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ORIGINAL ARTICLE

Biological nitrogen fixation in tropical dry forests with different legume diversity and abundance

Aleksandro Ferreira da Silva · Ana Dolores Santiago de Freitas · Tânia Lúcia Costa · Paulo Ivan Fernandes-Júnior · Lindete Míria Vieira Martins · Carolina Etienne de Rosália e Silva Santos · Kelly Alexsandra Souza Menezes · Everardo Valadares de Sá Barretto Sampaio

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Abstract Tropical dry forests have high diversity and plant abundance of potentially biologically nitrogen fixing (BNF) legume species, attributed to the ecological advantage of fixation. However, there are few estimates of N quantities annually fixed, hindering the understanding of factors that control BNF, like low phosphorus availability. The quantities of N fixed in three dry forest (caatinga) fragments of the semiarid Brazilian northeastern region with different legume plant proportions were determined and seedlings of *Mimosa tenuiflora* were grown with phosphorous fertilized soil from the fragments to verify if lack of fixation was due to the absence of rhizobia populations or P deficiency. The vegetation of all areas was dominated by legume plants, mainly potentially nodulating ones, despite the relatively high soil N availability. *M. tenuiflora* was the most abundant nodulating legume in all fragments, with annual leaf productions from 800 to 1400 kg ha^{-1}. BNF amounts were low (1.4, 18 and 3.6 kg ha^{-1} $year^{-1}$ in the mature caatinga of Petrolina and in the mature and regenerating caatingas of São João, respectively) considering the high proportions of potentially nodulating plants (33, 61 and 82% of total plant basal area), because 80, 10 and 70% of these plants were not fixing

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A. F. da Silva · A. D. S. de Freitas (✉) ·
T. L. Costa · C. Etienne de Rosália e Silva Santos
Departamento de Agronomia, Universidade Federal Rural
de Pernambuco, Rua Dom Manoel de Medeiros, s/n,
Recife, PE 52171-900, Brazil
e-mail: ana.freitas@pq.cnpq.br

A. F. da Silva
e-mail: aleks.agro@gmail.com

T. L. Costa
e-mail: tanialuciacosta@gmail.com

C. Etienne de Rosália e Silva Santos
e-mail: carolina.ssantos@ufrpe.br

P. I. Fernandes-Júnior
Embrapa Semiárido, BR 428, Km 152, Petrolina,
PE 56302-970, Brazil
e-mail: paulo.ivan@embrapa.br

L. M. V. Martins · K. A. S. Menezes
Departamento de Tecnologia e Ciências Sociais,
Universidade do Estado da Bahia, Avenida Edgard
Chastinet, s/n, Juazeiro, BA 48900-000, Brazil
e-mail: lindete.martins1@gmail.com

K. A. S. Menezes
e-mail: kelly_alexsandra@hotmail.com

E. Valadares de Sá Barretto Sampaio
Departamento de Energia Nuclear, Universidade Federal
de Pernambuco, Avenida Prof. Luís Freire, 1000, Recife,
PE 50740-540, Brazil
e-mail: esampaio@ufpe.br

and those fixing had only 20–46% of their N derived from the atmosphere. Since the pot grown seedlings nodulated abundantly, the low BNF could not be explained by absence of microsymbionts but likely to low symbiosis efficiency due to relatively high N and low P availability.

Keywords ^{15}N natural abundance · Leaf biomass · Semiarid · Symbiosis · Rhizobia

Introduction

Dry forests comprise more than 40% of tropical forests across the world (Murphy and Lugo 1995), covering more than 1 million km^2 in the Americas and Africa (Miles et al. 2006). They are among the most disturbed native vegetation (Murphy and Lugo 1986; Maass 1995; Banda et al. 2016), in that almost all remnant fragments have some anthropic action (Miles et al. 2006). In Brazil, caatinga, the dry forest of the semiarid northeastern region, is a mosaic of vegetation with different regeneration periods interspaced by 2% undisturbed remnants (Sampaio 2010). In the last decade, the area covered by caatinga has increased (Aide et al. 2013), since agricultural activities have decreased due to their low profitability (Sampaio 2010).

Leguminosae is the most species rich family in dry forests (Gentry 1995; Crews 1999; Queiroz 2006). The capacity to fix atmospheric nitrogen by symbiotic association with rhizobia bacteria is the distinct ecological feature of this family, although not common to all species (Allen and Allen 1981; Sprent 2009). Native species frequently obtain more than 80% of their N through symbiotic fixation (Teixeira et al. 2006; Freitas et al. 2010; Andrews et al. 2011). In spite of the importance of legume species, knowledge of the variables that control the ecological role of symbiotic fixation is still incomplete. An intriguing question is why nodulating legumes are abundant in lowland tropical forest, which have a relatively high soil N supply—indeed that it may function as an excess nutrient there (Vitousek et al. 2002), and relatively scarce in temperate forests, which are N limited (Crews 1999; Houlton et al. 2008). This question can be extended to tropical dry forests, that would have higher relative N availability to the plants than humid places (Aranibar et al. 2004; Swap et al. 2004; Freitas et al. 2015). Tropical dry forests have higher ^{15}N enrichment than humid forests (Aranibar et al. 2004; Swap et al. 2004; Freitas et al. 2015), implying that they are a system with a more open N cycle, with a higher prevalence of N losses, therefore a relative excess of N (Högberg 1997; Handley et al. 1999; Swap et al. 2004; Ometto et al. 2006).

Considering that tropical dry forests have a high proportion of legume plants and these plants may have high fixation, tropical dry forest could be considered a presumed hotspot of N fixation. However, until recently, the only published results of the N quantities symbiotically fixed in tropical dry forest were those of Sylla et al. (2002), in the Sahelian region of Senegal (Western Africa, 400 mm annual rainfall and 27 °C annual average temperature), and of Souza et al. (2012), in the Brazilian caatinga. In Senegal, two dry forests with high legume plant densities (mainly *Pterocarpus lucens* Lepr. ex Guill. and Perr.) fixed 29 and 11 kg N ha^{-1} year^{-1} (Sylla et al. 2002). In the caatinga (600 mm and 28 °C), contrary to expectations, fixation was absent in a forest regenerating for less than 38 years after agriculture abandonment, in spite of its high proportion (>57%) of leaf biomass of nodulating legume plants (mainly *Mimosa tenuiflora* (Willd.) Poir.) in relation to the total. In a mature caatinga, the proportions of N derived from the atmosphere were high (>60%) but the leaf biomass of the legume plants were less than 12% of the total, resulting in fixation of only 6 kg ha^{-1} (Souza et al. 2012). This last value is similar to those reported for tropical humid forests (Roggy et al. 1999), which have higher total N stocks.

One reason for the lack of data on the amounts of fixed N in different vegetation types is the complexity of the task, which involves measurements of both the total biomass produced in a certain period and the proportion of fixed N in this biomass. In the absence of direct measurements, indirect methods, such as simulation modeling, have been used to estimate fixation in tropical humid forests (Rastetter et al. 2001; Vitousek et al. 2002; Wang et al. 2007; Houlton et al. 2008; Cleveland et al. 2010). These estimates are based on the proportion of the potentially fixing species biomass in relation to the total aboveground biomass of the vegetation but they fail to consider that the presence of a potentially fixing plant does not necessarily guarantee that fixation is occurring.

Absence of fixation has been reported in several tropical dry areas (Ndiaye and Ganry 1997; Faye et al. 2007; Freitas et al. 2010; Souza et al. 2012) and could be explained by absence of native symbiotic bacteria in the soil, incapacity of the bacteria to infect the plants and/or low fixation efficiency due to soil nutrient deficiencies. However, most of the studies have not clearly identified which are the causes of the absence of fixation. Low P in relation to N availability may limit fixation (Crews 1993; Vitousek et al. 2002, 2013; Pons et al. 2007) and soils in the caatinga area are low in P (Silveira et al. 2006). Ample populations of nodulating bacteria are common in the soils of the regions where the legume species are native (Bala et al. 2003; Faye et al. 2007). However, there is little information on the spontaneous nodulation and on the natural occurrence of rhizobia populations able to form symbiosis with caatinga potentially nodulating legume species (Reis et al. 2010; Freitas et al. 2014).

Considering the scarcity of estimates of N fixation in tropical dry forests, the objectives of this research were: (1) to determine the quantities of N fixed in three caatinga forest fragments with different legume plant proportions; (2) to evaluate the proportion of N derived from fixation within the population of different legume species; (3) to relate the proportion of N derived from fixation to leaf N and P concentrations; and (4) to verify if any absence of fixation was due to the absence of native rhizobia populations or soil phosphorus deficiency.

Materials and methods

Two caatinga forest fragments were selected in the municipality of São João, in the Agreste mesoregion, and one in the municipality of Petrolina, in the Sertão mesoregion, of Pernambuco state, Brazil (Table 1). One area in each of the municipalities was regenerating for as long as local people could remember, at least 40 years in Petrolina and 50 years in São João, and were considered as remnants of mature vegetation, while the other area in São João was regenerating for 10 years, after being intensively used as pasture for dairy cattle.

Ten 400 m^2 plots were established in each area. In each plot, all plants with stem diameter at breast height (1.3 m above ground level) of one of the stem branches $\geq$3 cm were identified and had all their stem branch circumferences measured. The circumferences were transformed into diameters and basal areas; the basal areas of branched plants were summed and the equivalent average diameter of the plant was calculated. For each species, the plant density and the sum of the basal areas were calculated for each plot and for each area. Species diversity of each area was calculated using the Shannon diversity (H') and the Pielou equitability (J') indices (Brower and Zar 1984). The leaf biomass (L_B) of each plant was estimated using the equivalent stem diameter (D) and the equations used by Silva and Sampaio (2008) and Souza et al. (2012):

$$L_B = 0.0681\,D^{1.5829}, \quad \text{for species with large sizes}$$

$$L_B = 0.0180\,D^{1.845}, \quad \text{for species with small sizes.}$$

In each plot leaf samples were collected from up to five plants belonging to the following classification: (1) target species, all those legume species with known nodulation and N_2 potential fixation capacity (Sprent 2009); and (2) reference species, non-legume species and legume species known as non-nodulating species (Sprent 2009). The samples were composed of 10–20 healthy, fully expanded leaves per plant, cut in the outer part of the canopy, between 1.5 and 2 m above ground level. One composite soil sample was also collected from each plot, mixing 10 single samples taken randomly from the superficial soil layer (0–20 cm). The soil samples were analyzed (Table 2) following the procedures recommended by Embrapa (1997, 2009). The carbon and nitrogen concentrations in soil samples were assessed by dry combustion method in a LECO TruSpec CN (Leco, USA) equipment.

The plant samples were dried and ground to fine powder. A sub-sample was placed in a capsule and loaded into a ThermoQuest-Finnigan Delta Plus isotope ratio mass spectrometer (Finnigan-MAT; CA, USA), interfaced with an Elemental Analyzer (Carlo Erbamodel 1110; Milan, Italy) at the Laboratory of Isotope Ecology (CENA-USP, Brazil) to obtain their nitrogen isotope ratio and their total nitrogen content. The stable isotope ratios were measured relative to internationally recognized standards. Internal reference materials (atropine, yeast and soil standard no. 502–308 from LECO Corporation) were

Table 1 Main characteristics of the municipalities of São João, in the Agreste mesoregion and Petrolina, in the Sertão mesoregion, semiarid of Pernambuco State, Brazil

Characteristics	São João	Petrolina
Coordinates	08°52′32″S and 36°22′00″W	09°23′39″S and 40°30′35″W
Altitude (m a.s.l.)[a]	716	380
Mean annual rainfall (mm)	885	577
Mean annual temperature (°C)	21.1	26.3
Soil type	Regolithic Neosol	Red-yellow Argisol

[a] Meters above the sea level

Table 2 Soil attributes in two caatinga in the municipality of São João (regenerating for 10 years and >50 years) and in one caatinga vegetation in the municipality of Petrolina (regenerating for >40 years), semiarid of Pernambuco State, Brazil

Soil atribute	São João		Petrolina >40 years
	10 years	>50 years	
pH (1:2.5 water)	5.45 a	5.25 b	5.07 b
N (%)	0.06 a	0.05 a	0.05 a
C (%)	0.72 a	0.78 a	0.64 a
C/N	12.0 a	15.6 a	13.7 a
P (mg dm^{-3})	6.93 a	6.74 a	4.12 a
Ca^{2+} ($cmol_c$ dm^{-3})	1.80 a	1.31 a	1.33 a
Mg^{2+} ($cmol_c$ dm^{-3})	1.60 a	0.87 b	0.65 b
Na^{+} ($cmol_c$ dm^{-3})	0.12 a	0.05 b	0.02 b
K^{+} ($cmol_c$ dm^{-3})	0.54 a	0.39 a	0.34 a
Al^{3+} ($cmol_c$ dm^{-3})	0.17 a	0.31 a	0.34 a
Sand (g kg^{-1})	818 b	864 a	836 ab
Silt (g kg^{-1})	112 a	74 a	97 a
Clay (g kg^{-1})	70 a	62 a	67 a

Averages followed by equal letters in the lines do not differ significantly by the Tukey test at 5% probability level

included in every analytical run. The concentrations of ^{15}N were expressed in δ units in relation to the international standard (atmospheric N_2), based on the equation $\delta = (R_{sample}/R_{standard} - 1) \times 1000$. To determine the P concentrations in leaves, subsamples were digested in sulfuric acid and hydrogen peroxide (Thomas et al. 1967) and total P was determined by colorimetry according to the method described in Embrapa (2009).

Atmospheric nitrogen fixation by potentially nodulating legume was assumed to have had occurred when the $\delta^{15}N$ difference between the average value of the reference species in the area and the nodulating legume leaf value of each plant was >2‰ (Freitas et al. 2010, 2015). This difference has been established as a safe value to account for variations in both reference and nodulating legume $\delta^{15}N$ signals (Boddey et al. 2000), although lower values have been adopted (1‰, Nardoto et al. 2014). Assuming this difference, the highest $\delta^{15}N$ signal of a plant assumed to be fixing was lower than the lowest $\delta^{15}N$ signal of any reference plant, in each area.

The proportion of N derived from atmosphere (Ndfa) in the plant was calculated using the following formula (Shearer and Kohl 1986):

$$\%\text{Ndfa} = \left[\left(\delta^{15}N_{(reference)} - \delta^{15}N_{(target)}\right) / \left(\delta^{15}N_{(reference)} - B\right)\right] \times 100$$

where $\delta^{15}N_{(reference)}$ is the average $\delta^{15}N$ value of all reference species in the area; $\delta^{15}N_{(target)}$ is the $\delta^{15}N$ value of each target plant in the area; and B is the $\delta^{15}N$ value of the species when grown with atmospheric N_2 as the sole N source (no soil N). The B value of −1.24‰, determined by Reis et al. (2010) for *Mimosa caesalpiniifolia* Benth, was used in our calculations.

The amount of N in the foliar biomass of each species was calculated by multiplying its biomass (the sum of the leaf biomass of all plants of the species) by the average N concentration of the species in each plot. The amount of annually fixed N in each area was estimated by multiplying the amount of N in the foliar biomass of the species by the proportion of plants with clear evidence of biological nitrogen fixation (BNF) of the species and by average %Ndfa of these fixing plants.

To verify the occurrence of native rhizobia in the soil of the areas, *M. tenuiflora* (Willd.) Poir. seedlings were cultivated under greenhouse conditions, for 112 days, in pots with 500 g of soil from each area, replicated thrice. Seeds were disinfected with ethanol (70% v/v—3 min) and sodium hypochlorite (1% v/v—3 min), rinsed five times with sterile distilled water and then sown in the pots. The pots received 100 mL of nutrient solution without nitrogen (Hoagland and Arnon 1938) every week until harvest. Aboveground plant biomass and number and biomass of root nodules were determined.

Data from soil analysis obtained in the field experiment were tested for normality and variance of homogeneity and then submitted to analysis of variance, considering plot values as replicates and a completely randomized design. Averages of the three areas were compared by the Tukey test at 5% probability level. Data of leaf N and P concentrations and N:P ratio were submitted to analysis of variance considering a 3 × 2 factorial design (three areas and two plant groups: non nodulating and nodulating species); averages were compared by the Tukey test. Data from total plant density, total basal areas and total biomass were analyzed by *t* test at 5% probability level comparing the mature vegetation of both municipalities and also the two areas of São João. Comparisons of the same plant variables and pair of areas were made separately for nodulating legume, non-nodulating legume, and non-legume species and for *M. tenuiflora,* using the *t* test. Foliar $\delta^{15}N$ values of each nodulating species, in each area, were compared to those of a group composed of all reference plants of the same area using the *t* test. Data of *M. tenuiflora* foliar P and N concentrations, of plants in each area, were regressed against their $\delta^{15}N$ signals. 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% probability level. Nodule numbers were transformed by $(x + 1)^{1/2}$.

Results

In all three fragments, legume plants comprised a large proportion of the total plant density, basal area and leaf biomass (Table 3). Among the legume species, most belonged to potentially N_2 fixing species, which accounted for 33, 61 and 82% of the total plant basal area, in the mature caatinga of Petrolina and in the mature and regenerating caatingas of São João, respectively. *M. tenuiflora* (Willd.) Poir. was the most important nodulating species in all three fragments, and the only nodulating species present both areas in São João. In Petrolina, two more nodulating species were found, *Anadenanthera macrocarpa* (Benth.) Brenan and *Mimosa stipulacea* (Benth.) Ducke, besides two non-nodulating legume species, *Bauhinia cheilantha* (Bong.) Steud. and *Poincianella microphylla* (Mart. ex G. Don) L.P. Queiroz.

In the mature caatinga of São João, non-nodulating legume species (two *Senegalia* species) had similar plant density as the non-legume species, but with smaller plants, resulting in a contribution of just over 10% of the basal area and 14% of the total leaf biomass (Table 3). In the regenerating caatinga of this municipality, besides the large density of the potentially nodulating *M. tenuiflora*, non-nodulating legume species (*Senegalia* sp. and *Poincianella pyramidalis* (Tul.) LP Queiroz had almost three times the density of non-legumes, but a lower basal area and total leaf biomass.

In addition to the differences in proportions of legume species, the three caatinga fragments differed in plant density and basal area, except for the absence in significant difference in total leaf biomass between the two São João fragments (Table 3). The Petrolina fragment had the lowest tree and shrub density and also the smallest leaf biomass. On the other hand, it had the highest diversity and lower dominance of species (Table 4), with 21 shrub and tree species (Table 3). In São João, 16 species and nine families were found in the mature caatinga and nine species and seven families in the regenerating caatinga (data are presented as electronic supplementary material).

All trees in the three fragments had high leaf N concentrations, with no difference between nodulating

Table 3 Phytosociological parameters in two caatinga vegetation in the municipality of São João (regenerating for 10 years and >50 years) and in one caatinga vegetation in the municipality of Petrolina (regenerating for >40 years), semiarid of Pernambuco State, Brazil

Species	Density (plant ha^{-1})			Basal area (m^2 ha^{-1})			Leaf biomass (kg ha^{-1})		
	São João		Petrolina	São João		Petrolina	São João		Petrolina
	10 years	>50 years	>40 years	10 years	>50 years	>40 years	10 years	>50 years	>40 years
Nodulating legumes	572 B	877 Aa	260 b	3.55 A	3.68 Aa	3.32 b	1487 A	1448 Aa	943 b
	(88.4)[a]	(55.4)	(23.8)	(82.2)	(60.7)	(33.0)	(88.2)	(61.0)	(36.2)
Anadenanthera macrocarpa	–	–	15	–	–	0.39	–	–	96
Mimosa stipulacea	–	–	35	–	–	0.14	–	–	52
Mimosa tenuiflora	572 B	877 Aa	210 b	3.55 A	3.68 Aa	2.79 b	1487 A	1448 Aa	795 b
Non-nodulating legumes	55 B	330 Aa	182 b	0.23 B	0.63 Ab	1.37 a	82 B	322 Ab	431 a
	(8.5)	(20.9)	(16.6)	(5.3)	(10.4)	(13.6)	(4.9)	(14.0)	(16.6)
Bauhinia cheilantha	–	–	17	–	–	0.05	–	–	20
Senegalia sp. 1	–	147	–	–	0.29	–	–	150	–
Senegalia sp. 2	25	182	–	0.13	0.34	–	43	172	–
Poincianella microphylla	–	–	165			1.32	–	–	411
Poincianella pyramidalis	30	–	–	0.10	–	–	40	–	–
Non legumes	20 B	375 Ab	652 a	0.54 B	1.75 Ab	5.38 a	116 B	603 Ab	1227 a
	(3.1)	(23.7)	(59.6)	(12.5)	(28.9)	(53.4)	(6.9)	(25.0)	(47.2)
Total	647 B	1582 Aa	1094 b	4.32 B	6.06 Ab	10.07 a	1685 A	2373 Ab	2601 a

Averages followed by the same small capital letter comparing the two areas in São João and by the same small letter comparing the older regenerations in São João and Petrolina are not significantly different by the T test at 5% probability level

[a] Numbers in brackets are proportions (%) of the total of the area

Table 4 Shannon diversity (H′) and Pielou equability (J′) indices in two caatinga vegetation in the municipality of São João (regenerating for 10 years and >50 years) and in one caatinga vegetation in the municipality of Petrolina (regenerating for >40 years), semiarid of Pernambuco State, Brazil

Local	H′	J′
São João, 10 years	0.53	0.24
São João, >50 years	1.60	0.57
Petrolina, >40 years	2.30	0.75

and non nodulating species, and low leaf P concentrations (Table 5). Therefore, in general, their N:P ratio were always high in both non-nodulating and nodulating species, and still higher in Petrolina.

The amount of N contained in the leaf biomass was much more influenced by the biomass than by the N concentration in each fragment and it was higher in Petrolina than in the São João fragments (Table 6). The N stocked within nodulating species in Petrolina was equivalent to 26% of the total N stored in the leaves of all species. Above 88% of N in the regenerating fragment in São João was due to leaves of *M. tenuiflora*. In the mature fragment, the contribution of *M. tenuiflora* was proportionally lower (63% of the total N accumulated in leaves), but 2 kg ha^{-1} greater than in the regenerating fragment.

Table 5 N and P concentrations and N:P ratio in leaves of non-nodulatinga species and potentially nodulating legume species of two caatinga vegetation in the municipality of São João (regenerating for 10 years and >50 years) and in one caatinga vegetation in the municipality of Petrolina (regenerating for >40 years), semiarid of Pernambuco State, Brazil

Local/species	N (g kg^{-1})	P	N:P
São João, 10 years	28.57 A	1.09 AB	26.52 B
Non nodulating species	29.57	1.07	27.77
Nodulating legume species	27.88	1.09	25.65
São João, >50 years	31.70 A	1.20 A	27.22 B
Non nodulating species	32.20	1.19	28.17
Nodulating legume species	30.38	1.23	24.73
Petrolina, >40 years	29.66 A	1.00 B	32.85 A
Non nodulating species	29.46	1.05	31.85
Nodulating legume species	29.91	0.95	34.14

Averages followed by the same capital letter are not significantly different by the Tukey test at 5% probability level. The interaction of area and plant group was not statistically significant

The non-nodulating legume and non-legume species were enriched in ^{15}N in the three fragments, with little variation between species and between plants of the same fragment. In São João, the reference species used to estimate BNF in the mature area were *Commiphora leptophloeos* (Mart.) JB Gillett, *Lippia gracilis* Schauer, *Sapium* cf. *glandulosum* and *Senegalia* sp., with a global δ^{15}N average of 8.40 ± 1.40‰. In the regenerating area, the reference species were *Ziziphus joazeiro* Mart., *P. pyramidalis* (Tul.) LP Queiroz and *Senegalia* sp., with an average of 8.01 ± 1.04‰. In Petrolina, *Manihot pseudoglaziovii* Müll. Arg., *C. leptophloeos* and *P. microphylla* (Mart. Ex G. Don) L.P. Queiroz were collected as reference plants (δ^{15}N average of 12.26 ± 1.45‰).

The nodulating species in the São João mature fragment had a wide range of leaf δ^{15}N values, about 90% of them with values more than 2‰ lower than the average value of reference species (Table 6). Therefore, for 10% of the plants there is no clear evidence of fixation. The average proportion of nitrogen derived from atmosphere (%Ndfa) in the 90% fixing-plants was 46%, corresponding to a N amount symbiotically fixed of 18 kg ha^{-1}. In the regenerating fragment, only 30% of the nodulating plants (*M. tenuiflora*) had clear evidence of BNF, with an average Ndfa of 29%, equivalent to 3.6 kg ha^{-1}. In Petrolina, less than 22% of potentially nodulating species plants (all *Mimosa* sp.) differed by more than 2‰, averaging between 22 and 24% Ndfa and corresponding to 1.36 kg ha^{-1} (Table 6).

Leaf N and P concentrations of *M. tenuiflora*, the nodulating species common to all three fragments, were negatively correlated with leaf ^{15}N abundance in plants of the two São João fragments (Figs. 1, 2), but this relation was weak in plants of Petrolina fragment. Therefore, the higher the fixation the higher N and P leaf concentrations.

The low BNF rates, in relation to the proportions of potentially nodulating legume species, found in the three caatinga fragments could not be explained by the absence of microsymbionts. Native bacteria populations capable of nodulating *M. tenuiflora* were present in soil samples of the three fragments, as evidenced by the abundant nodule formation in the pot-grown seedlings of this species (Table 7). Those grown in pots with soil from the regenerating caatinga of São João had larger and more abundant nodules and probably were also more effective in fixation, since

Table 6 Total nitrogen in the leaf biomass (kg ha^{-1}), variation of the ^{15}N signal among plants of the species, proportion of plants of the species that were fixing (proportion, %), average proportion of N derived from atmosphere of plants that were fixing (%Ndfa) and fixed nitrogen (kg ha^{-1}) in leaves of two caatinga vegetation in the municipality of São João (regenerating for 10 years and >50 years) and in one caatinga vegetation in the municipality of Petrolina (regenerating for >40 years), semiarid of Pernambuco State, Brazil

Local/species	Total N (kg ha^{-1})	δ^{15}N (‰)			Fixing-plants		
		Min	Max	Average	Proportion (%)	Ndfa (%)	Fixed N (kg ha^{-1})
São João, 10 years							
Non fixing species	5.83	7.36	10.30	8.00 ± 1.04	–	–	
Mimosa tenuiflora	41.44	4.12	8.56	6.80 ± 1.33 ns	30	29	3.60
Total	47.27				–		3.60
São João, >50 years							
Non fixing species	30.13	6.61	11.0	8.40 ± 1.40	–	–	
Mimosa tenuiflora	43.90	1.84	6.96	4.26 ± 1.77*	90	46	18.17
Total	70.03				–		18.17
Petrolina, >40 years							
Non fixing species	80.85	10.32	13.17	12.26 ± 1.45[a]	–	–	
Mimosa tenuiflora	24.38	8.19	15.62	11.55 ± 2.17 ns	22	24	1.28
Mimosa stipulacea	1.42	9.49	11.17	10.40 ± 0.85 ns	30	20	0.08
Anadenanthera macrocarpa	2.99	11.83	15.58	13.71 ± 2.66 ns	0	–	0
Total	109.64					–	1.36

[a] Averages significantly different (*) or not (ns) from reference plants averages, by the T test at 5% probability level

these seedlings produced three times more biomass than those grown in the soils collected from the matures caatingas of both municipalities.

Discussion

The dominance of the vegetation of all areas by legume species, mainly potentially nodulating ones (Table 3), is typical of Neotropical dry forests (Pennington et al. 2000; Souza et al. 2012; Guedes et al. 2012). Caatinga vegetation is more closely related to the dry forests of Central Brazil than to other Neotropical dry forests and both have the highest species richness among these dry forests (Banda et al. 2016). The proportions of legume leaf biomass and nodulating legume leaf biomass in relation to the total leaf biomass were greater than those commonly found in tropical rainforests (Roggy et al. 1999; Gehring et al. 2008), even in the mature caatinga of Petrolina, which had the lowest legume dominance among the three sites (53% of legume leaf biomass and 36% of nodulating leaf biomass). The lower legume dominance in Petrolina support the observation that this dominance decreases as the total species richness and diversity increases (Murphy and Lugo 1986), even with a higher number of legume species, because the increase in richness and diversity of all other families outpaces that of Leguminosae.

The lower total species richness and diversity of dry tropical forests compared to those of tropical humid forests has been attributed to the environmental filter imposed by lower water availability (Murphy and Lugo 1986), while the higher dominance of legumes would be a consequence of their advantage in acquiring nitrogen through biological fixation (Allen and Allen 1981; Sprent 2009). Although valid in a broad sense, these generalizations must be tinted by the complexity of factors involved in the structuring of vegetations (Vitousek et al. 2002, 2013). The Petrolina caatinga fragment have a higher richness despite its lower total and worse distributed rainfall but its surrounded by a predominance of caatinga vegetation while the two São João fragments are surrounded by a large area of pasture and agriculture fields and their lower richness could point to the island effect (Higgs

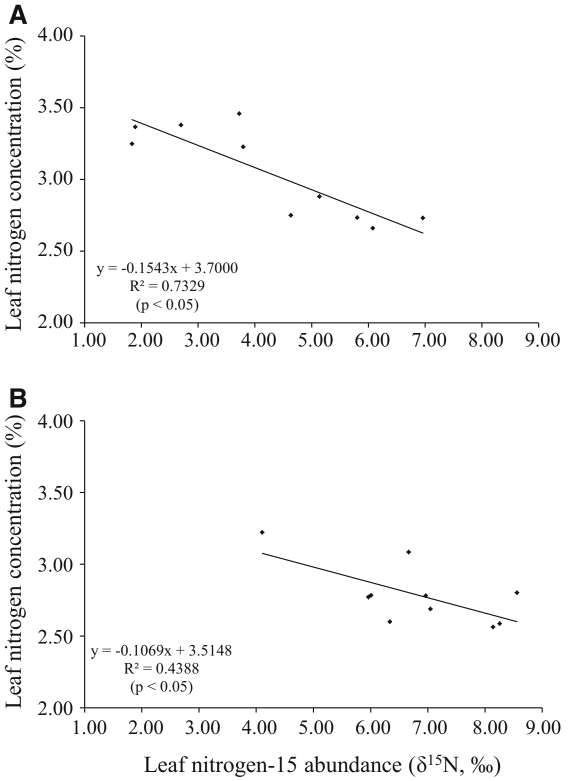

Fig. 1 Nitrogen concentration of leaves of *Mimosa tenuiflora* as a function of nitrogen-15 abundance ($\delta^{15}N$) in two caatinga vegetation in the municipality of São João (**a** regenerating for >50 years, **b** regenerating for 10 years)

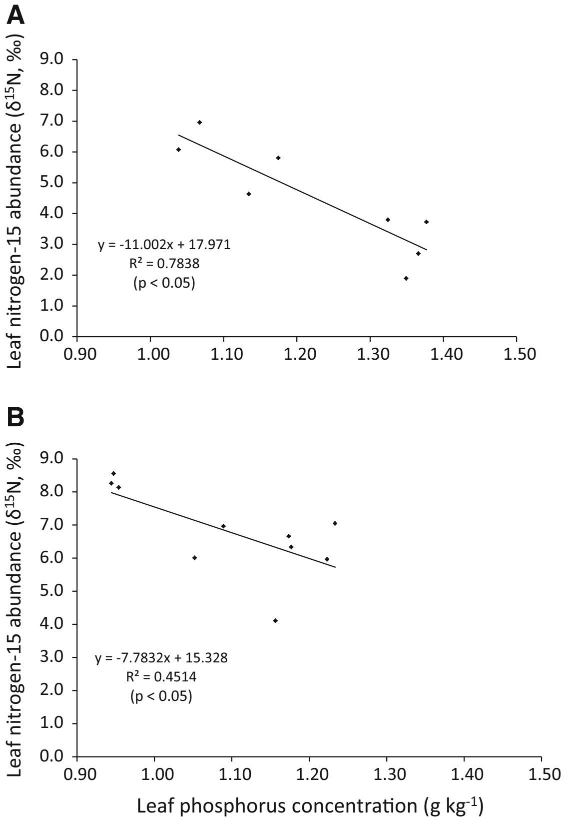

Fig. 2 Nitrogen-15 abundance ($\delta^{15}N$, ‰) of leaves of *Mimosa tenuiflora* as a function of phosphorus concentration of leaves in two caatinga vegetation in the municipality of São João (**a** regenerating for >50 years, **b** regenerating for 10 years)

1981; Medina et al. 2015). However, low availability of one or more nutrients could also be the cause, possibly micronutrients since differences of soil extractable macronutrients were relatively small among areas (Table 1).

The very high dominance of one legume species in the regenerating caatinga of São João confirms the usual observation that *M. tenuiflora* dominates the initial regeneration stages in many caatinga areas (Sampaio et al. 1998; Pereira et al. 2003; Lima et al. 2013). This pioneer species, occurring in dry areas from Brazil to Mexico (Queiroz 2009), can have high BNF (Freitas et al. 2010), in association with beta proteobacterial rhizobial symbionts (Bontemps et al. 2010; Reis et al. 2010).

The hypothesis that the legume dominance is due to their biological N fixation is based more on the obvious advantage of tapping another source of N apart from the soil than on actual measurements of the amounts of fixed N. In fact, these measurements are extremely scarce in tropical areas (Roggy et al. 1999; Sylla et al. 2002; Souza et al. 2012; Andrews et al. 2011; Nardoto et al. 2014). The hypothesis is challenged by different sources of evidence: (1) nitrogen availability, in general, is relatively high in tropical ecosystems (Vitousek and Matson 1988), despite the low total organic N soil concentrations, diminishing the ecological advantage of fixation; (2) the few measurements in tropical dry forests indicate low amounts of fixed N and in some cases, including in caatinga, even absence of fixation (Sylla et al. 2002; Souza et al. 2012); and (3) many highly abundant legume species in tropical dry forest are non-nodulating ones (Sampaio et al. 1998; Lima et al. 2013), indicating that the success of legume species may relate to other competitive abilities and not just to the ability to fix N (Vargas et al. 2015). It is likely that the nodulating legume species employ a facultative

Table 7 Nodulation and above ground biomass of *Mimosa tenuiflora* seedlings grown in pots with soil collected from two caatinga vegetation in the municipality of São João (regenerating for 10 years and >50 years) and in one caatinga vegetation in the municipality of Petrolina (regenerating for >40 years), semiarid of Pernambuco State, Brazil

Local	Nodules			Aboveground biomass (g plant^{-1})
	Number plant^{-1}	Biomass (mg plant^{-1})	Average nodule biomass (mg nodule^{-1})	
São João, 10 years	33 a	49 a	1.47 a	4.47 a
São João, >50 years	14 b	10 b	0.71 a	1.59 b
Petrolina, >40 years	15 b	13 b	0.98 a	1.53 b

Means followed by equal letters in the lines do not differ significantly by the Tukey test, at 5% probability level

strategy and only fix nitrogen under conditions of low nitrogen availability (Hedin et al. 2009).

Our results seem to support this facultative strategy, down to the level of individual plants, as has been reported in other areas (Boddey et al. 2000; Leblanc et al. 2007). The δ^{15}N signals of the plants belonging to potentially nodulating species varied from values much below those of the reference species, evidence of fixation, to values within the range of the reference species, indicating no fixation. As has been reported for caatinga (Freitas et al. 2015), the δ^{15}N signals of the reference species were high (8–12‰) with low variation (1.04–1.45‰ of standard deviation), conditions that facilitate detection of BNF and enhances the accuracy of the ^{15}N technique (Boddey et al. 2000). The estimations reveal that about 20% of the potentially nodulating *Mimosa* sp. plants were fixing in Petrolina while in São João the proportion increased to 30% in the regenerating caatinga and 90% in the mature caatinga (Table 6). These proportions are reflected in the estimates of the amounts of fixed N, higher in the mature caatinga of São João (18 kg ha^{-1} year^{-1}) than in the regenerating caatinga (3.6 kg ha^{-1} year^{-1}) and the mature caatinga of Petrolina (1.4 kg ha^{-1} year^{-1}). The amount in the mature caatinga of São João is the highest already reported for a caatinga site (Souza et al. 2012) and also higher than those of other tropical forests (Roggy et al. 1999; Nardoto et al. 2014), while the amounts for the other areas suggest a minimal advantage in fixation.

These estimates were made considering the δ^{15}N signal of each potentially nodulating plant and adopting the conservative hypothesis that fixation was only occurring if this signal was 2‰ lower than the average signal of references. If the estimations of fixation were made only for those nodulating species which had average δ^{15}N signal significantly lower than the average of the reference species, the amount fixed in the mature São João area (18.8 kg ha^{-1}) would be almost the same as using our criteria (18.2 kg ha^{-1}, Table 6) and those of the other two areas would be zero. The estimates would be slightly higher if all legume plants with δ^{15}N signal lower than the lowest δ^{15}N signal of any reference plant was considered as fixing because the lowest value of the reference plant was less than 2‰ different from the average signal of all reference plants, and therefore a few more legume plants would be considering as fixing but with low %Ndfa. Upper limits of fixation could be calculated if this safeguard condition was relaxed and the proportion of fixed N in each plant was estimated by a direct comparison of its δ^{15}N signal to the average δ^{15}N signal of all reference plants. The amounts would increase to 18.9, 8.3 and 2.3 kg ha^{-1} year^{-1}, for the mature and regenerating caatingas of São João and the mature caatinga of Petrolina, respectively. They are relatively small increases, despite the assumption that almost all potentially nodulating plants are fixing because the proportions of N derived from fixation (%Ndfa) decreases as the signal of the legume plant approach the average of the reference plants. Therefore, even these upper limits of fixation confirm that in some caatinga areas biological fixation has a small contribution to the soil–plant ecosystem and to many individual legume plants.

The causes of these low contributions and even absence of fixation are not very clear, as has been pointed out in other areas (Ndiaye and Ganry 1997; Faye et al. 2007; Souza et al. 2012). Variability among fixing plants is an expected result (Boddey et al. 2000) and has been observed even in agroforestry systems, subject to more controlled conditions (Leblanc et al.

2007; Martins et al. 2015). However, the large variation in the proportions of fixing plants among sites indicate that a local cause is involved. It is unlikely that absence or low fixation in the field was due to absence of native microsymbionts (Bala et al. 2003; Faye et al. 2007; Freitas et al. 2014) and the pot grown *M. tenuiflora* plants nodulated abundantly. Thus, it is more likely that nutritional factors were responsible for the low symbiosis performance.

BNF is controlled by N and P availability, being restricted when N availability is high and P availability is low (Crews 1993, 1999; Rastetter et al. 2001; Vitousek et al. 2002; van Groenigen et al. 2006). This control is so important that it is included in mathematical models that estimate BNF (Wang et al. 2007; Houlton et al. 2008; Cleveland et al. 2010). In general, N:P ratios above 16 indicate that the plants are P limited (Aerts and Chapin 2000; Güsewell 2004; Reich and Oleksyn 2004) and they varied from 24 to 34 in our three areas (Table 6). Higher N availability in relation to P could be part of the explanation of the lower symbiosis efficiency in Petrolina than in the mature fragment of São João. Although all three fragments had low soil P availability (Table 2), P seems to be more limiting than N in Petrolina than in the mature fragment of São João because: (1) almost all trees in Petrolina had lower foliar P concentrations and higher N:P ratios (mainly the potentially nodulating species) than in the mature fragment of São João (Table 5), where most of the legume plants were fixing N; (2) the non-fixing species of Petrolina had higher foliar δ^{15}N abundance than those of the other two areas (12.3 vs. 8.0‰ and 8.4‰), implying that they are in a system with a more open N cycle, with a higher prevalence of N losses (Högberg 1997; Handley et al. 1999; Swap et al. 2004; Ometto et al. 2006; Freitas et al. 2015); and (3) rainfall is scarcer and concentrated in a shorter period in Petrolina than in São João, possibly leading to a mismatch between the time when most of the N is mineralized and the time of the highest N uptake by the plants, resulting in excess of mineral N in the beginning of the raining season.

However, the relation of BNF and N:P ratio is complex, since symbiosis confers an advantage in the acquisition of P by plants (Houlton et al. 2008), possibly because fixing plants could invest in production of N phosphatases (Wang et al. 2007), maintaining higher leaf P concentrations. In São João, plants of *M. tenuiflora* with higher BNF (lower δ^{15}N) had higher N (Fig. 1) and also higher P concentrations (Fig. 2) and, particularly in the mature area, had lower leaf N:P ratio than non-fixing plants (Table 5). Also, the differences in the proportions of fixing plants could result from the effects of nutrients other than P, but these effects have received much less attention that that of P (Vitousek et al. 2002, 2013; Pons et al. 2007) and there is no information in relation to the subject in caatinga areas.

Conclusion

Although the vegetation of all three caatinga fragments were dominated by legume species, mainly potentially nodulating ones, the biologically fixed nitrogen amounts were low. This occurred because only part of the potentially nodulating plants were fixing and those fixing had low proportions of their N derived from the atmosphere. The lack of fixation could not be explained by absence of microsymbionts in the soil because pot grown seedlings nodulated abundantly. The low symbiosis efficiency in the field could be due to high N relative to low P availability, reflected in the high leaf N:P concentration ratios.

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