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AGRICULTURAL MICROBIOLOGY

Symbiosis of rhizobia with *Gliricidia sepium* and *Clitoria fairchildiana* in an Oxisol in the pre-Amazon region of Maranhão State

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ABSTRACT. Gliricidia (*Gliricidia sepium*) and sombreiro (*Clitoria fairchildiana*) have been recommended for agroforestry systems and reforestation of degraded areas due to their fast growth and symbiosis with rhizobia. However, little is known about native populations that nodulate these species. The objective of this study was to evaluate the phenotypic and genetic diversity of nitrogen-fixing bacteria isolated from nodules of gliricidia and sombreiro in alley cropping systems located in the pre-Amazon region of Maranhão State and to confirm their nodulation ability. Nodules were field collected from 20 plants of each species. The isolated strains were characterized morphologically, their 16S rRNA gene was partially sequenced, and their symbiotic ability was authenticated in siratro (*Macroptilium atropurpureum*). Despite being in the same climate and soil conditions, gliricidia and sombreiro are nodulated by different rhizobia genera, with *Rhizobium* predominant in gliricidia and *Bradyrhizobium* in sombreiro. Endophytic strains also colonized nodules in the field. Approximately 60% of *Rhizobium* strains did not nodulate siratro, whereas all *Bradyrhizobium* strains did. Native strains isolated from gliricidia nodules had low efficiency, and only four strains isolated from sombreiro nodules were efficient in siratro. These results highlight the importance of symbiotic relationships in the regulation of biological nitrogen fixation.

Keywords: *Bradyrhizobium*; *Rhizobium*; Papilionoideae; tree legumes; acid soil.

Simbioses de rizóbios com *Gliricidia sepium* e *Clitoria fairchildiana* em um Oxisol na região da Pré-Amazônia do Estado do Maranhão

RESUMO. Gliricídia (*Gliricidia sepium*) e sombreiro (*Clitoria fairchildiana*) têm sido recomendadas para uso em sistemas agroflorestais e reflorestamento de áreas degradadas devido ao seu rápido crescimento e simbiose com rizóbios. No entanto, pouco é conhecido em relação às populações nativas que nodulam estas espécies. O objetivo deste estudo foi avaliar a diversidade genética e fenotípica das bactérias fixadoras de nitrogênio isoladas de nódulos de gliricídia e sombreiro em sistemas *alley cropping* localizados na região pré-Amazônica do Estado do Maranhão, e autenticar sua capacidade de nodulação. Foram realizados: coleta dos nódulos em campo, isolamento, caracterização cultural, sequenciamento parcial do gene 16S rRNA e autenticação da capacidade simbiótica das estirpes em siratro (*Macroptilium atropurpureum*). Apesar de estarem sob as mesmas condições edafoclimáticas, gliricídia e sombreiro são colonizados por distintos gêneros de rizóbios, com predominância de *Rhizobium* em gliricídia, e *Bradyrhizobium* em sombreiro. Estirpes endofíticas também foram encontradas colonizando os nódulos. Cerca de 60% das estirpes de *Rhizobium* não nodularam siratro e todas de *Bradyrhizobium* nodularam esta espécie. Todas as estirpes nativas isoladas de nódulos de gliricídia apresentaram baixa eficiência, e somente quatro de sombreiro foram eficientes em siratro. Estes resultados ressaltam a importância das relações simbióticas no manejo da fixação biológica de N_2.

Palavras-chave: *Bradyrhizobium*; *Rhizobium*; Papilionoideae; leguminosas arbóreas; solo ácido.

Introduction

The pre-Amazon region in the state of Maranhão is characterized by high temperatures and heavy rainfall. These conditions, in combination with the fragile soil that originated from sedimentary rocks, are unfavourable for the maintenance of sustainable agricultural systems. The alley cropping system has been proposed to overcome this problem. In this system, leguminous trees are intercropped with agricultural crops and are periodically pruned, and the branches are used as mulch and green manure. Due to their rapid growth, high biomass production, rusticity, and biological nitrogen fixation (BNF) capabilities, two leguminous tree species that have been recommended

for this system in the region are gliricidia (*Gliricidia sepium* (Jacq.) Steud.) and sombreiro (*Clitoria fairchildiana* R.A. Howard).

BNF can be carried out by free-living, associative, or symbiotic bacteria. Among the symbiotic bacteria, the nitrogen-fixing Leguminosae-nodulating bacteria (NFLNB), also known as rhizobia, stand out due to their economic and ecological importance. NFLNB occur in structures known as "nodules" on plant roots or, in some cases, on the stems of leguminous plants. Inside the nodule, bacteria can transform atmospheric N_2 into ammonia (NH_3), a form of nitrogen that can be utilized by the plants, thereby totally or partially supplying the N demand of the crops. Gliricidia and sombreiro are tree legumes that can establish symbiosis with rhizobia. Due to this ability, and the other characteristics described above, these legumes have a high capacity for use in agroforestry systems and in the rehabilitation of degraded land, especially in areas with poor and fragile soil.

Gliricidia is a medium-size leguminous tree that is abundant throughout its natural habitat in Mesoamerica. It is of great commercial and economic interest for tropical regions since it can serve several functions, including crop shading, supplying firewood, hedges, animal forage, and green manure, as well as stabilizing soil and agroforestry systems (Simons & Stewart, 1994). Sombreiro is native to Brazil and is found mainly in the dense ombrophilous forest of the Amazon in secondary formations, but may also grow in open and disturbed habitats. This species is widely used in agroforestry systems and in landscaping and has a potential for use in the recovery of degraded areas (Aguiar, Freitas, Carvalho, Monroe, & Moura, 2011).

Although gliricidia and sombreiro are important for agroforestry and for soil recovery of degraded lands, there is little information available on the symbiotic ability and diversity of the native populations of nitrogen-fixing nodulating bacteria (NFNB) in these legume species in the pre-Amazon region. The aim of this study was to evaluate the phenotypic and genetic diversity of nitrogen-fixing nodulating bacteria in gliricidia and sombreiro in an alley cropping system in the pre-Amazon region of Maranhão State and to confirm the nodulating ability of the strains obtained in siratro (*Macroptilium atropurpureum*).

Material and methods

Area characterization and nodule sampling

Nodules were collected in a village in the municipality of Brejo, MA, in the pre-Amazon region of Maranhão (3°38' S, 42°58' W), in a 1.41 ha alley cropping system. The climate is classified as Aw (Köppen), humid equatorial and hot. The soil is classified as an Oxisol (Soil Survey Staff, 1993), with the following chemical characteristics: O.M. (g dm^{-3}) - 30.6; pH ($CaCl_2$) - 4.7; and P (resin), K, Ca, Mg, H+Al, and Al - 20.2, 0.92, 26.5, 9.6, 54.8, and 2.1 mg dm^{-3}, respectively. No inoculant was used in the area. Thus, the rhizobia populations are considered native.

To establish the alley cropping system, 2 t ha^{-1} of lime with a total neutralizing power of 50% was applied as a soil conditioner and 300 kg ha^{-1} of triple superphosphate was used as a fertilizer. In 2011, the leguminous trees *Gliricidia sepium* and *Clitoria fairchildiana* were planted in the area, with 2.5 x 0.5 m between plants, in 42 x 70 m plots. One plot was left without the leguminous plants (Figure 1). These species were intercropped with agricultural crops of mulato grass (*Brachiaria* sp. hybrid cv. Mulato) and corn (cv. AG 7088) starting in 2013. These crops received 400 kg ha^{-1} NPK 04-20-20 + 7 kg Zn ha^{-1} as fertilizer and, after sowing, a topdressing of 40 kg ha^{-1} N in the form of urea on days 15 and 40 following the corn planting. Thirty-six kg ha^{-1} of K_2O in the form of potassium chloride was also applied during the first topdressing.

At least 10 nodules per plant were collected from 40 plants (20 sombreiro plants and 20 gliricidia plants) in June 2014. The nodules were washed in running water, dried on paper towels, and packed into hermetically sealed vials containing cotton and silica gel for storage until use. In the field, five nodules of each species were evaluated for internal colour; all nodules were red.

Isolation and phenotypic characterization of strains

Nodules were isolated by hydration in sterile distilled water for 30 minutes, disinfection in alcohol for 30 seconds and in sodium hypochlorite (3%) for 3 minutes, and by washing six times with sterile distilled water. The nodules were subsequently macerated in plates containing culture media 79 (Fred & Waksman, 1928), also known as YMA (Vincent, 1970). The media had a pH of 6.8 and contained bromothymol blue. The macerate nodule material was spread in streaks to obtain single colonies, and the bacteria were incubated at 28°C. Pure colonies were harvested and morphologically characterized.

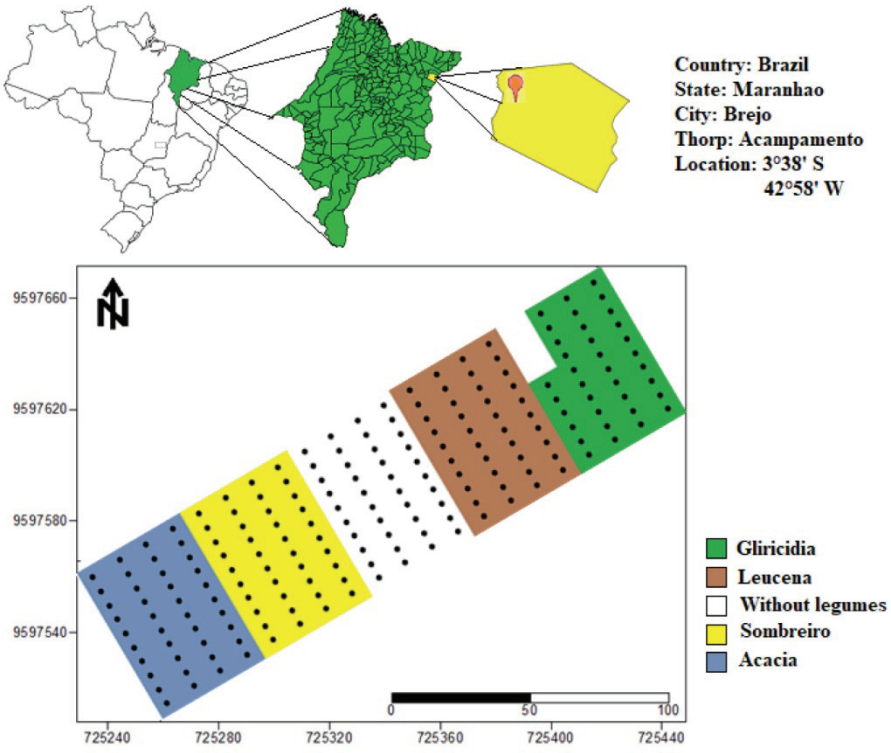

Figure 1. Location and diagram of the experimental area. Different colours indicate legume species that make up the alley cropping system. In all the plots, leguminous trees are intercropped with corn and mulato grass; in the plot without legumes, only corn and grass are cultivated. In this study, only gliricidia and sombreiro were sampled. Black dots are georeferenced and indicate the points where the soil samples were collected. The follow up of the results regarding periodic collections of soil in the georreferenced points are objective of other studies and are not included in this work.

Strains were isolated, choosing the biggest nodules, until approximately 40 strains per species were obtained. Cultures were characterized in the culture medium YMA using bromothymol blue. The following characteristics were analysed: time of growth - fast (1-3 days), intermediate (4-5 days), slow (6-10 days), very slow (more 10 days); and change in pH (acid, alkaline, or neutral). According to this characterization, 25 gliricidia strains and 18 sombreiro strains, representative of the phenotypic groups obtained, were selected for identification by partial sequencing of the 16S rRNA gene and for analysis of nodulating capability in siratro.

DNA extraction and partial sequencing of the 16S rRNA gene

Strains selected for partial sequencing of the 16S rRNA gene were grown at 28°C in solid media 79. After confirming of the purity of the strains, DNA was extracted with the alkaline lysis method (Niemann, Puehler, Tichy, Simon, & Selbitshka, 1997).

Partial amplification parameters, primer sequences, cycle conditions, and sequencing protocol of the 16S rRNA gene were previously described by Costa et al. (2013). The quality of the sequences was analysed with the BioNumerics 7.1 software (Applied Maths, Austin, TX, USA). Sequences were compared to those deposited in the GenBank database (National Center for Biotechnology Information - NCBI) using the BLAST.

The sequences obtained in this study were deposited in the GenBank database (National Center for Biotechnology Information - NCBI) under the accession numbers KX555372 through KX555414, for the 16S rRNA gene.

Strain authentication and efficiency

Strains selected for sequencing were also subjected to authentication by making use of siratro (*Macroptilium atropurpureum*), a highly promiscuous species, which grows rapidly and is characterized by abundant seed availability and easy handling. Siratro seeds were scarified on the surface and disinfected by immersion in H_2SO_4 for 20 minutes, followed by several washes with sterile distilled water, and subsequent soaking in sterile distilled water for 30 minutes. Seeds were germinated in Petri dishes with cotton and sterilized filter paper, soaked in sterile distilled water.

The experiment was carried out for 45 days in a greenhouse with a completely randomized

experimental design, with three replications. The treatments were: control without inoculation and with low mineral N concentration (5.25 mg L^{-1}); control without inoculation and with high mineral N concentration (52.5 mg L^{-1}); two controls inoculated with efficient reference strains for siratro [UFLA04-212 (*Bradyrhizobium* sp.) (Florentino, Guimarães, Rufini, Silva, & Moreira, 2009), and SEMIA 656 (*Bradyrhizobium* sp.), authorized by the Ministry of Agriculture, Livestock and Supply for the production of inoculants for siratro]; and samples inoculated individually with the strains under study. All samples inoculated with test or control strains received a low mineral N concentration. The experiment was carried out in long neck bottles (500 mL) containing sterile Hoagland solution (Hoagland & Arnon, 1950) at ¼ strength.

At the end of the experiment, the following assessments were made: number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), and relative efficiency compared to the treatment with a high mineral N concentration (RENC). RENC was calculated according to the following formula: RENC (%) = SDM of the treatment inoculated with the strain / SDM of the treatment with a high N concentration x 100.

Two nodules per treatment were separated and re-isolated from strains exhibiting effective nodulation for comparison of the phenotypic characteristics in relation to the inoculated strain.

To meet the statistical assumptions, the growth parameters SDM, NDM, and NN of gliricidia in the first stage were transformed using the equation: $\sqrt{X} + \sqrt{(X+1)}$. In the second stage, all growth parameters of gliricidia were transformed with the equation: $\sqrt{(X+0.5)}$. For sombreiro, SDM, root and shoot ratio (R/S), NDM, and NN were transformed using $\sqrt{(X+0.5)}$ in the first stage, and NDM was transformed in the second stage. Treatment means were compared by the Scott-Knott test at a 5% probability using the Sisvar 5.3 statistical analysis software (Ferreira, 2011).

Results and discussion

Isolation and phenotypic characterization of strains

All nodules used for isolation were visually healthy with a red internal colour. A total of 83 strains were obtained: 42 from gliricidia and 41 from sombreiro. According to the phenotypic characterization, strains isolated from gliricidia nodules exhibited rapid or intermediate growth as well as an acidic, neutral, or alkaline pH reaction with bromothymol blue in the YMA medium. Strains isolated from sombreiro nodules were more variable in phenotype. Among these strains, distinct phenotypes were observed, including fast, intermediate, slow, and very slow growth, with the predominance of intermediate growth and an alkaline pH reaction with bromothymol blue in the YMA medium.

Phenotypic characterization of the gliricidia strains showed low diversity. The predominance of fast growth and a neutral or acidic pH reaction among the isolated gliricidia strains is due to the large number of genetically identified strains in the genus *Rhizobium*, in which these characteristics are prominent, and due to the presence of endophytes within the nodules, which usually exhibit fast growth (Jaramillo et al., 2013).

Several authors have reported on the ability of gliricidia to establish symbiosis only with fast-growing rhizobia, and it has been proposed that gliricidia preferentially achieves effective symbiosis with strains of this type (Turk & Keyser, 1992; Moreira, Gillis, Pot, Kersters, & Franco, 1993; Melchor-Marroquín, Vargas-Hernández, Ferrera-Cerrato, & Krishnamurthy, 1999; Thiao, Neyra, Isidore, & Sylla, 2004; Florentino et al., 2014). However, the strains were not genetically identified in these studies.

The greater number of intermediate-growing strains, as well as slow-growing strains, among sombreiro isolates corroborates their identification in the *Bradyrhizobium* genus. However, Moreira et al. (1993) reported on strains exhibiting both fast and slow growth in sombreiro grown on different substrates in a nursery.

Identification by 16S rRNA gene partial sequencing

Forty-three strains were selected for identification by partial sequencing of the 16S rRNA gene (25 from gliricidia and 18 from sombreiro). Among gliricidia, most strains belong to the *Rhizobium* genus; however, native *Mesorhizobium* sp. and *Methylobacterium* sp. were also isolated (Table 1). Non-nodulating strains were also found colonizing gliricidia nodules and included the following genera: *Massilia*, *Klebsiella*, *Cryseobacterium*, *Bacillus*, and *Bosea* (Table 1). Strains isolated from gliricidia in the field, in the nursery, and under controlled conditions have already been identified as *Rhizobium* in south-eastern Brazil (Moreira, Haukka, & Young, 1998) and by other authors in various countries and continents (McInroy et al., 1999; Bala & Giller, 2001; Zurdo-Piñeiro et al., 2004; Degefu, Wolde-Meskel, & Frostegard, 2013). In addition to *Rhizobium*, fast-growing strains of other genera, such as *Sinorhizobium*, *Mesorhizobium*, *Alorhizobium*, and/or *Agrobacterium*, have been found by other authors in

Table 1. Culture, identification based on partial sequencing of the 16S rRNA gene, and mean values of shoot dry matter (SDM), root dry matter (RDM), root and shoot ratio (R/S), relative efficiency compared to the controls with high mineral N concentration (RENC), nodule dry matter (NDM), and number of nodules (NN) of bacterial strains isolated from *Gliricidia sepium* nodules cultivated in an alley cropping system in the pre-Amazon region of Maranhão, and authenticated regarding nodulation in *Macroptilium atropurpureum*. Values for two non-inoculated controls, one with high mineral N concentration (52.5 mg L^{-1}) and one with low mineral N concentration (5.25 mg L^{-1}), and two inoculated controls (UFLA 04-212 and SEMIA 656). Experiment were divided into two stages. The sequences of the strains were compared based on the most similar sequences found in the GenBank.

STAGE 1												
Treatment	Nodulation[1]	Culture Characteristics[2]	16S rRNA extension (pb)	Most similar sequences found in the GenBank			SDM[3]	RDM	R/S	RENC	NDM[3]	NN[3]
				Species	Accession number	Similarity (%)	g plant^{-1}			%	mg plant^{-1}	plant^{-1}
52.5 mg de N L^{-1}	-	---	---	---	---	---	0.65a[6]	0.33a	0.51b	100a	0.000c	0b
5.25 mg de N L^{-1}	-	---	---	---	---	---	0.11d	0.12b	1.10a	18c	0.000c	0b
UFLA04-212[5]	+	S/AL	---	*Bradyrhizobium* sp.	---	---	0.27b	0.18b	0.69b	41b	0.058a	29a
SEMIA 656	+	S/AL	---	*Bradyrhizobium* sp.	---	---	0.21c	0.11b	0.51b	33b	0.040b	30a
UFLA01-889	+	R/N	598*	*Rhizobium* sp.	JX855240.1	99	0.10d	0.09b	0.89b	15c	0.000c	2b
UFLA01-890	+	R/N	1236	*Rhizobium* sp.	JX855240.1	99	0.10d	0.13b	1.27a	16c	0.001c	2b
UFLA01-891	-	R/AL	1322	*Massilia* sp.	JX566630.1	99	0.11d	0.10b	0.95b	17c	0.000c	0b
UFLA01-892	+	R/N	760*	*Rhizobium* sp.	JX855240.1	99	0.08d	0.13b	1.65a	13c	0.001c	6b
UFLA01-893	+	R/N	1293	*Rhizobium* sp.	KM253159.1	99	0.09d	0.11b	1.22a	14c	0.001c	2b
UFLA01-895	-	R/N	843*	*Rhizobium* sp.	JX855240.1	99	0.10d	0.12b	1.21a	15c	0.000c	0b
UFLA01-896	-	R/AC	564*	*Klebsiella* sp.	DQ316102.1	100	0.09d	0.12b	1.4a	14c	0.000c	0b
STAGE 2												
52.5 mg N L^{-1}	-	---	---	---	---	---	1.36a[4/6]	0.58a[4]	0.43b[4]	100a	0.000c[4]	0b[4]
5.25 mg N L^{-1}	-	---	---	---	---	---	0.11c	0.12c	1.13a	8c	0.000c	0b
UFLA 04-212[5]	+	S/AL	---	*Bradyrhizobium* sp.	---	---	0.43b	0.23b	0.54b	32b	0.044b	36a
SEMIA 656	+	S/AL	---	*Bradyrhizobium* sp.	---	---	0.37b	0.15c	0.39b	27b	0.048a	32a
UFLA01-887	-	R/AL	439*	*Massilia* sp.	JX566630.1	99	0.08c	0.08c	0.86b	6c	0.000c	0b
UFLA01-888	-	R/AL	1324	*Chryseobacterium* sp.	JF327645.1	98	0.08c	0.12c	1.60a	6c	0.000c	0b
UFLA01-897	-	R/N	1313	*Rhizobium* sp.	JX855240.1	88	0.11c	0.14c	1.36a	8c	0.000c	0b
UFLA01-898	-	R/N	795	*Rhizobium* sp.	JX855240.1	99	0.09c	0.09c	1.07a	6c	0.000c	0b
UFLA01-900	+	R/N	1303	*Rhizobium* sp.	JX855239.1	99	0.11c	0.14c	1.33a	8c	0.001c	2b
UFLA01-901	-	R/N	1266	*Rhizobium* sp.	JX855163.1	99	0.12c	0.11c	0.98a	9c	0.000c	0b
UFLA01-902	-	R/AC	933*	*Rhizobium* sp.	JX855163.1	99	0.07c	0.07c	0.84b	5c	0.000c	0b
UFLA01-903	-	R/AC	683*	*Bacillus* sp.	KJ879598.1	100	0.11c	0.13c	1.32a	8c	0.000c	0b
UFLA01-905	+	R/N	1285	*Rhizobium* sp.	JX855166.1	99	0.08c	0.10c	1.61a	6c	0.002c	4b
UFLA01-906	-	R/N	1292	*Rhizobium* sp.	JX855240.1	99	0.10c	0.09c	0.95a	8c	0.000c	0b
UFLA01-907	-	I/N	1345	*Mesorhizobium* sp.	EU584257.1	98	0.12c	0.13c	1.09a	9c	0.000c	0b
UFLA01-908	+	R/N	856*	*Rhizobium* sp.	JX855240.1	99	0.11c	0.14c	1.25a	8c	0.001c	3b
UFLA01-909	-	R/AL	724*	*Bosea* sp.	JX566529.1	100	0.09c	0.11c	1.23a	7c	0.000c	0b
UFLA01-910	-	R/AC	1227	*Rhizobium* sp.	JX855240.1	99	0.10c	0.14c	1.40a	8c	0.000c	0b
UFLA01-911	-	R/AC	1275	*Rhizobium* sp.	JX855240.1	99	0.12c	0.13c	1.09a	9c	0.000c	0b
UFLA01-912	-	R/AC	1264	*Rhizobium* sp.	GU433459.1	99	0.11c	0.12c	1.14a	8c	0.000c	0b
UFLA01-913	-	R/AL	1253	*Methylobacterium* sp.	AM910533.1	99	0.10c	0.12c	1.27a	7c	0.000c	0b
UFLA01-899	-	R/N	1287	*Rhizobium* sp.	JX855240.1	99	Nd	Nd	Nd	Nd	Nd	Nd

[1](+) presence and (-) absence of nodules in *M. atropurpureum*. [2]Cultural characteristics in solid YMA medium: time of appearance of isolated colonies - R-rapid (1-3 days), I-intermediate (4-5 days), S-slow (6-10 days), VS-very slow (over 10 days); pH reaction in YMA medium with bromothymol blue - AC-acid, N-neutral, AL-alkaline. *only forward sequence used. [3]Data transformed by $\sqrt{X} + \sqrt{X+1}$. [4]Data transformed by $\sqrt{X+0.5}$. [5]All strains received 5.25 mg N L^{-1}. [6]Means followed by the same letter in the same column are not statistically different according to the Scott-Knott test at 5%. Nd – not determined.

other countries (Acosta-Durán & Martinez-Romero, 2002; Bala, Murphy, & Giller, 2003; Bala & Giller, 2006) and by Moreira et al. (1998) in Brazil. However, *Mesorhizobium* was only found in this study. This is the first report of the occurrence of *Methylobacterium* in gliricidia nodules. All strains found to be symbiotic with sombreiro belong to the *Bradyrhizobium* genus (Table 2). The other strains identified are not recognized as nodulating and are distributed in the genera *Klebsiella*, *Bacillus*, *Paenibacillus*, *Arthrobacter*, and *Leifsonia* (Table 2). The presence of *Bradyrhizobium* in the nodules of this legume, cultivated on different substrates in the nursery, has been previously reported (Moreira et al., 1993; 1998). However, *Burkholderia* was also identified by 16S rRNA sequencing in the BR8005 and BR8006 strains (Moreira, 2008) identified as *Rhizobium* by Moreira et al. (1993), based on a profile analysis of total protein at a time when there were only three rhizobia genera with distinct culture characteristics. However, *Burkholderia* was not found in the present study.

Strains of the *Bacillus* and *Paenibacillus* genera have been found in legume plant nodules and are considered endophytic, although their role remains to be fully elucidated. Studies have demonstrated that strains of the *Bacillus* genus can coexist with *Bradyrhizobium* in nodules and can nodulate and biologically fix nitrogen (Li, Wang, Chen, & Chen, 2008; Costa et al., 2013). It has been demonstrated that bacteria belonging to the *Arthrobacter* and *Klebsiella* genera are found in the rhizosphere in association with roots, or live freely in the soil, and promote plant growth (Kloepper, Lifshitz, & Zablotowicz, 1989; Glick, 1995). In addition, *Arthrobacter* sp. may be associated with senescent nodules and may

Table 2. Culture, identification based on partial sequencing of the 16S rRNA gene, and mean values of shoot dry matter (SDM), root dry matter (RDM), root and shoot ratio (R/S), relative efficiency compared to the controls with high mineral N concentration (RENC), nodule dry matter (NDM), and number of nodules (NN) of bacterial strains isolated from *Clitoria fairchildiana* nodules cultivated in an alley cropping system in the pre-Amazon region, and authenticated regarding nodulation in *Macroptilium atropurpureum*. Sequences of the strains were compared based on the most similar sequences found in the GenBank.

STAGE 1												
Treatment	Nodulation[1]	Culture Characteristics[2]	16S rRNA extension (pb)	Most similar sequences found in the GenBank			SDM[3]	RDM	R/S[3]	RENC	NDM[3]	NN[3]
				Species	Accession number	Similarity (%)	g plant^{-1}			%	mg plant^{-1}	plant^{-1}
52,5 mg N L^{-1}	-	---	---	---	---	---	0,65a[5]	0,33a	0,51b	100a	0,000c	0c
5,25 mg N L^{-1}	-	---	---	---	---	---	0,11d	0,12b	1,10a	18d	0,000c	0c
UFLA 04-212[4]	+	S/AL	---	*Bradyrhizobium* sp.	---	---	0,27b	0,18b	0,69b	41b	0,059a	28a
SEMIA 656	+	S/AL	---	*Bradyrhizobium* sp.	---	---	0,21c	0,11b	0,51b	33c	0,040b	30a
UFLA01-967	-	R/AC	624*	*Klebsiella* sp.	KF411348.1	99	0,07d	0,10b	1,34a	11d	0,000c	0c
UFLA01-968	-	R/AL	1357	*Bacillus* sp.	KF254678.1	100	0,10d	0,12b	1,17a	15d	0,000c	0c
UFLA01-969	-	R/N	698*	*Paenibacillus* sp.	KF750627.1	99	0,06d	0,10b	1,73a	9d	0,000c	0c
UFLA01-971	+	R/AC	308*	*Arthrobacter* sp.	FR745407.1	99	0,10d	0,10b	1,03a	15d	0,001c	4c
UFLA01-972	-	R/AC	577*	*Paenibacillus* sp.	EU571197.1	99	0,08d	0,12b	1,52a	13d	0,000c	0c
UFLA01-975	-	R/AC	905*	*Leifsonia* sp.	FR750300.1	99	0,11d	0,14b	1,24a	17d	0,000c	0c
UFLA01-982	-	R/AC	286*	*Leifsonia* sp.	FR750300.1	100	0,10d	0,12b	1,26a	15d	0,000c	0c
UFLA01-984	+	VS/AL	1182	*Bradyrhizobium* sp.	KJ739927.1	100	0,15d	0,14b	0,89b	24d	0,038b	14b
STAGE 2												
52,5 mg de N L^{-1}	-	---	---	---	---	---	1,36a[5]	0,57a	0,42c	100a	0,000c[3]	0c
5,25 mg de N L^{-1}	-	---	---	---	---	---	0,11c	0,12c	1,13a	8c	0,000c	0c
UFLA 04-212[4]	+	S/AL	---	*Bradyrhizobium* sp.	---	---	0.43b	0,23b	0,54c	32b	0,043a	36a
SEMIA 656	+	S/AL	---	*Bradyrhizobium* sp.	---	---	0,37b	0,15b	0,39c	28b	0,048a	32a
UFLA01-959	+	I/AL	1253	*Bradyrhizobium* sp.	KC677617.1	99	0,21c	0,09c	0,48c	15c	0,037a	42a
UFLA01-960	+	I/AL	784*	*Bradyrhizobium* sp.	KF933597.1	99	0,24b	0,10c	0,44c	17b	0,029b	41a
UFLA01-961	+	I/AL	1233	*Bradyrhizobium* sp.	KF114645.1	99	0,12c	0,10c	0,83b	9c	0,030b	21b
UFLA01-963	+	I/AL	621*	*Bradyrhizobium* sp.	KF357613.1	100	0,10c	0,12c	1,14a	8c	0,016c	21b
UFLA01-964	+	I/AL	321[1]	*Bradyrhizobium* sp.	KR779522.1	100	0,19c	0,09c	0,52c	14c	0,039a	44a
UFLA01-965	+	I/AL	792*	*Bradyrhizobium* sp.	KF933597.1	99	0,10c	0,05c	0,50c	7c	0,011c	12b
UFLA01-976	+	I/AL	1222	*Bradyrhizobium* sp.	KF114634.1	99	0,31b	0,13c	0,41c	23b	0,064a	43a
UFLA01-980	+	S/AL	1220	*Bradyrhizobium* sp.	KF114634.1	99	0,34b	0,17b	0,49c	25b	0,071a	50a
UFLA01-981	+	VS/AL	804*	*Bradyrhizobium* sp.	JN085495.1	97	0,11c	0,10c	0,97b	8c	0,027b	32a
UFLA01-985	+	I/AL	770*	*Bradyrhizobium* sp.	KF114645.1	100	0,27b	0,11c	0,40c	20b	0,029b	20b

[1](+) presence and (-) absence of nodules in *M. atropurpureum*. [2]Cultural characteristics in solid YMA medium: time of appearance of isolated colonies - R-rapid (1-3 days), I-intermediate (4-5 days), S-slow (6-10 days), VS-very slow (over 10 days); pH reaction in YMA medium with bromothymol blue - AC-acid, N-neutral, AL-alkaline. *only forward sequence used. [3]Data transformed by √X+0.5. [4]All strains received 5.25 mg N L^{-1}. [5]Means followed by the same letter in the same column are not statistically different according to the Scott-Knott test at 5%.

influence nitrogen loss in these nodules (Webb et al., 2010). In contrast, the genera *Bosea*, *Chryseobacterium*, *Klebsiella*, and *Massilia* can act as growth promoters in plants by means of phosphate solubilization and phytohormone production, among other processes (Chen et al., 2006; Shiraishi, Matsushita, & Hougetsu, 2010; Marra et al., 2012). The genus *Leifsonia*, includes endophytic bacteria found in plant roots, such as in ginseng (Qiu et al., 2007). However, none of the endophytic strains isolated from gliricidia or sombreiro nodules stimulated growth in siratro. Both phenotypic characterization and identification by partial sequencing of the 16S rRNA gene indicated that gliricidia and sombreiro are colonized by different NFLNB groups. This shows that the host exclusively determines whether the nodules are occupied by *Rhizobium* or *Bradyrhizobium* strains. Considering that both leguminous trees were sampled from the same area, it can be inferred that this response is likely related to host specificity to legume species with native NFLNB strains.

Strain authentication and efficiency

In both experiments, controls with a low mineral N concentration and without inoculation did not nodulate at any stage, indicating the absence of contamination in the experiments. Controls with a high mineral N concentration displayed superior nodulation values ($p < 0.05$) to all the tested strains and to the other controls for SDM, RDM, and RENC in both stages (Tables 1 and 2).

Considering SDM, RDM, and RENC, there were no strains isolated from gliricidia that were efficient at N_2 fixation with siratro, since all values were similar ($p > 0.05$) to that of the control with a low mineral N concentration for the parameters mentioned above (Table 1). The R/S ratio for most strains tested was superior ($p < 0.05$) to the controls with a high mineral N concentration, to the controls inoculated with the reference strains (UFLA 04-212 and SEMIA 656) and to the strains UFLA01-889, UFLA01-891, UFLA01-887, and UFLA01-902. There were no strains with a similar nodulation ($p > 0.05$) to that of the reference strains (UFLA 04-212 and SEMIA 656). Regarding the 17 *Rhizobium*

sp. strains, only seven nodulated siratro, and the *Mesorhizobium* sp. strain was unable to do so. Strains of species that are not recognized as nodulating were unable to do so, as expected (Table 1).

Regarding the SDM and RENC in sombreiro, the strains UFLA01-960, UFLA01-976, UFLA01-980, and UFLA01-985, which belong to the *Bradyrhizobium* genus, and the reference strains (UFLA 04-212 and SEMIA 656) showed a superior efficiency ($p < 0.05$) to the other strains (Table 2). For the R/S, eight of the 18 strains tested were similar ($p > 0.05$) to the controls with a low mineral N concentration. The other strains displayed superior values ($p < 0.05$) to the controls with a high mineral N concentration and to the reference strains (Table 2). For the NDM and NN, several strains were similar ($p > 0.05$) to the reference strains. All strains not recognized as nodulating were unable to do so, except for UFLA01-971, which was identified in the *Arthrobacter* genus. For RDM, only the UFLA01-980 strain displayed similar values ($p > 0.05$) to those of the reference strains. The remaining strains had values that were similar ($p > 0.05$) to the controls with low mineral N concentration (Table 2).

The siratro species was used to authenticate the nodulation ability of the strains, since it is a promiscuous species, i.e., it can be nodulated with various rhizobia genera and species (Lima et al., 2009), and because it also produces an abundance of seeds during several seasons, which are viable for long time periods. In addition, siratro seeds and plants are easy to manage in a greenhouse. This is not the case for the species studied here, whose seeds are not available during all seasons and also quickly lose viability. Although several studies have authenticated gliricidia strains in their original host (Florentino et al., 2014; Bala & Giller, 2006; Turk & Keyser, 1992; McIroy et al., 1999; Melchor-Marroquim et al., 1999; Acosta-Durán & Martinez-Romero, 2002; Thiao et al., 2004), no studies have been conducted to authenticate the strains in sombreiro, or to analyse the *Nif* and *Nod* genes in the strains in either species. Although siratro can be nodulated with every strain in the nodulating genera in the present study, the legume was nodulated with all the strains isolated from sombreiro nodules (belonging to the *Bradyrhizobium* genera), but it was not nodulated with some *Rhizobium* strains, nor with the *Methylobacterium* strain isolated from gliricidia, nor with the *Mesorhizobium* strain isolated from gliricidia. These results demonstrated that the use of siratro as a species for the authentication of strains is relatively limited, since it is not able to be nodulated with all species/strains in the same genus, for example, *Rhizobium* and *Mesorhizobium*. Nodulation in siratro authenticated various strains as being able to nodulate; however, the low efficiency of these strains with siratro cannot be extrapolated to their relationship with the original host, which should be investigated in further studies. In addition, the symbiotic preference of *Rhizobium* for gliricidia and of *Bradyrhizobium* for sombreiro should be considered in selecting efficient strains for these species in future studies.

The inability to be nodulated with most endophytic strains was expected, as there are few reports of nodulation for these genera, and typically, these studies do not yield conclusive data. Effective nodulation with a strain of the *Arthrobacter* genus may be explained by the possibility of horizontal gene transfer, encoding the nodulation of rhizobia species in the soil (Shiraishi et al., 2010). However, this aspect should be investigated in more detail.

Conclusion

Gliricidia is preferentially nodulated by rapid-growing strains of the *Rhizobium* genus, while sombreiro is nodulated by strains of *Bradyrhizobium*.

Endophytic strains of the genera *Cryseobacterium*, *Massilia*, *Bacillus*, *Bosea*, and *Klebsiella* were found in gliricidia nodules, while the genera *Klebsiella*, *Bacillus*, *Paenibacillus*, *Arthrobacter*, and *Leifsonia* were found in sombreiro nodules. Among these strains, only *Arthrobacter* exhibited effective nodulation in siratro.

To our knowledge, this is the first report of the isolation of *Methylobacterium* from gliricidia nodules.

All native strains isolated from gliricidia showed a low nodulation efficiency in siratro, and only four strains isolated from sombreiro were efficient in siratro.

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