ASSESSMENT OF N₂ FIXATION BY THREE Arachis pintoi ECOTYPES USING THE ISOTOPE DILUTION TECHNIQUE

Tropical and Subtropical Agroecosystems

[DETERMINACION DE LA FIJACION DE N₂ POR TRES ECOTIPOS DE Arachis pintoi USANDO LA TECNICA DE DILUCION DE ISOTOPOS]

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ABSTRACT.

The nitrogen derived from N₂ fixation was measured by the ¹⁵N 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 ¹⁵N-enriched solution at 5 atom % ¹⁵N. Dry matter yield was highest (P≤0.001) 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≤0.001) 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% ¹⁵N in excess of legume shoots were lower (0.027 ± 0.01) with BS, compared to NS group (0.052 ± 0.002). The N₂ 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 N₂ fixation than the native strains.

Key words: Arachis pintoi ecotypes; reference plants; Bradyrhizobium strain; ¹⁵N isotope.

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 N₂ 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 N₂ fixation for the different agricultural systems, including the extensive, uncultivated tropical savannas used for grazing, mentioned that annual N₂ fixation inputs in pastures and fodder legumes were 12–25 million tones. However, the benefits of this N₂ 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 N₂ 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 unknown 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 N₂ 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 ¹⁵N isotope 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 N₂ fixation.

MATERIALS AND METHODS

The experiment was set up in the greenhouse at Imperial College at Wye (University of London), to assess symbiotic N₂ fixation by the Arachis pintoi ecotypes CIAT 17434, 18744 and 18748 using the ¹⁵N 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, δ¹⁵N of 7.5 ‰, while values for Ca, K and Mg were 16.5, 0.8 and 0.9 cmol kg⁻¹ soil⁻¹, respectively. P was 30.9 μg g⁻¹ 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 1⁻¹). In between, pots were watered with deionized water.

Before sowing, the soil/sand mixture of every pot received a ¹⁵N-enriched solution of K¹⁵NO₃ at a rate of 1 kg N ha⁻¹ at 5 atom% ¹⁵N. A volume of 250 ml pot⁻¹ taken from the diluted ¹⁵N solution was used to wet the mixture, which was thoroughly mixed. The pots were then sown with A. pintoi ecotype or reference plant. Subsequently ¹⁵N applications were made after each harvest using the same volume on the pot’s surface, in order to avoid depletion of plant available ¹⁵N (Boddey et al., 2008).

After germination A. pintoi ecotypes were inoculated with the Bradyrhizobium strain CIAT 3101 (19 x 10⁷ 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 ‰ δ¹⁵N 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 °C 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 forced-
hot-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% 15N 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 N2 fixed by the A. pintoi ecotypes using the 15N isotope dilution technique, the equation defined by McAuliffe et al. (1958) was applied:

\[ P=1 - (\text{atom} \% \frac{15N \text{ excess leg}}{\text{atom} \% 15N \text{ excess ref}}) \times 100 \]

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

\[ \text{Atom}\% \frac{15N \text{ excess}}{15N \text{ in air N}_2} = (\text{atom} \% \frac{15N \text{ sample}}{15N \text{ in air N}_2}) \]

Where atom % 15N in air N2 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 ≤ 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 37.8 ± 7.1 and 56.8 ± 7.1 g dry matter yield (DMY) pot\(^{-1}\) (P ≤ 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 15N 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 15N 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 ≤ 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% 15N in legumes with Bradyrhizobium strain CIAT 3101 showed lower values compared with the grasses, indicating an active process of 15N isotope dilution throughout the experiment (Figure 1). On the contrary, A. pintoi ecotypes inoculated with native strains had high atom% 15N values indicating a poor N\(_2\) fixation. Viera-Vargas et al. (1995) reported 15N 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 15N 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\textsubscript{2} fixation on soybean plants grown in soil enriched with \textsuperscript{15}N (20 atom% \textsuperscript{15}N excess), inoculated with Bradyrhizobium commercial strains. Plants showed \textsuperscript{15}N enrichments from 0.0091 to 0.0149 atom% \textsuperscript{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 \textsuperscript{15}N enrichment compared to reference plants used.

The values of atom% \textsuperscript{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% \textsuperscript{15}N values were similar between both inoculation groups (0.052 ± 0.002). At the first harvest shoot atom% \textsuperscript{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 ≤ 0.001). Shoot atom% \textsuperscript{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 \textsuperscript{15}N values with averages of 0.073 ± 0.001 and 0.034 ± 0.004 atom% \textsuperscript{15}N in excess for native and improved strain, respectively.

Atom% \textsuperscript{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 ≤ 0.001). The interaction harvest/reference plant was statistically different at P ≤ 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\textsuperscript{-1}) of three ecotypes of Arachis pintoi over five harvests using native or an improved Bradyrhizobium strain.

<table>
<thead>
<tr>
<th>Strain</th>
<th>1\textsuperscript{(1)}</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native strain</td>
<td>2.4 ±0.4</td>
<td>9.7±0.6</td>
<td>3.9±1.3</td>
<td>1.5±0.2</td>
<td>2.2±0.6</td>
<td>19.7±0.7</td>
</tr>
<tr>
<td>CIAT 3101</td>
<td>3.0 ±0.5</td>
<td>16.8±1.9</td>
<td>18.9±5.8</td>
<td>8.1±5.8</td>
<td>7.1±2.6</td>
<td>53.9±5.2</td>
</tr>
<tr>
<td>Average harvest/strain</td>
<td>2.7±0.2</td>
<td>13.3±1.0</td>
<td>11.4±2.1</td>
<td>4.8±1.3</td>
<td>4.7±0.8</td>
<td>36.9±0.7</td>
</tr>
</tbody>
</table>

\textsuperscript{(1)} Means in the same column followed by the same superscript letter are not significantly different (P ≤ 0.001).
\textsuperscript{(2)} Means with same letters within row are not statistically different (P ≤ 0.001).

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>1\textsuperscript{(1)}</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native strain</td>
<td>75 ±5</td>
<td>243±13</td>
<td>47±5</td>
<td>26±3</td>
<td>48±8</td>
<td>439±20</td>
</tr>
<tr>
<td>CIAT 3101</td>
<td>107 ±8</td>
<td>625±56</td>
<td>361±54</td>
<td>221±60</td>
<td>225±39</td>
<td>1538±117</td>
</tr>
<tr>
<td>Average harvest/strain</td>
<td>91±5</td>
<td>434±49</td>
<td>204±42</td>
<td>123±36</td>
<td>136±27</td>
<td>988±128</td>
</tr>
</tbody>
</table>

\textsuperscript{(1)} Means in the same column followed by the same superscript letter are not significantly different (P ≤ 0.001).
\textsuperscript{(2)} Means with same letters within row are not statistically different (P ≤ 0.001).

Ref. plant

<table>
<thead>
<tr>
<th>Species</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. hypogaea</td>
<td>69</td>
<td>102</td>
<td>89</td>
<td>29</td>
<td>33</td>
<td>324±66</td>
</tr>
<tr>
<td>B. arrecta</td>
<td>179</td>
<td>171</td>
<td>214</td>
<td>78</td>
<td>134</td>
<td>776±126</td>
</tr>
<tr>
<td>B. brizantha</td>
<td>62</td>
<td>130</td>
<td>76</td>
<td>37</td>
<td>76</td>
<td>382±31</td>
</tr>
<tr>
<td>C. nlemfuensis</td>
<td>172</td>
<td>131</td>
<td>16</td>
<td>52</td>
<td>50</td>
<td>422±45</td>
</tr>
<tr>
<td>Average by Harvest \textsuperscript{(3)}</td>
<td>121±18</td>
<td>134±9</td>
<td>99±33</td>
<td>49±6</td>
<td>73±11</td>
<td>476±57</td>
</tr>
</tbody>
</table>

\textsuperscript{(1)} Means in the same column followed by the same superscript letter are not significantly different (P ≤ 0.001).
\textsuperscript{(2)} Means with same letters within row are not statistically different (P ≤ 0.001).
\textsuperscript{(3)} Means in the same column followed by the same letter are not significantly different (P ≤ 0.001).
The amount of N\textsubscript{2} fixed was higher in plants inoculated with strain CIAT 3101 regardless of the reference plant used (P ≤ 0.001) (Figure 2). N\textsubscript{2} fixed estimates were also statistically different (P ≤ 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 ≤ 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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{2} fixation values found by Suarez et al. (1992) with A. pintoi were similar (0.63) to our results. They mentioned that N\textsubscript{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\textsubscript{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\textsubscript{2} fixed**

Arachis pintoi ecotypes inoculated with native strains fixed on average only 9 % of the N\textsubscript{2} fixed by the same plants with the improved strain (P ≤ 0.01) (Figure 3). Similarly, the interaction Bradyrhizobium strain-reference plant was statistically different (P ≤ 0.001). The effect of the reference plant on the amount of N\textsubscript{2} fixed was highly significant (P ≤ 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\textsubscript{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 Sylvetsers-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*.

<table>
<thead>
<tr>
<th>Ref. Plants</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. hypogaea</em></td>
<td>0.028</td>
<td>0.04</td>
<td>0.08</td>
<td>0.07</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>(0.0009)</td>
<td>(0.001)</td>
<td>(0.004)</td>
<td>(0.004)</td>
<td>(0.0008)</td>
</tr>
<tr>
<td><em>B. arrecta</em></td>
<td>0.031</td>
<td>0.063</td>
<td>0.10</td>
<td>0.12</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>(0.0004)</td>
<td>(0.002)</td>
<td>(0.002)</td>
<td>(0.007)</td>
<td>(0.007)</td>
</tr>
<tr>
<td><em>B. brizantha</em></td>
<td>0.025</td>
<td>0.06</td>
<td>0.12</td>
<td>0.11</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>(0.001)</td>
<td>(0.006)</td>
<td>(0.005)</td>
<td>(0.006)</td>
<td>(0.004)</td>
</tr>
<tr>
<td><em>C. nlemfuensis</em></td>
<td>0.037</td>
<td>0.06</td>
<td>0.10</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>(0.002)</td>
<td>(0.001)</td>
<td>(0.0002)</td>
<td>(0.008)</td>
<td>(0.006)</td>
</tr>
</tbody>
</table>

(1) Means in the same column (harvest) followed by the same superscript letter are not significantly different (P ≤ 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.
CONCLUSIONS

The dry matter yields in legumes depended to a large extent on the effectiveness of the *Rhizobium* strains to fix N\textsubscript{2}. The improved *Bradyrhizobium* strain CIAT 3101 was more efficient in fixing N\textsubscript{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\textsubscript{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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