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ASSESSMENT OF N_2 FIXATION BY THREE *Arachis pintoi* ECOTYPES USING THE ISOTOPE DILUTION TECHNIQUE

[DETERMINACION DE LA FIJACION DE N_2 POR TRES ECOTIPOS DE *Arachis pintoi* USANDO LA TECNICA DE DILUCION DE ISOTOPOS]

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ABSTRACT.

The nitrogen derived from N_2 fixation was measured by the ^{15}N isotope dilution method, in three *Arachis pintoi* ecotypes: CIAT 17434, 18744 and 18748, using a *Bradyrhizobium* strain (BS) CIAT 3101, and native strains (NS) from Veracruz, Mexico, under greenhouse conditions. The reference plants were: a non-nodulating *Arachis hypogaea*, *Brachiaria arrecta*, *Brachiaria brizantha* and *Cynodon nlemfuensis.* Plant material received a ^{15}N-enriched solution at 5 atom % ^{15}N. Dry matter yield was highest ($P\leq0.001$) in *A. Pintoi* ecotypes+BS (53.9 ±5.2 g pot^{-1}), compared to *A. Pintoi* ecotypes+NS (19.7 ± 0.7 g pot^{-1}). There were significant differences ($P\leq0.001$) in N yield between *A. Pintoi* ecotypes+NS (439 ± 19.6 mg pot^{-1}) and *A. Pintoi* ecotypes+BS (1538 ± 117.0 mg pot^{-1}). Values of atom% ^{15}N in excess of legume shoots were lower (0.027 ± 0.01) with BS, compared to NS group (0.052 ± 0.002). The N_2 fixed was greater than 50% in the three *A. Pintoi* ecotypes+ BS, using grasses as reference plant. *Arachis pintoi* ecotypes CIAT 17434 and CIAT 18744 fixed more N than ecotype CIAT 18748; and the improved *Bradyrhizobium* strain CIAT 3101 was more efficient in N_2 fixation than the native strains.

Key words: *Arachis pintoi* ecotypes; reference plants; *Bradyrhizobium* strain; ^{15}N isotope.

RESUMEN

Se midió la fijación de N_2 por la técnica de dilución de isótopos de N^{15} en tres ecotipos de *Arachis pintoi*: CIAT 17434, 18744 y 18748, usando la cepa *Bradyrhizobium* CIAT 3101 (BS) y cepas nativas (NS) de Veracruz, Mexico, en invernadero. Las plantas de referencia fueron: *Arachis hypogaea* (no-nodulante), *Brachiaria arrecta, Brachiaria brizantha* y *Cynodon nlemfuensis.* El material vegetal recibió una solución de N^{15} enriquecida con 5% de átomos de N^{15}. El rendimiento de materia seca fue más alto ($P\leq0.001$) en los ecotipos *A. pintoi*+ BS (53.9 ±5.2 g maceta^{-1}), comparado con los ecotipos *A. pintoi*+NS (19.7 ± 0.7 g maceta^{-1}). Se encontraron diferencias significativas ($P\leq0.001$) en rendimiento de N entre ecotipos *A. pintoi*+NS (439 ± 19.6 mg maceta^{-1}) y ecotipos *A. pintoi*+BS (1538 ± 117.0 mg maceta^{-1}). Los valores de % átomos de N^{15} en exceso en hojas y tallos de las leguminosas fueron más bajos (0.027 ± 0.01) con BS, comparado al grupo de NS (0.052 ± 0.002). El N_2 fijado fue mayor a 50% en los ecotipos *A. pintoi*+BS, usando las gramíneas como plantas de referencia. Los ecotipos *Arachis pintoi* CIAT 17434 y 18744 fijaron más N que el ecotipo CIAT 18748; y la cepa mejorada *Bradyrhizobium* CIAT 3101 fue más eficiente para fijar N_2 que las cepas nativas.

Palabras clave: Ecotipos de *Arachis pintoi*; plantas de referencia; cepa de *Bradyrhizobium*; isótopos de N^{15}.

INTRODUCTION

Savannas cover about 300 million hectares of South America, where soils, mainly Oxisols and Ultisols are of very low fertility and high acidity (Pereira, 1982). According to Boddey *et al*. (2004), tropical regions of Brazil sustain at least 80 million ha of pastures, principally *Brachiaria* spp, where they estimated that at least half of these pastures are degraded mainly due to lack of fertilization and overgrazing.

The use of legumes as N_2 fixers to improve soil fertility and consequently pasture productivity in the tropical areas has been proposed since 1970 (CIAT, 1974). Biological nitrogen fixation (BNF) is the key to sustainable agricultural systems in tropical soils, which are frequently deficient in N (Hungria and Vargas, 2000). Herridge *et al.* (2008) in a review about the more recent estimates of biological N_2 fixation for the different agricultural systems, including the extensive, uncultivated tropical savannas used for grazing, mentioned that annual N_2 fixation inputs in pastures and fodder legumes were 12–25 million tones. However, the benefits of this N_2 fixation to the system in many cases is not immediate as it requires improvement of other complementary factors such as the presence of effective *Bradyrhizobium* strains (Sanginga *et al*., 2000), and proper choice of persistent legumes in order to achieve the desired benefits (Giller and Cadisch, 1995).

The contribution of improved legumes as a forage component is more important when grown along with grasses, as grasses take advantage of the N fixed by the legume (Ledgard, 2001). New tropical forage legumes like *Arachis pintoi*, *Centrosema pubescens*, *Desmodium ovalifolium,* and *Stylosanthes guianensis* have been shown to improve pasture productivity. Research results obtained from the hot humid areas of Mexico (Cab-Jiménez *et al*., 2008; Castillo-Gallegos, 2003; Ascencio *et al*., 2005; Castillo *et al*., 2005) and from other parts of Latin America (Ciro *et al*., 2004; Behling-Miranda, 2003) showed that *Arachis pintoi* has the potential to be grown in association with grasses, and that an accurate quantification of the N_2 that can be symbiotically fixed by this legume is important in order to determine their value and suitability in improving tropical animal production systems and maintaining or improving N levels in the soil (Chalk and Ladha, 1999).

The *Bradyrhizobium* strain CIAT 3101 has been identified as a successful strain to inoculate *A. pintoi* ecotypes (Purcino *et al*., 2000; Thomas, 1994). However, the role of native rhizobial strains in the promotion of an effective nodulation is unknow till date (Thomas, 1994; Pinto *et al*., 1999; Melchor-Marroquin *et al*., 1999; Castro *et al*., 1999). Mendez and Mayz (2000) indicated that tropical soils contain a great diversity of native rhizobial strains that could fix N_2 required by legume or proportionate new isolates to produce effective inoculants.

The objectives of this study were to quantify the BNF contribution by three *A. pintoi* ecotypes, using the ^{15}Nisotope dilution technique, and to determine the most appropriate *Rhizobium* strain for the growth and N accumulation for the above mentioned ecotypes. Therefore, both improved and native rhizobial strains were included in this experiment to gain knowledge about their potential to promote N_2 fixation.

MATERIALS AND METHODS

The experiment was set up in the greenhouse at Imperial College at Wye (University of London), to assess symbiotic N_2 fixation by the *Arachis pintoi* ecotypes CIAT 17434, 18744 and 18748 using the ^{15}N isotope dilution method and four reference plants: a non-nodulating *Arachis hypogaea, Brachiaria arrecta, Brachiaria brizantha* and *Cynodon nlemfuensis.* The soil used for this study was a sandy loam (11, 19, 24 and 46 % clay, silt, fine and coarse sand, respectively) and had a pH of 6.8, total N % of 0.38, δ ^{15}N of 7.5 $^{0}/_{00}$, while values for Ca, K and Mg were 16.5, 0.8 and 0.9 cmol kg soil^{-1}, respectively. P was 30.9 μg g soil^{-1}. The soil was air dried and sieved through a 2 mm sieve and combined with 1/3 of sand. Sterilized pots (sodium hypochlorite-water, 4-20) were filled with 2 kg of the sand-soil mixture.

Sterilized (0.2% mercuric chloride) seeds of the three cultivars of *A. pintoi,* A. *hypogaea* and *B. brizantha* were used, and vegetative materials (stolons) for the other species were collected from plant stock materials. Seeds or stolons were sown in pots (17.5 x 17.5 x 12.5 cm) and after seedling emergence, they were thinned to 2 plants per pot. Legumes and reference plants were treated with an N-free nutrient solution (200 ml twice a week) and a micronutrients-stock solution (0.5 ml l^{-1}). In between, pots were watered with deionised water.

Before sowing, the soil/sand mixture of every pot received a ^{15}N-enriched solution of $K^{15}NO_3$ at a rate of 1 kg N ha^{-1} at 5 atom% ^{15}N. A volume of 250 ml pot^{-1} taken from the diluted ^{15}N solution was used to wet the mixture, which was thoroughly mixed. The pots were then sown with *A. pintoi* ecotype or reference plant. Subsequently ^{15}N applications were made after each harvest using the same volume on the pot's surface, in order to avoid depletion of plant available ^{15}N (Boddey *et al*., 2008).

After germination *A. pintoi* ecotypes were inoculated with the *Bradyrhizobium* strain CIAT 3101 (19 x 10^7 cells pot^{-1}) or with a mixture of native rhizobial strains obtained from a soil solution (1:5 soil/distilled water) prepared from a soil of Veracruz, Mexico (7.7 $^{0}/_{00}$ δ ^{15}N enrichment), under native vegetation. Each pot was supplied with 4 ml aliquot of supernatant for each strain and repeated 4 times. Inside greenhouse, temperature and air humidity were maintained at around 26 ºC and 70%, respectively, throughout the entire experiment.

The study consisted of five plant growth cycles of 73 days duration. After 73 days, plants were harvested and the shoot dry weights were measured for legumes and grasses. Shoots were dried at 40 °C in a forcedhot-air oven to a constant weight, weighed and ground to a fine powder (<1 mm) using a micro hammer mill. The ground samples were then sub-sampled, weighed into tin capsules and analyzed for %N and atom% ^{15}N using a stable isotope mass spectrometer (Europa Scientific 20-20, Crewe, UK), coupled to a C/N analyzer (Roboprep). All samples were 4 determined in duplicate.

To calculate the proportion (*P*) of N_2 fixed by the *A. pintoi* ecotypes using the ^{15}N isotope dilution technique, the equation defined by McAuliffe *et al*. (1958) was applied:

P=1 – (atom % ^{15}N excess leg/atom % ^{15}N excess ref) x 100

The ^{15}N enrichment of plant samples was expressed as atom% ^{15}N excess, according to the following equation:

Atom% ^{15}N excess = (atom % ^{15}N sample - atom % ^{15}N in air N_2)

Where atom % ^{15}N in air N_2 is 0.3663.

A randomized experimental design, with a split-plot arrangement was used, with strains as main plots, and legumes and reference plants as sub-plots, replicated four times. Statistical analyses of data were conducted and differences among treatments were compared by least significant difference (LSD) at $P \leq 0.001$.

RESULTS AND DISCUSSION

A. pintoi ecotypes inoculated with the *Bradyrhizobium* strain yielded more dry matter on a proportion of 63% than the other group. Highly significant differences were observed for interactions strain x harvest (Table 1). The best ecotypes, with CIAT 3101 strain were: 17434 and 18748, with cumulative DMY of 67.6 ± 7.1 and 56.8 ± 7.1 g dry matter yield (DMY) pot^{-1} ($P \leq 0.05$) respectively, compared to 18744 with 37.8 ± 7.1 g pot^{-1}. At each harvest, DMY was always higher for plants inoculated with improved strain of *Bradyrhizobium*, although at the first harvest no significant variation existed between the two groups. The reason for the decline in DMY inspite of high N_2 fixation was not clear.

In a pot experiment, Chu *et al*. (2004) evaluated the N_2 fixation of *Arachis hypogaea* L., Zhenyuanza 9102 in a monocropping system, applying 15, 75 and 150 kg N ha^{-1} using a ^{15}N isotope dilution method. They recorded shoots DMY of 6.8±0.9, 8.2±0.8 and 7.3±0.7 g plant^{-1}, respectively for each level of N applied. These figures are similar to the values obtained in this experiment in harvests 2 and 3, but lowest in harvests 1, 4 and 5. Under field conditions, Suarez *et al.* (1992) found reductions in DMY for non-inoculated *A. pintoi* associated with a native pasture in Colombia using the "isotope ^{15}N dilution" technique. The DMY at first harvest was 4.5 g plant^{-1} but the following yields were approximately 2.3, 2.0 and 1.7 g plant^{-1} at 22, 25 and 28 weeks, respectively. This was a decline of 62% of the DMY obtained at the first harvest, similar to the reduction observed in the group of ecotypes inoculated with native strains in this experiment.

The N produced by the legumes inoculated with the strain CIAT 3101 was higher than the native strain (Table 2). Differences between these two groups averaged 220 mg N pot^{-1} harvest^{-1}, which is 71% more N for the improved strain group. This was due to the effect of inoculation with the improved strain CIAT 3101, that allowed a better N_2 fixation and hence more N content. The highest level of N was recorded in the second harvest, probably because the plants reached physiological maturity on that date. Chu *et al*. (2004) reported highest values on *Arachis hypogaea* L., Zhenyuanza 9102: 195, 237 and 228 mg N plant^{-1}, for 15, 75 and 150 kg N ha^{-1} compared to the range of 53.5 to 180.5 mg N plant^{-1}obtained here on the five harvests with *Bradyrhizobium* strain CIAT 3101. Plants used by Chu *et al*. (2004) were fertilized with higher levels of N, compared to the present study.

Among the reference plants *B. arrecta* was largest and had a significantly ($P \leq 0.001$) higher N yield compared to other reference plants. *Arachis hypogaea*, *B. brizantha* and *C. nlemfuensis* produced 48 % of the amount of N produced by *B. arrecta*. After the second harvest the N yields decreased to a range of 49 to 99 mg N pot^{-1} between third and fifth harvests. In Colombia, Suarez *et al*. (1992) reported that *A. pintoi* (not inoculated) produced less total N yield (340 mg plant^{-1}) than the reference plant *B. decumbens* (970 mg plant^{-1}). In our experiment, *A. pintoi* ecotypes inoculated with native strain were (on average) similar to the amount of N produced by the reference plants. However, *A. pintoi* ecotypes inoculated with *Bradyrhizobium* strain CIAT 3101 produced highest N content.

The values of atom% ^{15}N in legumes with *Bradyrhizobium* strain CIAT 3101 showed lower values compared with the grasses, indicating an active process of ^{15}N isotope dilution throughout the experiment (Figure 1). On the contrary, *A. pintoi* ecotypes inoculated with native strains had high atom% ^{15}N values indicating a poor N_2 fixation. Viera-Vargas *et al.* (1995) reported ^{15}N enrichment values for *Centrosema* hybrid Itaguai, *Galactia striata* and *D. ovalifolium* of 0.07, 0.09 and 0.13 % respectively. These legumes were considered as high N_2 fixers by the fact that they showed ^{15}N enrichment to be considerably lower than that of the reference plants: *B. brizantha*, *P. maximum* and *B. arrecta* with 0.25, 0.28 and 0.16 %, respectively. Guimaraes *et al*. (2008) assessed the N_2 fixation on soybean plants grown in soil enriched with ^{15}N (20 atom% ^{15}N excess), inoculated with *Bradyrhizobium* commercial strains. Plants showed ^{15}N enrichments from 0.0091 to 0.0149 atom% ^{15}N excess. This range is much smaller than the values obtained in this study. They mentioned that soybean plants obtained very high proportions of their N from BNF due to the lower ^{15}N enrichment compared to reference plants used.

The values of atom% ^{15}N in excess found in shoots of legumes with the improved strain were generally lower (0.027 ± 0.01) than those of the native strains group (0.052 ± 0.002). At the first harvest shoot atom% ^{15}N values were similar between both inoculation groups (0.024 ± 0.001 and 0.024 ± 0.0004) after which the differences were more pronounced ($P \leq 0.001$). Shoot atom% ^{15}N values of *A. pintoi* ecotypes were not significantly different (0.04±0.003, 0.04±0.003 and 0.04 ± 0.0029 on average). In almost all treatments shoots at the third harvest had highest ^{15}N values with averages of 0.073 ± 0.001 and 0.034 ± 0.004 atom% ^{15}N in excess for native and improved strain, respectively.

Atom% ^{15}N excess in shoots of reference plants varied between species and there was an interaction of species by harvest (Table 3). The range (first to fifth harvest) was from 0.1 to 0.09 % ($P \leq 0.001$). The interaction harvest/reference plant was statistically different at $P \leq 0.05$, and *B. brizantha* showed highest (0.12 %) and lowest (0.02 %) averages at the third and first harvests, respectively.

Table 1. Average (and standard errors of difference) shoot dry matter yield (g pot^{-1}) of three ecotypes of *Arachis pintoi* over five harvests using native or an improved *Bradyrhizobium* strain.

	Shoot dry matter yield (g pot^{-1})					
	harvest					
Strain	1[(1)]	2	3	4	5	Total
Native strain	2.4 ^{a}±0.4	9.7 ^{b}±0.6	3.9 ^{b}±1.3	1.5 ^{b}±0.2	2.2 ^{b}±0.6	19.7^b±0.7
CIAT 3101	3.0 ^{a}±0.5	16.8^a±1.9	18.9^a±5.8	8.1 ^{a}±5.8	7.1 ^{a}±2.6	53.9^a±5.2
Average [(2)] harvest/strain	2.7 ^{b}±0.2	13.3^a±1.0	11.4^a±2.1	4.8 ^{b}±1.3	4.7 ^{b}±0.8	36.9±0.7

[(1)]Means in the same column followed by the same superscript letter are not significantly different ($P \leq 0.001$).
[(2)]Means with same letters within row are not statistically different ($P \leq 0.001$).

Table 2**.** Average (and standard errors of difference) N content (mg pot^{-1}) of three ecotypes of *Arachis pintoi* over five harvests using native or an improved *Bradyrhizobium* strain, and four reference plants.

	harvest					
Strain	1[(1)]	2	3	4	5	Total
Native strain	75 ^{a} ±5	243 ^{b} ±13	47 ^{b} ±5	26 ^{b} ±3	48 ^{c} ±8	439 ^{b} ±20
CIAT 3101	107 ^{a} ±8	625 ^{a} ±56	361 ^{a} ±54	221 ^{a} ±60	225 ^{c} ±39	1538 ^{a} ±117
Average [(2)] harvest/strain	91 ^{c} ±5	434 ^{a} ±49	204 ^{b} ±42	123 ^{c} ±36	136 ^{c} ±27	988 ±128
Ref. plant						
A. hypogaea	69	102	89	29	33	324 ^{b} ±66 [3]
B. arrecta	179	171	214	78	134	776 ^{a} ±126
B. brizantha	62	130	76	37	76	382 ^{b} ±31
C. nlemfuensis	172	131	16	52	50	422 ^{b} ±45
Average by Harvest [(3)]	121^c ±18	134 ^{c} ±9	99 ^{bc} ±33	49 ^{a} ±6	73 ^{ab} ±11	476±57

[(1)]Means in the same column followed by the same superscript letter are not significantly different ($P \leq 0.001$).
[(2)]Means with same letters within row are not statistically ($P \leq 0.001$) different.
[(3)]Means in the column or row followed by the same letter are not significantly different ($P \leq 0.001$).

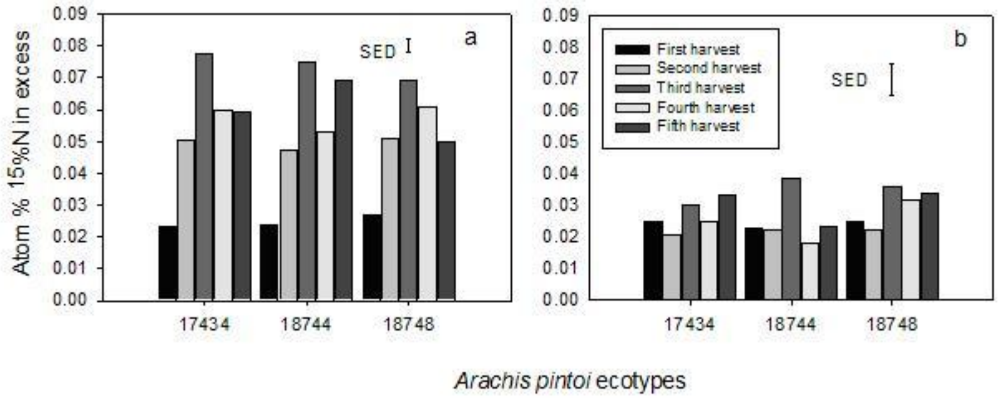

Figure 1. Values of atom % ^{15}N excess of shoots of three *Arachis pintoi* ecotypes inoculated with native strains a) or CIAT 3101 *Bradyrhizobium* strain b) over five harvests. SED represents standard error of difference.

The amount of N_2 fixed was higher in plants inoculated with strain CIAT 3101 regardless of the reference plant used ($P \leq 0.001$) (Figure 2). N_2 fixed estimates were also statistically different ($P \leq 0.001$) using *A. hypogaea* or grasses as reference plants. *Arachis hypogaea* showed a very poor performance than legumes inoculated with native strains; and the opposite occurred with *Bradyrhizobium* CIAT 3101 strain, but always with lower proportion (0.41 ± 0.03 %) compared to grasses (0.56 ± 0.03) ($P \leq 0.01$). No statistical significance was observed for the interactions strain-ecotypes, reference plant-ecotypes neither strain-ecotypes-reference plants. The effect of the improved strain CIAT 3101 was very evident, and N_2 fixation values were higher than those of legumes inoculated with the mixture of native strains. In the former group, using *A. hypogaea* as a reference plant the pasture legumes fixed proportionately 0.26 less N_2 than when using grasses. In ecotypes inoculated with the strain CIAT 3101 the level of fixation showed only slight variations and fixation was nearly constant from the second to fifth harvest with 0.64, 0.67, 0.71 and 0.59, respectively.

Since results under glasshouses conditions are not always comparables to field performance, some observations about field situations must be done. The results of *A. pintoi* CIAT 17434 obtained by Thomas *et al.* (1997) using the same *Bradyrhizobium* strain CIAT 3101 were higher than the values reported here. They recorded 0.82 and 0.68 of N_2 fixation in Oxisols (clay loam) of high and low fertility, respectively, averaged for three reference plants. In our case and for the same strain the three ecotypes recorded 0.60 of N_2 fixation throughout the last four harvests. They also reported high values under the same conditions for *Centrosema acutifolium* (0.94) and *Stylosanthes capitata* (0.87). Likewise, the proportion of N derived from fixation on the soil of high fertility was always above 0.8 during the three years that the experiment lasted. However, contrary to the findings of this experiment, Purcino *et al*. (2003) tested under field conditions in two sites of Brazil, the response of *A. pintoi* ecotype BRA 031143 (CIAT 22160) to inoculation with selected rhizobia strains, and found that *Bradyrhizobium* strain CIAT 3101 was ineffective for this legume, in terms of dry matter yield and total shoot nitrogen, compared to MGAP13, NC230 and NC70 strains. In one of the two sites, they attributed the poor response from the CIAT 3101 strain to the already high level of N content in the soil.

The N_2 fixation values found by Suarez *et al.* (1992) with *A. pintoi* were similar (0.63) to our results. They mentioned that N_2 fixed by *A. pintoi* could be higher in a soil with less available N, but no references about the N soil content were cited in this report. N_2 fixation is also likely to be high in our results after the legume establishment due to the restricted soil volume and hence mineral N supply capacity in the pots.

Amount N_2 fixed

Arachis pintoi ecotypes inoculated with native strains fixed on average only 9 % of the N_2 fixed by the same plants with the improved strain ($P \leq 0.01$) (Figure 3). Similarly, the interaction *Bradyrhizobium* strain-reference plant was statistically different ($P \leq 0.001$). The effect of the reference plant on the amount of N_2 fixed was highly significant ($P \leq 0.001$) averaging 35 ± 18, 113 ± 12, 108 ± 13 and 107 ± 12 for *A. hypogaea*, *B. arrecta*, *B. brizantha* and *C. nlemfuensis*, respectively showing that N_2 fixation estimates were lowest with *A. hypogaea* as a reference plant. The ecotype 17434 inoculated with CIAT 3101 fixed more N_2 mostly due to its high levels of N content. No interactions were observed for ecotype-reference plant as well as strain-reference plant-ecotype.

These contrasting estimates were mainly due to differences in the percentage of N_2 fixation, although the effect of reference plants was also evident. N uptake (atmospheric N_2 and soil N) and other, growth factors such as dry matter yield accumulation were increased due to the amounts of N_2 fixed by the group of ecotypes inoculated with the improved strain. These observations are confirmed by Sylvetser-Bradley *et al*. (1988) who found that *A. pintoi* had marked responses in N content when inoculated with strain CIAT 3101.

Table 3**.** Shoot atom %^{15}N excess of the reference plants *Arachis hypogaea*, *Brachiaria arrecta*, *Brachiaria brizantha* and *Cynodon nlemfuensis*.

	Shoot atom % ^{15}N excess				
	Harvest				
Ref. Plants	1	2	3	4	5
A. hypogaea	0.028 [(1)] [a] (0.0009) [(2)]	0.04 [b] (0.001)	0.08 [a] (0.004)	0.07 [b] (0.004)	0.05 [a] (0.00008)
B. arrecta	0.031 [a] (0.0004)	0.063 [a] (0.002)	0.10 [a] (0.02)	0.12 [a] (0.007)	0.09 [a] (0.007)
B. brizantha	0.025 [a] (0.001)	0.06 [a] (0.006)	0.12 [a] (0.005)	0.11 [a] (0.006)	0.08 [a] (0.004)
C. nlemfuensis	0.037 [a] (0.002)	0.06 [a] (0.001)	0.10 [a] (0.0002)	0.06 [b] (0.008)	0.08 [a] (0.006)

[(1)]Means in the same column (harvest) followed by the same superscript letter are not significantly different ($P \leq 0.001$).
[(2)]Values in brackets are standard error of means.

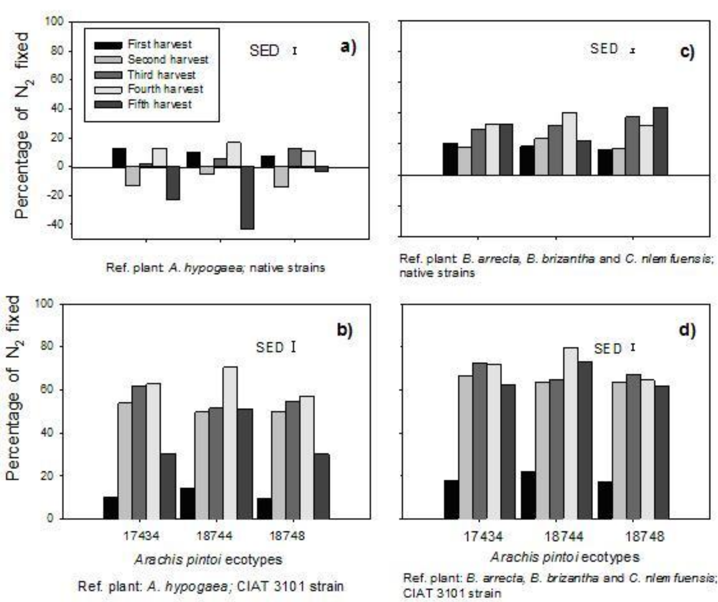

Figure 2. Proportion of nitrogen fixed by *Arachis pintoi* ecotypes with a) and b) non-nodulating *A. hypogaea,* or c) and d) average of three grasses as reference plants and two *Bradyrhizobium* strains, during five harvests. SED represents standard error of difference.

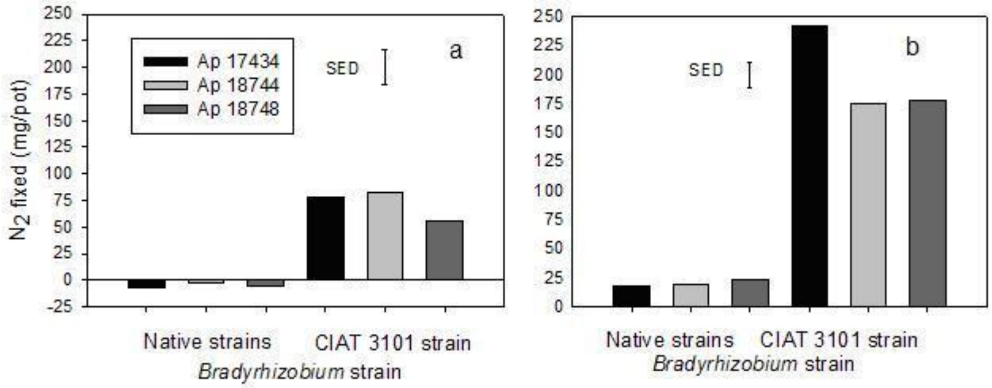

Figure 3**.** Amount of nitrogen fixed by three *Arachis pintoi* ecotypes using non-nodulating *Arachis hypogaea* a) or the average of three grasses b) as reference plants, and two *Bradyrhizobium* strains. Cumulative values over five harvests. SED represents standard error of difference.

CONCLUSIONS

The dry matter yields in legumes depended to a large extent on the effectiveness of the *Rhizobium* strains to fix N_2. The improved *Bradyrhizobium* strain CIAT 3101 was more efficient in fixing N_2 than the native strains. The ecotypes *Arachis pintoi* CIAT 17434 and CIAT 18744 fix more N than the ecotype CIAT 18748. Also, grasses *B. arrecta*, *B. brizantha* and *C. nlemfuensis* could be considered as the best reference plants to estimate N_2 fixation. On the other hand, native strains from the North-Central region of Veracruz, Mexico are not effective to fix atmospheric nitrogen under the conditions used in this experiment.

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