evaluated for their capacity to form nodules on *A. pintoi* cv. Amarillo and ecotype BRA 031143. Two hundred and twenty-five strains were from the Soil Microbiology Laboratory Collection of the North Carolina State University, 3 strains were from Centro Internacional de Agriculture Tropical (CIAT, Colombia), and 2 strains were from Universidade Federal de Minas Gerais (UFMG, Brazil). Most of the 230 *Bradyrhizobium* sp. strains used in this study were isolated from nodules collected in South America from wild peanuts from the centres of diversity of the peanut principally from Brazil, Argentina, Bolivia, and Paraguay. These had previously been tested for effectiveness with *A. hypogaea* (spanish and virginia types) and cross-inoculated with siratro (*Macroptilium atropurpureum*), cowpea, mungbean, and pigeon pea. Before planting, seeds of cv. Amarillo

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were surface-sterilized by soaking in 70 g L^{-1} of $Ca(OCl)_2$ (Elkan *et al.*, 1981) and seeds of the ecotype BRA 031143 were surface-sterilized by soaking in a solution containing 35 g L^{-1} of $Ca(OCl)_2$ and 5 g L^{-1} of NaOCl. After gently shaking for 10 min in these solutions, the seeds were washed eight times with sterile water and pre-germinated in sterilized vermiculite. After germination, the seedlings were transplanted to 180-mL plastic bottles containing sterilized vermiculite as substrate, inoculated with 1 mL suspension of the appropriate rhizobial strain, and capped with plastic to avoid contamination. Rhizobial cultures for seedling inoculations were grown in an orbital incubator at 28°C until log phase (about 10^8 cells mL^{-1}). As controls, the same strains were tested on siratro plants. The experiment was conducted in a growth chamber with 16 h of light at 28°C and 8 h of darkness at 22°C. After six weeks of growth, the plants were harvested and nodule colour intensity was graded according to a scale of zero (no pink colour) to three (intense pink colour). The growth of all 230 strains were also evaluated in petri dishes, in solid HM-Salts medium (Elkan, 1995), in an incubator at 28°C for 5–8 days.

Test for N-fixing capacity

The N-fixation efficiency of strains graded 2 and 3 according to their pink colour was further tested using the same *A. pintoi* genotypes, cultivated in modified Leonard jars (Wacek and Alm, 1978) filled with a sterile mixture of 1:1 sand–vermiculite. For this experiment, seeds were surface-sterilized and pre-germinated as described before. Each jar was planted with one seedling inoculated with 10 mL of a solution containing approximately 10^8 cells mL^{-1} of the appropriate rhizobial strain. Uninoculated jars and jars fertilized with mineral nitrogen [70 parts per million (ppm) N as KNO_3] were used as controls. Every week, all jars received 200 mL of a Norris and Date (1976) nutrient solution without N modified by Vargas (pers. commun.) for optimum *A. pintoi* growth. The medium is shown in Table 1. The experiment was carried out in a greenhouse and the experimental design was a randomised complete block design, each treatment in triplicate. After eight weeks of growth, the plants were harvested and separated into roots and shoots. Nodules were removed from the roots and counted. Dry weight was determined for shoots, roots, and nodules. Shoot nitrogen content was determined according to the standard Kjeldahl method. Strain effectiveness was calculated according to the equation proposed by Date *et al.* (1993) (100 × inoculated plant DM/N fertilized plant DM) with N-fixing effectiveness classified as ineffective <35%; lowly-effective 35–50%; effective 50–80%; and highly effective >80%. An analysis of variance was done for each variable and the mean values were analysed by the Scott–Knott method (1974).

Table 1 Modified Norris and Date medium used for growth for *Arachis pintoi*

Ingredient	Concentration
KCl	0.001 M
KH_2PO_4	0.005 M
K_2HPO_4	0.005
$MgSO_4$ $7H_2O$	0.001 M
$CaSO_4$ $2H_2O$	0.007 M
$CuSO_4$ $5H_2O$	0.01 ppm Cu
$ZnSO_3$ $7H_2O$	0.025 ppm Zn
$MnSO_4$ $4H_2O$	0.25 ppm Mn
$(NH_4)_6$ Mo_7O_{24} $4H_2O$	0.0025 ppm Mo
H_3BO_3	0.125 ppm B
$FeC_6H_5O_7$	0.3 ppm Fe

Strain screening for tolerance to low pH

The most effective strains for each *Arachis* material were tested for their capacity to grow at arbitrarily selected pH levels (6.8, 6.0, 5.5, 5.0, 4.5, and 4.0) in solid HM-Salts medium (Elkan, 1995) in petri dishes, for 6–8 days. Additionally, seedlings of each genotype were grown in vermiculite pots, inoculated with the selected strain, and irrigated every other day with the modified Norris (1964) solution without N, and with pH properly adjusted according to treatments. Nitrogen and non-inoculated control treatments were included. The experiment was carried out in the growth chamber under the same conditions described before. After six weeks, the plants were harvested and the same parameters as measured in the greenhouse experiment were recorded. The data obtained were subjected to statistical analysis and means were ranked according to Duncan's Multiple Range Test at the 5% level.

Strains competitiveness in a Cerrado soil

Strains selected were further studied at EPAMIG in Sete, Lagoas, Brazil. Ecotype BRA 031143 plants inoculated with strains NC 70, NC 229, NC 230, NC 502.3, NC 656.1, and TAL 295 were grown in pots containing 180 cm^3 of a Cerrado soil. The soil was previously limed to 50% cation exchange capacity and fertilized with macro- (except N) and micro-nutrients. Rice husk was added to immobilize soil N and two controls with and without N as NH_4NO_3 (300 mg kg^{-1} soil) were included. As the soil used in this experiment was not sterilized, the treatment receiving no inoculation and no N fertilization gives an indication of the effectiveness of the native rhizobia population. This experiment was carried out as a randomised complete block design, with treatments in triplicate. After 12 weeks of growth, the plants were harvested and shoot DM yield, shoot per cent N, and shoot N content determined. Means were ranked according to Duncan's Multiple Range Test at the 5% level.

Results and Discussion

Preliminary screening

From the 230 strains tested for nodulating *A. pintoi*, 48 strains were selected for their capacity to form pink-coloured nodules on plants cultivated in Leonard jars. Among them, 33 strains originated from centres of diversity of the genus *Arachis* in South America (Elkan *et al.*, 1981) and were identified as NC, three strains were from the NIFTAL TAL 295, TAL 1000, and TAL 1371, three strains were obtained from the USDA-Beltsville (3G4b4, 3G4b5, and 3G4b20), three were miscellaneous cowpea strains (SMS-2, Tha 201, and Tha 205), one strain was from the Nitragen Co. (8b4), three strains were from CIAT (CIAT 2138, CIAT 3101, and CIAT 3806), and two strains were from UFMG-Brazil (MGAVI9 and MGAC2).

Test for N-fixing capacity

Response to inoculation varied between the two *A. pintoi* genotypes, as previously observed for other *Arachis* species (Elkan *et al.*, 1980, 1995). Some strains were effective for ecotype BRA 031143 but ineffective for cv. Amarillo (Figure 1). According to the protocol proposed by Date *et al.* (1993) to classify strains effectiveness, 19% were classified as highly effective, 25% as effective, 17% as lowly effective, and 39% as ineffective for the ecotype BRA 031143. Strain CIAT 3101 considered effective for *A. pintoi* (Thomas, 1993) was classified as highly effective for ecotype BRA 031143 but not for cv. Amarillo. For cv. Amarillo, strains CIAT 3101 and NC 656.1 were classified as lowly effective and all others as ineffective. Although all 48 strains were capable of nodulating both genotypes, most strains were classified as either lowly effective or ineffective. These results are in accordance with previous studies (Thomas, 1993; Oliveira *et al.*, 1996, 1997), which determined that *Arachis* spp are promiscuous ineffective legumes. The effect of inoculation on BRA 031143 shoot DM (Figure 2) showed that 20 strains produced DM yield comparable to the N treatment. Cultivar Amarillo was much more responsive to N fertilization than ecotype BRA 031143 with the N control producing more DM for all the strains (Figure 3). However, 21 strains produced significantly more DM yield with cv. Amarillo than control plants not fertilized with mineral-N. On both genotypes, the addition of N fertilizer increased DM yield but not shoot per cent nitrogen. Thirty-three strains increased shoot per cent nitrogen of ecotype BRA 031143 (Figure 3) and 37 strains of cv. Amarillo (Figure 4) when compared to the mineral-N treatment. For BRA 031143, 22 strains produced shoot nitrogen content comparable to plants which received mineral-N (Figure 5). For cv. Amarillo, although 22 strains produced shoot N, with values significantly higher than the control plants which did not receive mineral-N, none of the strains tested produced sufficient shoot nitrogen content comparable with the mineral-N treatment (Figure 6).

Figure 1 Inoculation of *Arachis pintoi* with *Bradyrhizobium* strain 3G4b20. This strain was effective for ecotype BRA 031143, but not for cv. Amarillo

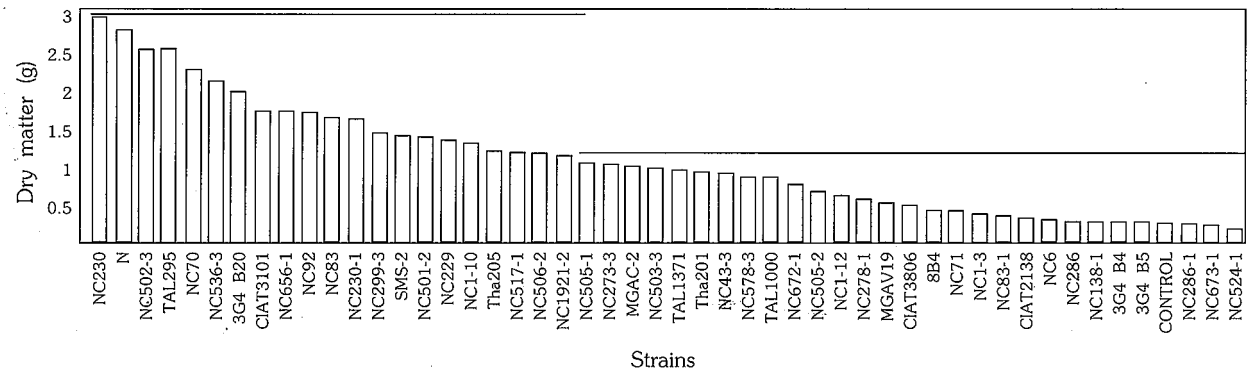

Figure 2 Effect of inoculation on shoot dry matter yield of *Arachis pintoi* BRA 031143. Treatment means covered by the same line are not significantly ($P > 0.05$) different by the Scott–Knotts test

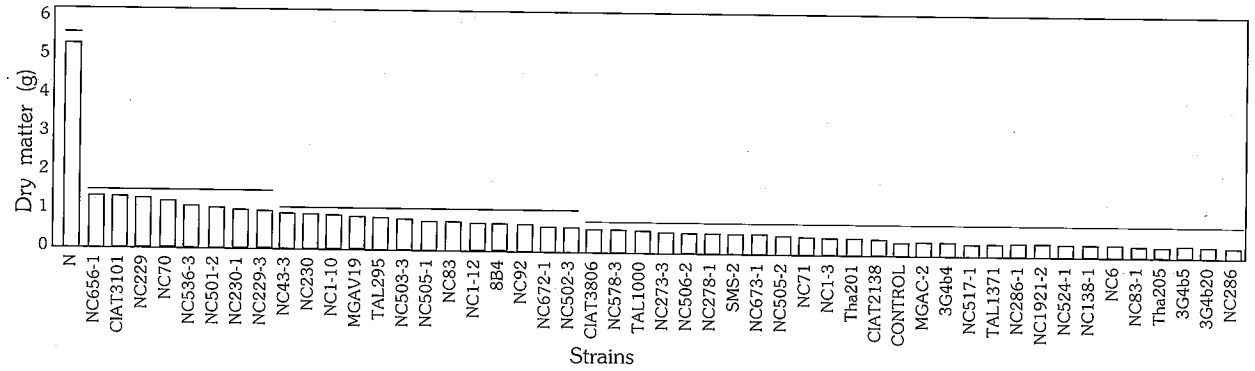

Figure 3 Effect of inoculation on shoot dry matter yield of *Arachis pintoi* cv. Amarillo. Treatment means covered by the same line are not significantly ($P > 0.05$) different by the Scott–Knotts test

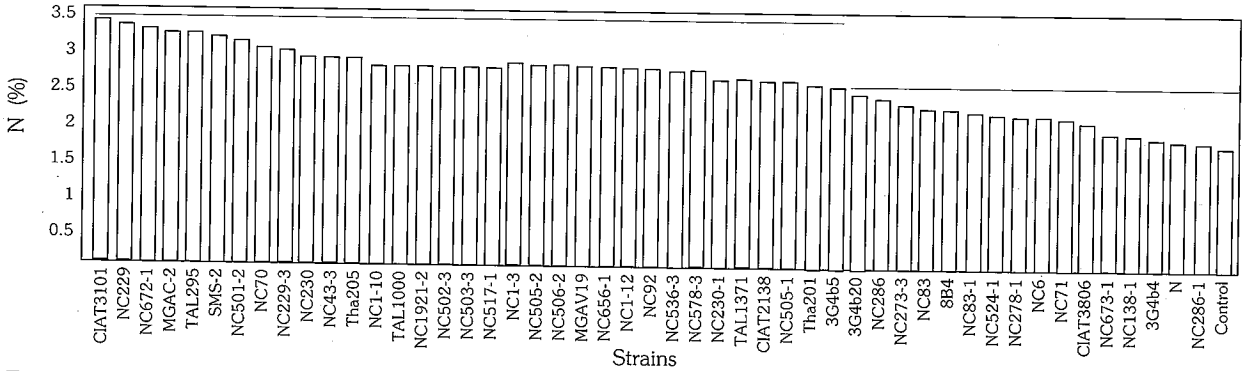

Figure 4 Effect of inoculation on shoot per cent nitrogen of *Arachis pintoi* BRA 031143. Treatment means covered by the same line are not significantly ($P > 0.05$) different by the Scott–Knotts test

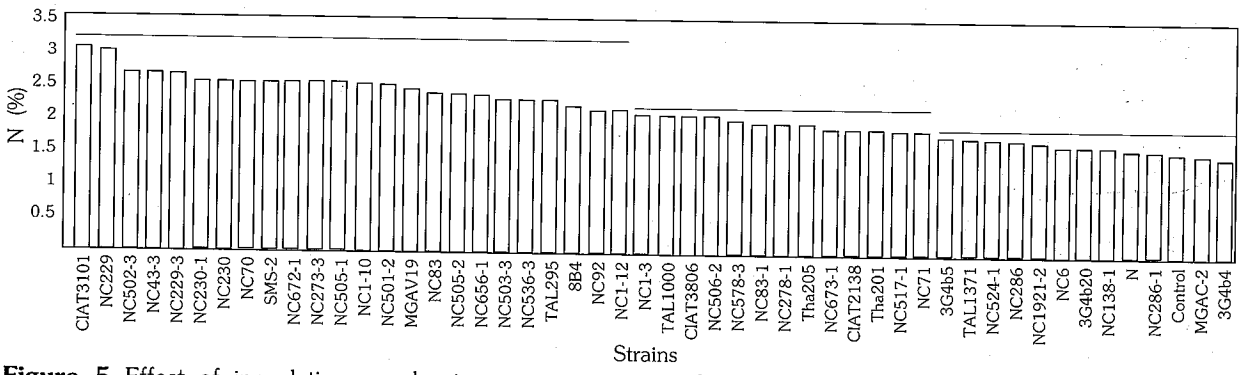

Figure 5 Effect of inoculation on shoot per cent nitrogen of *Arachis pintoi* cv. Amarillo. Treatment means covered by the same line are not significantly ($P > 0.05$) different by the Scott–Knotts test

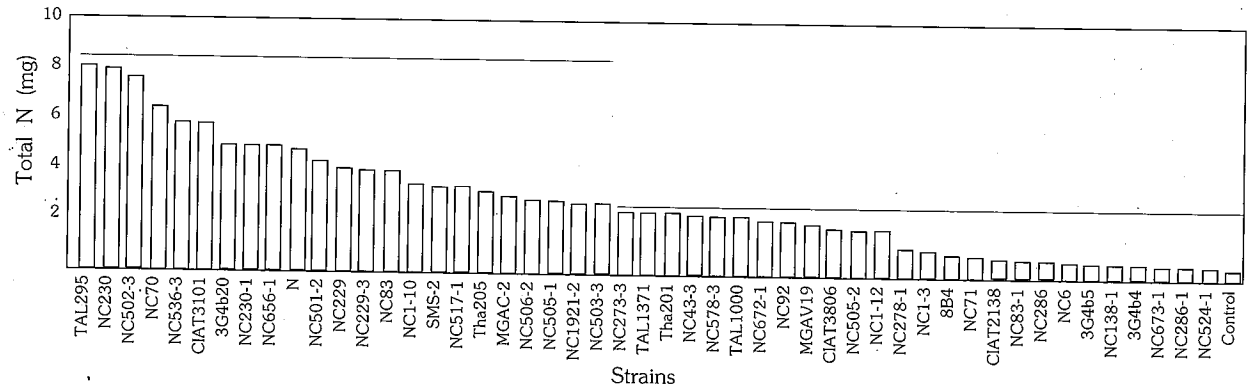

Figure 6 Effect of inoculation on shoot nitrogen content of *Arachis pintoi* BRA 031143. Treatment means covered by the same line are not significantly ($P > 0.05$) different by the Scott–Knotts test

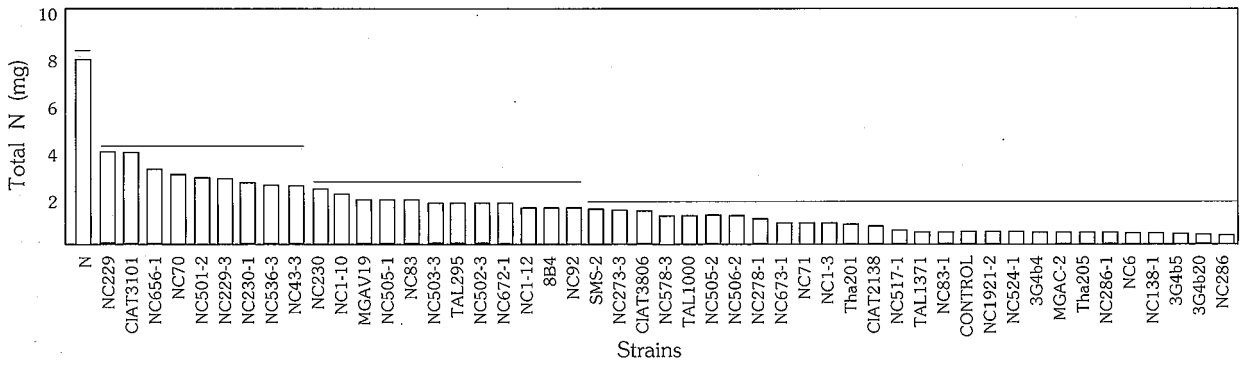

Figure 7 Effect of inoculation on shoot nitrogen content of *Arachis pintoi* cv. Amarillo. Treatment means covered by the same line are not significantly ($P > 0.05$) different by the Scott–Knotts test

Strain screening for tolerance to low pH

As maximum growth of *A. pintoi* is reduced when cultivated at soil pH below 5.4 (Rao and Kerridge, 1993), the objective of this experiment was to identify strains capable of forming effective nodules at low pH. It was observed that all strains tested—NC 70, NC 230, NC 502.3, NC 656.1, CIAT 3101, and TAL 295, grew well when pH ranged from 4.5 to 6.8 but only strains CIAT 3101 and NC 656.1 grew well at pH 4.0. Both genotypes nodulated abundantly and did not differ ($P > 0.05$) in DM yield, shoot per cent nitrogen, and shoot nitrogen content when cultivated for six weeks in a growth chamber and irrigated with N-free solution ranging between 4.5 and 6.8 (data not shown).

Strains competitiveness in Cerrado soil

Because cv. Amarillo showed less response to inoculation than ecotype BRA 031143, the experiment to screen for strain competitiveness in the Cerrado soil was done with BRA 031143. These results are summarised in Table 2. Field trials have demonstrated that uninoculated BRA 031143 plants produced significantly more shoot DM yield and protein content than cv. Amarillo (Andrade *et al.*, 1997; Viana *et al.*, 1998). All strains evaluated in this experiment—NC 70, NC 229, NC 230, NC 502.3, NC 656.1, and TAL 295, produced significantly more shoot DM yield than control plants not fertilized with mineral-N, with values comparable to the mineral-N treatment. As the non-fertilized plants formed nodules with the native rhizobia of the Cerrado soil, these results clearly indicated that these selected strains were more effective than the native rhizobia (Table 1). Strains NC 229 and NC 656.1 produced higher shoot per cent nitrogen than the control plants (mineral-N and native rhizobia), whereas, except for strain TAL 295, all others produced more shoot nitrogen content than the native rhizobia (non-fertilized plants). It is noteworthy that strain NC 229 produced significantly more shoot nitrogen content than the N-fertilized treatment, with values nearly three-fold higher than those observed for the non-fertilized (native rhizobia) plants.

Table 2 Effect of *Arachis pintoi* BRA 031143 inoculation with selected *Bradyrhizobium* strains on shoot dry matter yield, per cent protein, and shoot total N in a Cerrado soil

Treatment	Shoot dry matter (g)	Shoot N (%)	Shoot N content (mg)
NC 70	3.975 a[1]	2.737 b	10.85 b
NC 229	4.004 a	3.597 a	14.42 a
NC 230	4.266 a	2.427 bc	10.35 b
NC 502.3	3.912 a	2.460 bc	9.77 b
NC 656.1	3.028 bc	3.413 a	10.28 b
TAL 295	3.660 ab	2.270 bc	7.991 bc
With nitrogen	3.757 ab	2.693 bc	10.18 b
Without nitrogen	2.484 c	2.190 c	5.437 c

[1]Means of three replications. Similar letters in the same column do not differ significantly ($P > 0.05$) by Duncan's Multiple Range Test

Arachis pintoi shares the property of nodulation promiscuity with *A. hypogaea* under field conditions, although effective nodulation is considerably more restrictive. It has been even more difficult to obtain effective nodulation with singles strain isolates as a prelude to improving N-fixation with this symbiosis. Only 48 out of the 230 peanut isolates were able to fix N in *A. pintoi* and about half of these had significantly greater shoot nitrogen than did the uninoculated control. However, inoculation with effective *Bradyrhizobium* strains resulted in a maximum of less than half of the total shoot nitrogen and about one-third of the shoot DM as that in the N control. ^{15}N isotope studies have shown that effective rhizobia supply about 60% of the total N in *A. hypogaea*, so the potential for improvement of this symbiosis exists. It is interesting to note that inoculation of ecotype *A. pintoi* BRA 031143 resulted in much greater response in DM yield and N accumulation than did *A. pintoi* cv. Amarillo using the same rhizobial strains. Although this

study was designed to obtain effective single-strain isolates, these results showed a significant specific cultivar × rhizobia interaction which can be exploited via plant breeding technology to further improve N-fixation efficiency. Lastly, several of the selected *Bradyrhizobium* strains in preliminary screening studies under field conditions not only outcompeted the native microflora, but also outperformed these with respect to plant yield and total N-fixation. Despite the fact that the question remains as to why *A. pintoi* is so much more specific than *A. hypogaea* in forming effective nodules, this study has demonstrated that it is possible to improve the symbiosis under field conditions by carefully selecting the correct *Bradyrhizobium* strains for inoculation. Based on these results, new experiments have been established to evaluate strains NC 70, NC 229, and NC 230 under field conditions on two Cerrado sites.

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