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Vegetation cover affects rhizobia-tree legume symbiosis in the semi-arid region of Brazil

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Biological nitrogen fixation (BNF) is the main form of introducing nitrogen into systems with low fertilizer input, which prevail in the semi-arid region of Brazil. BNF was evaluated in leucena and sabiá grown in samples of a soil collected under different vegetation cover: native vegetation; capoeira (area abandoned for the restoration); agricultural use; sabiá grove; and leucena grove. Nodulation was abundant in soil under all the types of cover. Leucena grown in soil under capoeira exhibited the highest dry biomass of nodules, while the same cover provided the lowest dry biomass of nodules for sabiá. Growth and shoot biomass were higher in plants grown in soil under caatinga. BNF was high in leucena and sabiá, reaching rates higher than 97% in plants grown in soil under capoeira. The highest amounts of fixed nitrogen (N) were found in leucena plants grown in soil under caatinga. It is the first estimate of the potential to fixed N for sabiá and leucena in soils of the semi-arid region, providing an initial estimate of the amounts of N that can be fixed in the field by these tree legumes in the Brazilian semi-arid.

Key words: Indigenous rhizobia, isotope, *Leucaena leucocephala* (Lam.) de Wit, *Mimosa caesalpinifolia* Benth, N-15 natural abundance method, tropical dry forest.

INTRODUCTION

Shifting cultivation, which is predominant in semi-arid tropical regions, causes a reduction in native vegetation cover due to successive cycles of deforestation, burning, farming and/or overgrazing, and abandonment of the area (Galindo et al., 2008; Menezes et al., 2012; Sousa et al., 2012). These practices may lead to the degradation

of natural resources and the reduction of soil fertility, caused by erosion, nutrient export by the harvesting of agricultural products and forage intake by animals (Nunes et al., 2012). Biological nitrogen fixation (BNF) has a key role in maintaining N stocks in these systems characterized by low fertilizer input (Freitas et al., 2015).

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The understanding of the diverse aspects that involve the symbiotic process between rhizobia and tree legumes (occurrence of native rhizobia, nodulation and efficiency of the symbiotic process) in soils of these regions is important to establish an adequate management of BNF.

There are too many limited studies that relate the various aspects of the rhizobia-legume symbiosis with the different types of vegetation cover found in these regions. The first factor to consider is that symbiosis is not established in the absence of native populations of bacteria capable of nodulating certain legume species. Microsymbiont (rhizobia) populations are generally abundant in soils of regions where legume species are native to (Bala et al., 2003). However, in several situations, non-native legumes used in agroforestry systems can also present effective symbiosis (Martins et al., 2015). Due to several edaphoclimatic factors not yet understood, it is also possible that symbiosis is not efficient even in the presence of compatible rhizobia populations (Faye et al., 2007; Silva et al., 2017). Both the growth of free-living rhizobia in soils as well as their ability to nodulate plants and fix nitrogen are sensitive to environmental conditions and may be dependent on soil quality. Different types of vegetation cover or management affect the diversity of rhizobia (Guimarães et al., 2012), and may favor more or less efficient populations differently (Calheiros et al., 2013; Santos et al., 2017).

Sabiá (*Mimosa caesalpinifolia* Benth.) and leucena (*Leucaena leucocephala* (Lam.) de Wit) are tree legumes widely used in the recovery of degraded areas and in agroforestry systems in the semi-arid region of Brazil (Silva et al., 2016). These species present characteristics of rusticity, rapid growth, high biomass production and especially the ability to establish symbiosis with rhizobia (Chaer et al., 2011). Soils of the Brazilian semi-arid region harbor populations of bacteria that nodulate *M. caesalpinifolia* and *L. leucocephala* and apparently vary in symbiotic efficiency, thus affecting nodulation and plant growth (Silva et al., 2016). However, there are no estimates of N rates fixed by leucena and sabiá in soils of the semi-arid region. Other tree legumes can obtain more than 50% of their nitrogen requirements through BNF in this region (Freitas et al., 2010).

The aim of this study was to estimate the efficiency of the rhizobial populations that nodulate sabiá and leucena in a soil of the Brazilian semi-arid region under different vegetation cover by determining symbiotic parameters and estimating the contribution of symbiosis to plant nitrogen nutrition.

MATERIALS AND METHODS

Seedlings of leucena (*L. leucocephala* (Lam.) de Wit) and sabiá (*M. caesalpinifolia* Benth.) were grown in pots containing samples (2.0 kg) of a Luvisolo Crômico (Embrapa, 2011) Luvisols or Aridisols in the FAO and American classifications, respectively located in the city of Serra Talhada (7°59'7" S and 38°17'34" W, 443 m altitude,

average annual rainfall of 686 mm and average annual temperature of 23.8°C), Brazil. The soil samples were collected in the surface layer (0.0-0.2 m) in areas located under the same soil patch, but with different vegetation cover: (1) native vegetation (preserved caatinga, the Brazilian dry forest, with no history of clear cutting for agricultural use); (2) capoeira (area abandoned for restoration of natural fertility after consecutive cycles of intercropped corn, *Zea mays* (L.), and cowpea, *Vigna unguiculata* (L.) Walp.); (3) agricultural use (intercropping of corn and cowpea); (4) sabiá grove installed 20 years ago; and (5) leucena grove installed 20 years ago. In each area, we obtained composite samples from 5 simple samples collected at randomly marked points in the field. The composite soil samples were air-dried, broken down, homogenized and passed through a 4 mm mesh sieve. In each composite sample, subsamples were collected to determine pH (water); P (Mehlich-1); exchangeable K⁺, Na⁺, Ca²⁺ and Mg²⁺, total organic carbon (TOC); and sand, silt and clay ratios (Table 1) (Embrapa, 2011).

A greenhouse experiment was conducted using a completely randomized design with three replicates in a 2 × 5 factorial arrangement (two tree legume species and five types of vegetation cover). Extra pots containing soil samples from the 5 areas with different vegetation cover were grown with *Bauhinia cheilantha* (Bong.) Steud. and *Senna spectabilis* (DC.) H.S. Irwin & Barneby, which are non-nodulating tree legumes (Allen and Allen, 1981) from the Caesalpinioideae sub-family, used as reference plants to estimate the contribution of BNF in leucena and sabiá.

Prior to sowing, the seeds of the legumes were subjected to thermal shock, with water at 80°C for 15 min, followed by immersion in water at room temperature for 12 h to break dormancy. The seeds were then disinfested with 95% ethyl alcohol for one minute, immersed in 1% sodium hypochlorite for 2 min and washed 10 times with distilled and sterile water. At the time of sowing, four seeds were sown in each pot with 2 kg of soil, and after seven days, one plant was left per pot.

Plants were harvested 100 days after planting. The plants were then separated into shoots and roots from which all nodules were collected. All material was dried in an oven at 65°C for 72 h and weighed to determine dry biomass and the number of nodules was counted. All samples of the plant shoots were ground to fine powder. A subsample was placed in a capsule and loaded into a Thermo Quest-Finnigan Delta Plus isotope ratio mass spectrometer (Finnigan-MAT; CA, USA) interfaced with an Elemental Analyzer (Carlo Erba model 1110; Milan, Italy) at the Laboratory of Isotope Ecology (CENA-USP, Brazil) to obtain the nitrogen isotope ratio and the total nitrogen content of these samples. Stable isotope ratios of nitrogen were measured according to the internationally recognized standards. Internal reference materials (atropine, yeast and soil standard no. 502 - 308 from LECO Corporation) were included in every analytical run. The concentrations of ¹⁵N were expressed in δ units in relation to the international standard (atmospheric N₂), based on the equation

$$\delta = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000$$

where R_{sample} and R_{standard} are the ratio ¹⁵N:¹⁴N of the sample and the standard (air), respectively.

Estimates of the percentage of nitrogen derived from the atmosphere (%Ndfa) were done whenever the δ¹⁵N signals of leucena and sabiá were significantly different from the mean signal of the reference species (Högberg, 1997). The equation proposed by Shearer and Kohl (1986) was used to calculate %Ndfa:

$$\%Ndfa = \left[\left(\frac{\delta^{15}N(\text{reference}) - \delta^{15}N(\text{fixing})}{\delta^{15}N(\text{reference})} \right) \times 100 \right]$$

where δ¹⁵N_(reference) is the mean value of δ¹⁵N of the reference plants

Table 1. Chemical and physical attributes of soil samples of Luvisolo Crômico under different types of vegetation cover.

Attribute	Vegetation Cover				
	Native Vegetation (caatinga) ¹	Capoeira ²	Intercrop ³	Sabiá grove ⁴	Leucena grove ⁵
pH in water (1:2.5)	7.22	7.17	7.03	6.36	6.34
P - Mehlich -1 (mg dm ⁻³)	51	54	52	55	61
K ⁺ - Mehlich - 1 (cmolc dm ⁻³)	0.61	0.56	0.71	0.57	0.7
Na ⁺ - Mehlich - 1 (cmolc dm ⁻³)	0.42	0.36	0.41	0.37	0.41
Ca ²⁺ - 1 mol L ⁻¹ (cmolc dm ⁻³)	4.02	2.78	2.68	3.81	3.68
Mg ²⁺ - 1 mol L ⁻¹ (cmolc dm ⁻³)	2.76	2.41	2.46	3.12	2.46
Al ³⁺ - 1 mol L ⁻¹ (cmolc dm ⁻³)	0	0	0	0	0
TOC (dag kg ⁻¹)	9.61	6.41	5.67	10.8	9.77
Sand (dag kg ⁻¹)	38	48	54	47	44
Silt (dag kg ⁻¹)	26	20	18	27	26
Clay (dag kg ⁻¹)	36	32	28	26	30

(1) Native vegetation (preserved caatinga, with no history of clearcutting for agricultural use); (2) capoeira (area abandoned for the restoration of natural fertility after cultivation with consecutive cycles of intercropped corn, *Zea mays* L., and cowpea, *Vigna unguiculata* (L.) Walp.); (3) area under agricultural use (intercropping of corn and cowpea); (4) area with sabiá grove installed 20 years ago; and (5) area with leucena grove installed 20 years ago.

(*B. cheilantha* and *S. spectabilis*) grown in pots containing soil collected under each of the 5 types of vegetation cover, $\delta^{15}\text{N}_{(\text{fixing})}$ is the mean value of $\delta^{15}\text{N}$ of each legume for each vegetation cover and B is the $\delta^{15}\text{N}$ value of the species when grown with atmospheric N₂ as the sole N source (no soil N). The B value of -1.24‰ determined by Reis et al. (2010) for *M. caesalpinifolia* Benth was used in the present calculations.

N content in each nodulating legume was determined by multiplying the content of the element by the corresponding biomass. The amount of N fixed in plant shoots was estimated by multiplying the value of %Ndfa by the N content of each fixing species.

Data from soil analysis were tested for normality and variance of homogeneity and then submitted to analysis of variance, considering subsample values as replicates and a completely randomized design. For the pot experiment, biomass and nodule data were submitted to analysis of variance, considering a completely randomized design, and comparing the averages by the Tukey test at 5%. Foliar $\delta^{15}\text{N}$ values of each nodulating species, in each area, were compared to those of all reference plants of the same area using the T test.

RESULTS AND DISCUSSION

The seedlings of leucena and sabiá produced the highest shoot biomass and accumulated the most N when grown in soil samples of the area with little anthropic influence, covered with native vegetation (caatinga) (Table 2).

Microsymbiont populations capable of nodulating both tree legume species are naturally established in the soil, regardless of vegetation cover (Table 3). However, different responses to the type of vegetation cover were observed for leucena and sabiá in terms of natural nodulation, although all the seedlings exhibited symbiotic nodules. The number of nodules (average of 84 nodules plant⁻¹) did not significantly differ between the sabiá seedlings grown in soil under the different types of

vegetation cover ($p \leq 0.05$). The smallest biomasses of nodules (40 mg) were found in plants grown in soil under intercropping and capoeira, and the largest biomass (300 mg) was formed in plants grown in soil under leucena grove.

Leucena nodulation was favored when the soil was covered with sabiá and impaired in plants grown in soil under corn and cowpea intercropping (Table 3). The largest nodules were formed by plants grown in soil under capoeira, but did not differ ($p \leq 0.05$) in size compared to the plants grown in soil under native vegetation (caatinga).

The mean $\delta^{15}\text{N}$ values in the leaves of the reference plants (*B. cheilantha* and *S. spectabilis*) were generally high and did not differ between plants grown in soils under different ($p \leq 0.05$) vegetation cover (Table 4). Regardless of the species and the vegetation cover of the soil in which they were grown, all legume plants were isotopically impoverished in relation to the reference plants in at least 3.3‰ (in sabiá seedlings grown in soil under caatinga). Thus, BNF contributed to nitrogen nutrition of both species when grown in soil samples under the five types of vegetation cover, and in most cases accounted for high proportions of plant nitrogen (up to 50%), reaching up to more than 90%. Between the species evaluated in this study, BNF was less important for nitrogen nutrition of *M. caesalpinifolia*, with an overall average of N derived from the atmosphere of 57% in contrast to 76% in *L. leucocephala*. The maximum proportion of fixed nitrogen in *M. caesalpinifolia* was approximately 97% in plants grown in soil under capoeira and only 38% in soil under corn and cowpea intercropping (Table 4).

The highest amounts of N were fixed in the symbiosis of leucena (average of 140 mg pot⁻¹), which was

Table 2. Shoot dry biomass, N content and accumulation in the biomass of two tree legumes grown in samples of Luvisolo Crômico under different vegetation cover.

Species	Vegetation Cover				
	Native vegetation (caatinga)	Capoeira	Intercrop	Sabiá grove	Leucena grove
Shoot dry biomass (g)					
<i>Leucaena leucocephala</i>	11.65 ^a	8.12 ^c	2.58 ^d	5.58 ^c	6.5 ^{bd}
<i>Mimosa caesalpinifolia</i>	8.98 ^a	4.23 ^b	4.17 ^b	6.48 ^{ab}	4.75 ^b
Total nitrogen content (%)					
<i>Leucaena leucocephala</i>	2.47 ^a	2.65 ^a	2.56 ^a	1.48 ^b	2.49 ^a
<i>Mimosa caesalpinifolia</i>	1.97 ^a	1.47 ^b	1.39 ^b	2.23 ^a	1.87 ^{ab}
Accumulation of total N in shoots (mg pot⁻¹)					
<i>Leucaena leucocephala</i>	296 ^a	221 ^{ab}	72 ^d	87 ^{cd}	166 ^{bc}
<i>Mimosa caesalpinifolia</i>	182 ^a	65 ^{bc}	62 ^c	149 ^{ab}	93 ^{bc}

Means followed by the same letter in the row for the different vegetation cover did not differ by the Tukey test ($p < 0.05$).

Table 3. Number and dry biomass of nodules of two tree legumes grown in samples of Luvisolo Crômico under different vegetation cover.

Species	Vegetation Cover				
	Native vegetation (caatinga)	Capoeira	Intercrop (corn + cowpea)	Sabiá grove	Leucena grove
Number of nodules					
<i>Leucaena leucocephala</i>	44 ^b	58 ^{ab}	31 ^b	115 ^a	68 ^{ab}
<i>Mimosa caesalpinifolia</i>	127 ^a	34 ^a	85 ^a	142 ^a	35 ^a
Dry biomass of nodules (mg)					
<i>Leucaena leucocephala</i>	650 ^{ab}	730 ^a	270 ^c	500 ^{bc}	460 ^{bc}
<i>Mimosa caesalpinifolia</i>	300 ^a	40 ^b	100 ^b	180 ^{ab}	190 ^{ab}
Specific mass of nodules (mg nodule⁻¹)					
<i>Leucaena leucocephala</i>	14.7 ^a	12.5 ^a	8.7 ^{ab}	4.3 ^b	6.7 ^b
<i>Mimosa caesalpinifolia</i>	2.3 ^{ab}	1.1 ^b	1.1 ^b	1.2 ^b	5.4 ^a

Means followed by the same letter in the row do not differ by the Tukey test ($P < 0.05$).

approximately more than twice the amount of N fixed by sabiá. Leucena plants grown in soil under caatinga and capoeira were the ones with the highest amounts of fixed N (245 and 218 mg pot⁻¹, respectively). For sabiá, the vegetation cover did not influence the amount of N fixed in plant shoots.

Nodulation and/or BNF efficiency may be constrained by various plant-related conditions, by the microsymbiont, and by soil and climate conditions affecting symbiosis. Rhizobia populations capable of nodulating legumes are generally abundant in soils of regions where the species are native to (Bala et al., 2003). The nodulation of sabiá, a native species of the Caatinga Biome, confirms this assumption and has already been observed in other

studies with soils of the region (Reis et al., 2010; Silva et al., 2016). Although leucena is an exotic species native to Central America, its nodulation evidences the presence of microsymbiont populations in the soils of the semi-arid region of Brazil. It is possible that the ability of this legume to establish symbiosis with a wide range of rhizobia species belonging to the *Rhizobium* (Pereyra et al., 2015), *Mesorhizobium* (Rangel et al., 2016), *Sinorhizobium* (Xu et al., 2013), *Bradyrhizobium* (Wang et al., 2006), *Allorhizobium* and *Cupriavidus* (Florentino et al., 2009) genera promotes a greater chance of nodulation in different soil and climatic conditions.

The presence of compatible rhizobia populations and the formation of nodules do not necessarily mean that

Table 4. Natural abundance of $\delta^{15}\text{N}$ (‰), nitrogen derived from the atmosphere (%Ndfa) and fixed nitrogen of tree legumes grown in samples of Luvisolo Crômico under different vegetation cover.

Species	Vegetation Cover				
	Native vegetation (caatinga)	Capoeira	Intercrop	Sabiá grove	Leucena grove
$\delta^{15}\text{N}$ (‰)					
Reference plants	7.26 ^a	6.32 ^a	5.87 ^a	5.59 ^a	6.63 ^a
<i>Leucaena leucocephala</i>	0.25*	-1.08*	1.37 *	3.37 *	-0.61*
<i>Mimosa caesalpinifolia</i>	3.98*	-1.94*	3.24 *	2.14 *	1.93*
Ndfa (%)					
<i>Leucaena leucocephala</i>	83 ^a	99 ^a	64 ^{ab}	42 ^b	92 ^a
<i>Mimosa caesalpinifolia</i>	39 ^b	97 ^a	38 ^b	51 ^b	27 ^b
Fixed N (mg pot⁻¹)					
<i>Leucaena leucocephala</i>	245 ^a	218 ^{ab}	51 ^c	37 ^c	153 ^b
<i>Mimosa caesalpinifolia</i>	75 ^a	63 ^a	27 ^a	86 ^a	51 ^a

Means followed by the same letter, lowercase in the row for the different vegetation cover did not differ by the Tukey test ($p < 0.05$).

symbiosis will be efficient (Faye et al., 2007; Silva et al., 2017). Both the growth of the free-living rhizobia in soils as well as their ability to nodulate plants and fix nitrogen are sensitive to environmental conditions and may be dependent on soil attributes, such as acidity, aluminium toxicity (Rufini et al., 2011), salinity (Medeiros et al., 2008), phosphorus (Suliman and Tran, 2015; Silva et al., 2017), and molybdenum availability (Matoso and Kusdra, 2014), high temperatures (Ferrari et al., 1967) and water availability (Zilli et al., 2013). As the samples were collected in adjacent areas with similar chemical and physical attributes (Table 1), differences in nodulation (Table 4) possibly occurred because the different types of vegetation cover or management affect the diversity of rhizobia (Guimarães et al., 2012) and may favor more or less efficient populations differently. Furthermore, the effect of vegetation cover on the microsymbiont populations varies according to the specificities of macrosymbiont (Silva et al., 2016). Leucena was more sensitive to the different vegetation cover, presenting almost three times the number of nodules when grown in soil covered with sabiá grove compared to soil under caatinga (Table 3). Thus, inoculation with previously selected bacteria species could be a strategic method in sites with low and ineffective compatible rhizobia population.

The number of nodules did not explain the proportions of nitrogen fixed by the plants. The lowest fixation rates in leucena plants grown in soils covered with sabiá grove (Table 2) contrast with the highest number of nodules (Table 3). In sabiá, the vegetation cover that provided the highest fixation rate (Table 4) was not the same that presented the highest nodulation (Table 2), indicating that most of the nodules were not effective. Small and non-

functional nodules represent a photoassimilate drain (Atkins, 1984). Therefore, the number of nodules may be a variable that inaccurately explains the efficiency of symbiosis.

The results of the isotopic signals allowed accuracy in the estimates of biological nitrogen fixation (Högberg, 1997). The signals of the reference plants were high in every vegetation cover evaluated in this study and differed by more than three $\delta^{15}\text{N}$ units from the fixing plants. The differences were more pronounced in *M. caesalpinifolia* with $\delta^{15}\text{N}$ values ranging from 2.63‰ (intercropping) to 8.26‰ (capoeira). This pattern of nitrogen isotopic composition was also found by Freitas et al. (2015) in tree plants of the Caatinga region. In these conditions, the effect of the isotopic discrimination associated with the biological nitrogen fixation process is very small, that is, the impact of using $B=0$ or $B=-1.24$ ‰ in estimates of %Ndfa is only of a few percentage units. Thus, for greater simplicity, we chose to present only the results using $B=-1.24$ ‰ (Table 3), which are more conservative, possibly slightly underestimating the amounts of fixed nitrogen.

The high contributions of biological nitrogen fixation to the tree legume seedlings contrasts with several estimates presented in the literature for legumes such as soybean (Oberson et al., 2007), cowpea (Adjei-Nsiah et al., 2008), peanut and faba bean (Herridge et al., 2008). It is known that biological fixation is an important process in acquiring nitrogen by the native tree species of the caatinga under field conditions (Teixeira et al., 2010; Sousa et al. 2012). For example, in natural fragments of caatinga, adult plants of *M. tenuiflora*, a species occurring in dry areas from Brazil to Mexico (Queiroz 2009), can derive more than 80% of their nitrogen nutrition from

atmosphere (Freitas et al., 2010), in association with beta proteobacterial rhizobial symbionts (Bontemps et al., 2010; Reis et al. 2010). However, absence of fixation in this species could not be explained by absence of microsymbionts but likely to low symbiosis efficiency due to relatively high N and low P availability (Silva et al., 2017).

There is no data in the literature on the fixation rates of *sabiá*. Data on fixation by exotic species grown in the Brazilian semi-arid region are very scarce. In agroforestry systems, *Gliricidia sepium*, a no native species, can also fix considerable amounts of N (N_{dda} > 50%) and contribute to 40 kg ha⁻¹ of leaves to the system (Martins et al., 2015).

Conclusions

L. leucocephala and *M. caesalpinifolia* have the capacity to abundantly nodulate in a Luvisolo Crômico of the Brazilian semi-arid region, but this capacity depends on the type of vegetation cover. Both species exhibited high biological nitrogen fixation capacity (values above 50% in most plants), which in some cases reached 99% of nitrogen derived from the atmosphere. However, fixation is less important for *M. caesalpinifolia*.

These information are important because it is the first estimate of the potential to fix N for *sabiá* and leucena in soils of the semi-arid region. In spite of being a study in pots, it provides an initial estimate of the amounts of N that can be fixed in the field by these tree legumes. It is undoubtedly different under field conditions, with all possible limitations, especially water availability, which can reduce the symbiotic potential and biomass production. However, high contribution of the fixed nitrogen to both legumes is an indicator of the great potential of these legumes to fix atmospheric N in agroecosystems in the semi-arid region of Brazil.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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