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Article

Rhizobial diversity in shrub-tree legume-based silvopastoral systems

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ABSTRACT: Silvopastoral systems based on tree legumes intercropped with forage grasses can harbor a high diversity of rhizobia, and these bacteria are good indicators of soil quality in several management systems. The objective of this work was to evaluate the morphophysiological, genetic and symbiotic diversity of cowpea [*Vigna unguiculata* (L.) Walp] rhizobia from soils under silvopastoral systems based on shrub-tree legumes. The experiment was performed in a randomized block design with three treatments and three replications, consisting of signalgrass (*Urochloa decumbens* Stapf.) intercropped with sabia (*Mimosa caesalpiniaefolia*); signalgrass intercropped with gliricidia (*Gliricidia sepium*) and single signalgrass. The samples were collected in the legume row (0 meter) and 4 and 8 meters away. Later, cowpea was used as a trap plant to capture the rhizobia. All strains were phenotypically characterized, authenticated, and genetically identified. Phenotypical characterization of the 431 isolates showed high diversity forming 69 groups at 100% similarity, of which 60 were able to nodulate cowpea during the authentication, and 36 presented relative efficiency superior or equal to the recommended bacteria for the crop. Most of the sequenced strains belonged to *Bradyrhizobium* (67%) and *Methylobacterium* (9%). *Leifsonia* (9%), *Cohnella* (6%), *Rhizobium* (3%), *Burkholderia* (3%), and *Paenibacillus* (3%) were also represented. Soils under silvopastoral systems harbor efficient rhizobia populations in cowpea with a high genetic diversity, which can be recommended for agronomic efficiency assays.

Key words: *Methylobacterium*, *Gliricidia sepium*, pasture, *Mimosa caesalpiniaefolia*, *Vigna unguiculata*.

INTRODUCTION

Silvopastoral systems integrate livestock, forage and shrub or tree species, with several economic benefits (Apolinário et al. 2015). If the arboreal component includes legumes, they also promote an increase on soil fertility including nutrient input, mainly from nitrogen, into deep soil layers (Apolinário et al. 2015) with significant effects up to 1 m depth (Lira Junior et al. 2020b). This is particularly important since a large part of tropical subhumid pastures, typically based on pure grasses and not fertilized with N, are submitted to various stages of degradation with major environmental impacts (Lima et al. 2018).

Gliricidia (*Gliricidia sepium* (Jacq.) Steud.) and sabia (*Mimosa caesalpiniifolia* Benth.) are tropical shrub-tree legumes that benefit grass pastures (Apolinário et al. 2015) and provide nutritive support to ruminants. These legumes have fast growth, high regeneration capacity, drought resistance (Mendes et al. 2013; Paula et al. 2015) and fix nitrogen when in symbiosis with diazotrophic microorganisms (Martins, J. C. R. et al. 2015; Martins, P. G. S. et al. 2015) at around 200 kg N·ha^{-1} yr^{-1} (Apolinário et al. 2015).

Soil microbial diversity can be affected by soil management and plant cover (Barros et al. 2018), and the silvopastoral system based on shrub tree legumes determines the structure of the total bacterial and the nitrogen-cycle bacteria communities, as shown by Barros et al. (2021). Soils under agriculture and agroforestry may harbor a high rhizobia genetic diversity due to increased nitrogen demand, stimulating the rhizobial population (Guimarães et al. 2012), and this biodiversity may be both a means to evaluate management system impacts (Lima et al. 2009) and a source of efficient rhizobial strains for use in inoculant production (Uzoh and Babalola 2018).

The effects of silvopastoral systems on rhizobial diversity have not been published up to now, to the best of our knowledge, although general effects of land use systems and changes on rhizobial diversity are widely known (Berza et al. 2021; Gnangui et al. 2021; Souza and Procópio 2021; Wang et al. 2021). This rhizobial diversity can be studied by either culture independent methods or by culture dependent ones, in this case typically using trap species. The selection of the trap species is a central point, due to the well-known specificity between legume and rhizobia.

While tropical legumes are generally considered promiscuous (Lira Junior et al. 2015), cowpea [*Vigna unguiculata* (L.) Walp] is particularly so, and nodulates with a large rhizobial range to varying degrees of efficiency (Ndungu et al. 2018), including both alpha and beta rhizobia (Castro et al. 2017; Lardi et al. 2017; Muindi et al. 2021), so it is frequently used as a trap plant species when one of the goals is to obtain the largest cross section possible of rhizobial biodiversity with a single legume species (Jaramillo et al. 2013).

The objective of this work was to determine phenotypical, genetic, and symbiotic diversity of cowpea [*Vigna unguiculata* (L.) Walp] rhizobia as an indicator of overall rhizobial diversity and silvopastoral systems effects upon it.

MATERIALS AND METHODS

Experimental area and design

Silvopastoral systems were established in 2011 at the experimental field of Instituto Agronômico de Pernambuco (IPA), Itambé, Pernambuco state, Brazil (7°25'S; 35°6'W, 190 m above average sea level), and the study station soil is classified as Ultisol Red-Yellow, according to Jacomine et al. (1973). The climate is AS' in the Köppen classification (i.e., hot and humid), with average rainfall and annual temperature of 1,300 mm·year^{-1} and 24 ºC, respectively. The area was continuously grazed by crossbred bovines (Holstein-Frísia-Zebu), with grazing density variable according to the objectives of animal experiments carried out in the area (Santos et al. 2020).

This experiment was conducted in a randomized block design with three treatments and three replications, in nine plots of 1 ha each (43.5 × 230 m), consisting of signalgrass (*Urochloa decumbens* Stapf.) intercropped with sabia (*Mimosa caesalpiniaefolia*) (B + S); signalgrass intercropped with gliricidia (*Gliricidia sepium*) (B + G) and single signalgrass (B). Legumes were planted in 14 double rows, spaced 15 × 1 × 0.5 m, with signalgrass in the rows among each double row.

Soil samples were collected in June 2016 to a 20-cm depth. Each intercropped plot was divided into three transects along, which samples were collected at a 0, 4 and 8-meter distance from the legume double row, to prepare a composite sample for each distance and each plot. Three samples were randomly collected in B treatment to form a composite soil sample at the same depth, totaling 21 samples. Soil chemical characterization is presented in Table 1.

Table 1. Soil chemical characteristics of the silvopastoral system in Itambé, Pernambuco, Brazil, 2016.

Distance (m)	pH Water 1:2.5	Ca	Mg	Al	Na	K	H + Al	P
		($cmol_c \cdot dm^{-3}$)						($mg \cdot dm^{-3}$)
				B + S				
0	5.0	4.68	1.20	0.43	0.06	0.24	8.31	8
4	5.1	3.62	2.42	0.50	0.07	0.16	7.92	11
8	5.3	4.32	1.88	0.31	0.06	0.17	7.41	6
				B + G				
0	5.2	4.43	1.17	0.41	0.06	0.23	7.60	9
4	5.5	4.68	1.90	0.15	0.08	0.30	6.75	7
8	5.6	4.60	2.43	0.20	0.05	0.24	6.48	6
				B				
	5.6	3.70	2.00	0.17	0.04	0.19	6.48	9

B + S: signalgrass intercropped with shrub-tree sabia; B + G: signalgrass intercropped with shrub-tree gliricidia; B: single signalgrass.

Rhizobia capture using cowpea as the trap species and morphophysiological characterization

Cowpea seeds (cultivar IPA-206) were superficially disinfected using 70% alcohol for 30 s, then submerged in 2.5% sodium hypochlorite and cleaned with ultrapure water. All seeds were placed in trays to germinate and incubated for two days at room temperature. Seedlings were moved to sterile 350-mL bottles filled with Hoagland solution without N (Hoagland and Arnon, 1950) at 1/4 strength with two 1.8-cm wide germitest paper tapes placed inside each bottle. Each seedling was inoculated with 1 mL of each decimal serial dilution from 10^{-1} to 10^{-9} from a soil sample in a sterile saline solution of 0.85% NaCl.

A completely randomized block design in triplicate was used, with two positive controls inoculated with strains BR 3267 (*Bradyrhizobium yuanmingense*) or BR 3262 (*Bradyrhizobium pachyrhizi*) (Leite et al. 2018) recommended for cowpea (Brasil 2011), and two uninoculated controls, one without nitrogen and one with mineral N (52.5 $mg{\cdot}L^{-1}$), totaling 513 experimental units. Both positive control strains are currently recommended for commercial rhizobial inoculant production, and according to Brazilian regulations, the usage of two or more strains for a given legume is recommended to increase inoculant effectiveness. After 35 days of inoculation, the plants were collected, the presence or absence of nodules was observed, and the rhizobia community density was estimated by the most probable number (Woomer et al. 1994).

Later, nodules were detached, counted, and stored in silica gel tubes, with 20 nodules from each sample randomly selected for isolation. The nodules were superficially disinfected in 70% ethyl alcohol for 30 s, and subsequently in 2.5% sodium hypochlorite for 5 min, followed by 10 washes in sterile distilled water (Vincent 1970). Nodules were macerated in Petri dishes with yeast-mannitol-agar (YMA) culture medium (Vincent 1970), with Congo red (0.25% in 0.2 N KOH). The plates were incubated at room temperature, and, after bacterial growth, successive streaking was carried out until obtaining pure colonies.

Pure isolates were phenotypically characterized based on development of colonies (fast: up to three days; intermediate: four or five days; slow: six to 10 days; and very slow: more than 10 days); media pH change (acid, neutral, and alkaline); mucus amount (little, moderate or abundant); size (< 1, 1 to 2 and > 2 mm) and color of the colonies (yellow, white, pink, and cream). A binary matrix was created from the cultural characteristics, and the isolates were grouped by the unweighted pair group analysis (UPGMA) method using PAST 3.18 (Hammer et al. 2001). For the diversity estimate, Shannon, Weaver diversity index (H), Simpson dominance index, and the Pielou equitability index (J) were also calculated using PAST 3.18 (Hammer et al. 2001).

Strain authentication and symbiotic efficiency

A randomly selected representative from each of the phenotypical groups at 100% similarity was used for authentication under greenhouse conditions, as well as the same controls of the first phase, although the non-inoculated control with mineral nitrogen received the equivalent to 50 $kg{\cdot}ha^{-1}$ N. The experiment consisted of 73 treatments in a completely randomized block design in triplicate, totaling 219 experimental units.

The planting was carried out in 200-mL disposable cups filled with a sand: vermiculite 2:1 (v:v) mix autoclaved. Three IPA-26 cultivar cowpea seeds were sown per cup, and later the plants were thinned to one plant per cup. The bacteria were grown under orbital shaking at 150 rpm for three days for fast growing and five days for slow growing strains. Inoculation was three days after planting using 2 mL of YMA medium per seed. Every three days, 30 mL of N-free Hoagland nutrient solution was applied. Irrigation was performed with sterile distilled water whenever necessary.

The plants were harvested 35 days after inoculation, and shoot dry mass (SDM), root dry mass (RDM), nodules number (NN) and nodule dry mass (NDM) were determined. Relative efficiency (RE) was calculated by shoot dry mass from inoculated treatments and shoot dry mass of the N-supplied control ratio. The mean values were organized by the Scott-Knott test at 5% probability, using the Sisvar 5.6 statistical program (Ferreira 2011).

Genetic diversity of the 16S rRNA gene

From the authentication experiment, the isolates with relative efficiency not different or higher than the recommended strains were selected for characterization of genetic diversity by total bacteria gene sequencing.

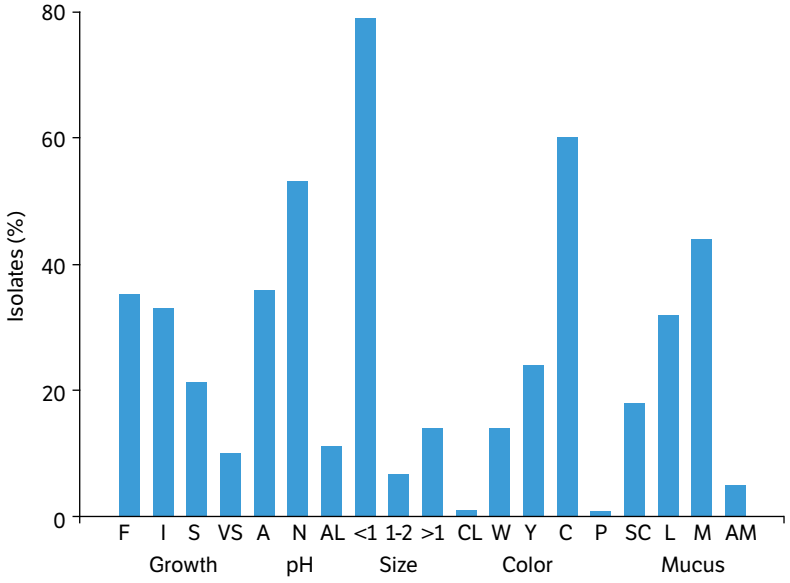
During the extraction of the genomic bacterial DNA (bead beating method), a little part of the bacterial colony was introduced in TE buffer solution, and cell lysis was carried out in 10% sodium dodecyl sulfate solution (SDS). The compound was stirred with phenol:chloroform and centrifuged at 12,000 rpm to obtain a supernatant, which was also centrifuged in the presence of isopropanol. The pellet formed was washed with ethanol, centrifuged, and then eluted in 50 µL of sterile water and stored at -20°C. The DNA obtained was visualized on a 1% electrophoresis agarose gel (Araújo et al. 2020).

The primers used during the amplification of the 16S rRNA gene were 27F (5'-AGAGTTTGACCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane 1991). Amplification was performed in a solution containing 2 µL of DNA, 2.5 µL buffer of polymerase chain reaction (PCR) 10x, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 0.4 mM of each primer, 1 U of Taq DNA polymerase and ultrapure water to a final volume of 50 µL. The entire amplification process was as follows: denaturation at 94°C for 5 min, 35 cycles of denaturation (94°C for 40 s), annealing (55°C for 40 s), extension (72°C for 1.5 min), and a final extension of 72°C, for 7 min. Then, the PCR products were sent to the Macrogen Laboratory in Korea. The 16S rRNA sequences identified were compared to those obtained within the EzBioCloud 16S-based ID (Yoon et al. 2017). A Neighbor-Joining phylogenetic tree was constructed using the Kimura 2-parameter method to compare the obtained 16S rRNA sequences with those from type strains strived from the EzBioCloud service (https://www.ezbiocloud.net/identify), by the MEGA 11 program (Tamura et al. 2021), applying a bootstrap with a minimum of 1,000 replications.

RESULTS AND DISCUSSION

Rhizobial communities' density ranged from 5.7×10^4 to 1.5×10^6 cell·g soil^{-1} without significant effect of intercropping or distance from the legume row.

We isolated 431 isolates, of which 35% showed rapid growth, 33% intermediate, 21% slow and 10% very slow; while 11% of the isolates alkalized the culture medium, 53% did not change the pH and 36% acidified the medium. Regarding colony color, cream (60%), yellow (24%), white (14%), pink (1%) and colorless (0.9%) bacteria were obtained. There was a predominance of bacteria capable of producing little to moderate mucus (44 and 32%) (Fig. 1). There was no significant effect of the silvopastoral system on the diversity (mean H = 2.3), dominance (mean D = 0.8) or equitability (mean J = 0.9) of the rhizobia.



YMA: yeast-mannitol-agar; F: fast growth; I: intermediate growth; S: slow growth; VS: very slow growth; A: medium acidification; N: none pH changed; AL: medium alkalization; CL: colorless; W: white; Y: yellow; C: cream; P: pink; SC: scare production mucus; L: low mucus production; M: moderate mucus production; AM: abundant mucus production.

Figure 1. Morphophysiological features of bacteria isolated in YMA culture medium under silvopastoral system soils in Itambé, Pernambuco, Brazil.

It was expected that the silvopastoral systems, based on shrub-tree legumes, would increase the density and diversity of rhizobia in relation to single signalgrass. In addition, it was expected that rhizobia diversity would be greater on the legume range soils (0 m) and it would decrease at an 8-m distance from the legume range. However, no significant differences were observed in the density and diversity of rhizobia, whose values were similar to those found in other studies (Lima et al. 2009; Castro et al. 2017).

It is known that rhizobia in the soil respond quickly to changes in land use (Ormeño-Orrillo et al. 2012). Hence, we assumed that five years of system implementation were sufficient to influence the soil rhizobia community. In addition, other research in the same experimental field and sampling year had already demonstrated that there was a significant increase in the abundance, spatial and temporal heterogeneity of diazotrophic microorganisms and ammonium-oxidizing bacteria (Barros et al. 2018; 2021), in the chemical attributes of the soil (Lima et al. 2018), in soil C and N stocks (Lira Junior et al. 2020b) and in soil organic matter quality (Lira Junior et al. 2020a).

One possible reason for the lack of response observed for rhizobial diversity is the choice of cowpea as the trap plant, since it is able to nodulate with background rhizobial populations predating systems, and thus not differentiate the legume species. We must also consider that both studies of Barros et al. (2018) and Barros et al. (2021) were based on culture independent methods, which tend to be more sensitive to environmental effects, though not feasible when evaluation of the rhizobial strains as to their efficiency is desired.

Isolates were grouped into 69 phenotypical groups at 100% similarity. Among the 69 isolates evaluated in the authentication and in the symbiotic efficiency experiments, 60 produced nodules and fixed nitrogen in cowpea (Table 2), confirming cowpea's promiscuity (Guimarães et al. 2012; Lira Junior et al. 2015) and its efficiency as a trap plant for rhizobia capture. Cowpea is a promiscuous grain legume that is normally nodulated by *Bradyrhizobium*, which exhibits slow growing (Zhang et al. 2011). It is known that sabia is preferentially associated with *Burkholderia* (Martins, P. G. S. et al. 2015), a betaproteobacteria, and it is possible that cowpea was not effective in differentiating the silvopastoral system from single signalgrass due to its preference for association with *Bradyrhizobium*, an alphaproteobacteria. A possible alternative would be the use of other legumes as trap plants, such as sabia or gliricidia.

Table 2. Nodule number (NN), nodule dry mass (NDM), root dry mass (RDM), shoot dry mass (SDM), and relative efficiency (RE) of cowpea inoculated with rhizobia isolated from soils under silvopastoral system in Itambé, Pernambuco, Brazil.

Strain	Origin of isolates	NN	NDM (mg)	RDM (g)	SDM (g)	RE (%)
43 R1.1	B + G	86.00 a	70 a	0.24 b	1.20 a	338.31 a
35 R3.3	B + G	66.66 a	70 a	0.33 a	1.06 a	297.19 a
42 R2.1	B + G	64.66 a	280 a	0.37 a	1.04 a	291.59 a
44 R1.1	B + G	62.00 a	60 a	0.30 a	1.01 a	284.11 a
51 R2.2	B + G	49.00 a	50 a	0.16 b	0.96 a	271.03 a
57 R3.1	B + G	61.33 a	70 a	0.22 b	0.94 a	263.55 a
65A R1.1	B + G	78.33 a	70 a	0.42 a	0.91 a	257.01 a
1 R3.2	B + G	50.00 a	50 a	0.22 b	0.91 a	256.07 a
35 R3.1	B + G	52.66 a	50 a	0.27 a	0.86 a	241.12 a
17A R2.2	B + G	66.00 a	50 a	0.23 b	0.86 a	242.99 a
11 R3.2	B + G	56.33 a	70 a	0.28 a	0.86 a	242.05 a
58 R3.2	B + G	62.66 a	90 a	0.26 a	0.82 a	230.84 a
36 R1	B + G	54.00 a	80 a	0.25 b	0.81 a	227.10 a
10 R2.1	B + G	35.66 a	70 a	0.19 b	0.77 a	216.82 a
2B R2.2	B + G	39.66 a	40 a	0.14 b	0.55 b	211.33 a
37 R1.1	B + G	53.33 a	50 a	0.35 a	0.70 a	197.19 a
12 R2.1	B + G	31.00 b	30 a	0.18 b	0.69 a	194.39 a
33 R2.2	B + G	25.00 b	40 a	0.30 a	0.63 b	178.50 b
44A R1.1	B + G	49.33 a	40 a	0.13 b	0.60 b	170.09 b
18 R3.1	B + G	29.33 b	50 a	0.18 b	0.60 b	168.22 b
11 R2.1	B + G	0.00 c	0.00 c	0.27 a	0.48 b	135.51 b

continue...

Table 2. Continuation...

Strain	Origin of isolates	NN	NDM (mg)	RDM (g)	SDM (g)	RE (%)
25C R1	B + G	0.00 c	0.00 c	0.30 a	0.46 b	128.97 c
17A R2.1	B + G	0.00 c	0.00 c	0.19 b	0.44 b	125.23 c
49 R3.6	B + G	17.00 b	30 a	0.18 b	0.42 c	119.62 c
51 R2 1	B + G	16.00 b	20 a	0.33 a	0.41 c	113.08 c
65B R1.1	B + G	0.00 c	0.00 c	0.31 a	0.36 c	100.93 c
49B R1.1	B + G	0.00 c	0.00 c	0.33 a	0.27 c	77.57 c
49 R2.2	B + G	0.00 c	0.00 c	0.22 b	0.19 c	55.13 c
97A R2.1	B + S	57.33 a	70 a	0.30 a	1.15 a	323.36 a
114C R1	B + S	55.33 a	70 a	0.29 a	1.07 a	300 a
83 R1.1	B + S	58.66 a	70 a	0.32 a	1.06 a	299.06 a
113 R1.1	B + S	62.66 a	80 a	0.34 a	1.01 a	283.18 a
90A R1	B + S	63.66 a	60 a	0.19 b	1.00 a	280.37 a
91B R1.2	B + S	43.33 a	70 a	0.29 a	0.98 a	274.76 a
81A R2.2	B + S	63.00 a	80 a	0.29 a	0.95 a	267.29 a
124 R2.1	B + S	58.66 a	60 a	0.22 b	0.90 a	253.27 a
89 R3.1	B + S	55.33 a	60 a	0.27 a	0.89 a	251.40 a
115 R3.1	B + S	57.33 a	60 a	0.18 b	0.89 a	251.40 a
113 R2.2	B + S	45.66 a	70 a	0.22 b	0.89 a	251.40 a
138A R1.2	B + S	50.33 a	50 a	0.31 a	0.89 a	249.53 a
141 R3.2	B + S	51.66 a	60 a	0.23 b	0.89 a	249.53 a
90B R1	B + S	62.33 a	50 a	0.37 a	0.88 a	246.72 a
97 R3.3	B + S	51.33 a	50 a	0.22 b	0.86 a	242.05 a
138A R3.1	B + S	56.00 a	60 a	0.28 a	0.85 a	239.25 a
130 R3.1	B + S	46.33 a	70 a	0.32 a	0.84 a	236.45 a
75 R2.2	B + S	61.33 a	40 a	0.26 a	0.80 a	224.30 a
108 R3.4	B + S	41.66 a	40 a	0.28 a	0.64 b	180.37 b
107D R2.1	B + S	46.00 a	50 a	0.21 b	0.64 b	180.37 b
107B R2.2	B + S	21.66 b	50 a	0.19 b	0.60 b	169.16 b
105 R1.1	B + S	15.33 b	40 a	0.22 b	0.55 b	156.07 b
89A R1.2	B + S	30.33 b	30 a	0.15 b	0.53 b	149.53 b
122 R3.7	B + S	11.66 b	60 a	0.24 b	0.51 b	143.92 b
138B R1.2	B + S	0.00 c	0.00 c	0.30 a	0.45 b	126.16 c
92B R2.1	B + S	0.40 c	0.00 c	0.00 a	0.23 c	115.23 c
74A R1.2	B + S	0.00 c	0.00 c	0.16 b	0.37 c	104.67 c
75 R3.2	B + S	0.00 c	0.00 c	0.34 a	0.34 c	96.26 c
130 R2.2	B + S	0.00 c	0.00 c	0.35 a	0.33 c	92.52 c
92A R2.2	B + S	0.00 c	0.00 c	0.31 a	0.32 c	89.71 c
91A R1.2	B + S	12.50 b	40 a	0.26 a	0.46 b	86.91 c
113 R1.2	B + S	20.33 b	20 a	0.32 a	0.26 c	72.89 c
145 R3.1	B	48.00 a	50 a	0.33 a	1.08 a	303.73 a
162 R3.2	B	64.66 a	60 a	0.31 a	0.97 a	271.96 a
154 R2.2	B	66.00 a	70 a	0.30 a	0.90 a	253.27 a
155 R1.1	B	75.00 a	80 a	0.30 a	0.94 a	264.48 a
147 R3.6	B	64.33 a	80 a	0.27 a	0.93 a	262.61 a
148 R1	B	61.66 a	60 a	0.24 b	0.81 a	228.03 a
147 R3.3	B	54.00 a	50 a	0.29 a	1.06 a	199.06 a
155A R2.1	B	24.33 b	40 a	0.21 b	0.44 b	125.23 c
146 R2	B	14.66 b	0.00 c	0.17 b	0.31 c	86.91 c
BR3262		50.66 a	90 a	0.30 a	1.03 a	290.65 a
BR3267		42.33 a	60 a	0.20 b	0.79 a	221.49 a
TA		0.00 c	0.00 c	0.12 b	0.22 c	61.68 c
TN		0.00 c	0.00 c	0.34 a	0.35 c	100.00 c
Mean a		56.45	47.6	0.3	0.92	255.96
Mean b		20.72	-	0.19	0.53	163.17
Mean c		0.00	-	-	0.31	98.88
VC%		39.7	90.88	35.91	24.34	24.5

B + S: signalgrass intercropped with shrub-tree sabia; B + G: signalgrass intercropped with shrub-tree gliricidia; B: single signalgrass.

Isolates 43 R1.1 and 65A R1.1 had the highest NN (86 and 78 nodules·plant^{-1}) (Table 2), while NN of 45 isolates did not differ statistically ($p > 0.05$) from the recommended strains BR 3267 and BR 3262 (Table 2). No significant difference was observed for NDM (Table 2). Thirty-nine isolates presented RDM values similar to the reference BR 3262 and to the nitrogen control, with no statistical difference ($p > 0.05$) between them. The SDM of 41 isolates did not differ from the recommended strains (Table 2). RE of 42 isolates did not differ from reference bacteria, and, while the difference was not significant, isolates 43 R1.1, 97A R2.1, 145 R3.1, 114C R1, 83 R1.1 and 35 R3.3 showed higher RE's than BR 3262, indicating a higher efficiency for these isolates (Table 2).

The silvopastoral system soils were not managed with fertilization or tillage since their implementation, keeping only livestock, so this management system favored rhizobia community development (Pires et al. 2018), of which 70% of the isolates that nodulated in the cowpea authentication experiment had RE equal or higher than the recommended strains BR3262 and BR3267. Rocha et al. (2017) observed that the land use change to pasture stimulated the multiplication of rhizobia in cowpea plants, and that plants were benefited in growth, especially in soil previously cultivated with grass crops. Soils cultivated for long periods with grasses are also known to maintain a rich rhizobial community and to establish symbiosis with succeeding legumes, providing a resilience effect (Lima et al. 2009; Wakelin et al. 2018).

A total of 36 strains with relative efficiency at least equal to those recommended were sequenced, but strains 141 R3.2, 91B R1.2 and 113 R2.2 had low quality sequences and were not considered. The obtained sequences ranged from 850 to 1,303 bp and exhibited 83.22 to 99.91% of identity with sequences deposited on EzBioCloud (Table 3). The phylogenetic relationships among the sequences are shown in Fig. 2. Most of the sequenced strains are *Bradyrhizobium* (67%), as observed in other studies (Guimarães et al. 2012; Jaramillo et al. 2013; Ndungu et al. 2018), but 16S rRNA sequencing was not sufficient to provide a good species-level resolution, and sequencing of other housekeeping genes is usually recommended to identify *Bradyrhizobium* species (Costa et al. 2019).

Table 3. Identity of cowpea rhizobial isolates from silvopastoral system soil in Itambé, Pernambuco, Brazil, based on the most similar sequences in EzBioCloud.

Strain	Origin	Cultural characteristics[a]	RE%[b]	Nucleotide number	Top-hit type strain on EzBioCloud	Similarity (%)
					Species	
43 R1.1	B + G	S/AL/SC	338.31	1,191	*Bradyrhizobium elkanii* USDA 76	96.50
97A R2.1	B + S	I/A/M	323.36	1,172	*Bradyrhizobium elkanii* USDA 76	99.06
145 R3.1	B	S/AL/L	303.73	1,168	*Bradyrhizobium huanghuaihaiense* CGMCC 1.10948	99.91
114C R1	B + S	VS/AL/SC	300.00	874	*Methylobacterium radiotolerans* JCM 2831	99.77
83 R1.1	B + S	S/AL/L	299.06	1,171	*Bradyrhizobium elkanii* USDA 76	98.89
35 R3.3	B + G	F/A/L	297.19	1,181	*Bradyrhizobium elkanii* USDA 76	96.50
42 R2.1	B + G	I/N/L	291.59	1,251	*Cohnella xylanilytica* MX15-2	96.88
44 R1.1	B + G	S/N/SC	284.11	1,122	*Rhizobium altiplani* BR10423	98.31
113 R1.1	B + S	I/N/SC	283.18	1,173	*Bradyrhizobium elkanii* USDA 76	99.57
90A R1	B + S	S/A/L	280.37	1,035	*Leifsonia shinshuensis* JCM 10591	92.37
51 R2.2	B + G	I/A/M	271.03	1,167	*Bradyrhizobium elkanii* USDA 76	98.11
81A R2.2	B + G	F/AL/L	267.29	863	*Methylobacterium longum* 440	83.22
155 R1.1	B	VS/A/L	264.48	1,303	*Cohnella plantaginis* YN-83	93.92
57 R3.1	B + G	I/N/L	263.55	1,197	*Bradyrhizobium elkanii* USDA 76	96.24
147 R3.6	B	VS/AL/L	262.61	1,169	*Bradyrhizobium elkanii* USDA 76	98.89
65A R1.1	B + G	S/N/SC	257.01	1,188	*Bradyrhizobium elkanii* USDA 76	98.61
1 R3.2	B + G	I/AL/M	256.07	1,170	*Bradyrhizobium elkanii* USDA 76	99.83
154 R2.2	B	F/A/SC	253.27	1,079	*Leifsonia shinshuensis* JCM 10591	98.61
124 R2.1	B + S	VS/N/SC	253.27	1,173	*Bradyrhizobium elkanii* USDA 76	96.92
115 R3.1	B + S	S/N/M	251.40	1,171	*Bradyrhizobium elkanii* USDA 76	99.66
89 R3.1	B + S	F/AL/SC	251.40	850	*Methylobacterium radiotolerans* JCM 2831	94.35
138A R1.2	B + S	VS/N/M	249.53	1,173	*Bradyrhizobium elkanii* USDA 76	99.14
90B R1.1	B + S	VS/N/M	246.72	1,170	*Bradyrhizobium elkanii* USDA 76	99.40
17A R2.2	B + G	F/N/M	242.99	1,113	*Burkholderia cepacia* ATCC 25416	99.91

continue...

Table 3. Continuation...

Strain	Origin	Cultural characteristics[a]	RE%[b]	Nucleotide number	Top-hit type strain on EzBioCloud	Similarity (%)
					Species	
11 R3.2	B + G	S/AL/L	242.05	1,172	*Bradyrhizobium elkanii* USDA 76	99.06
35 R3.1	B + G	I/AL/SC	241.12	1,171	*Bradyrhizobium elkanii* USDA 76	98.89
130 R3.1	B + S	I/N/M	236.45	1,173	*Bradyrhizobium elkanii* USDA 76	99.57
58 R3.2	B + G	S/N/L	230.84	1,179	*Bradyrhizobium elkanii* USDA 76	97.61
148 R1	B	VS/N/SC	228.03	1,170	*Bradyrhizobium elkanii* USDA 76	99.74
36 R1	B + G	S/A/SC	227.10	1,085	*Leifsonia shinshuensis* JCM 10591	98.68
75 R2.2	B + S	F/A/M	224.30	1,245	*Paenibacillus cineris* LMG 18439	98.14
147 R3.3	B	S/AL/L	199.06	1,194	*Bradyrhizobium elkanii* USDA 76	96.93
162 R3.2	B	I/N/M	271.96	1,171	*Bradyrhizobium elkanii* USDA 76	99.57

[a]F: fast growth; S: slow growth; I: intermediate growth; VS: very slow growth; AL: medium alkalination; A: medium acidification; N: none pH changed; SC: scarce production mucus; M: moderate mucus production; L: low mucus production; RE: relative efficiency; B + S: signalgrass intercropped with shrub-tree sabia; B + G: signalgrass intercropped with shrub-tree gliricidia; B: single signalgrass.

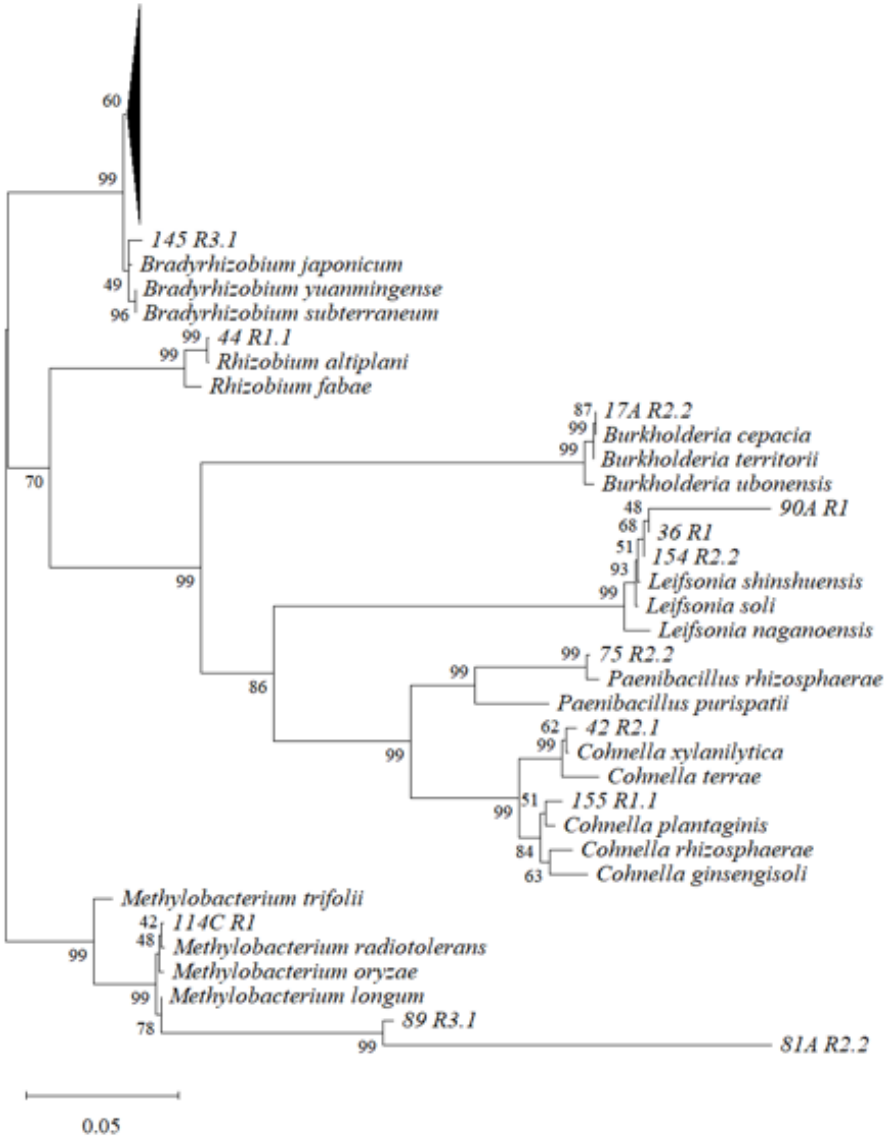


Figure 2. Neighbor-Joining phylogenetic tree of 16S rRNA sequences (950 nucleotides) of cowpea rhizobial isolates from silvopastoral system soil in Itambé, Pernambuco, Brazil. Bootstrap values (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method.

Besides *Bradyrhizobium* sp., strains were also identified as *Rhizobium* and *Burkholderia* genera, which have already been reported to nodulate cowpea in different soil types and management systems, such as São Francisco Valley soils (Leite et al. 2009), Amazonian soils under different land use systems (Guimarães et al. 2012), and rehabilitated soils revegetated with grasses (Castro et al. 2017), confirming the wide symbiotic compatibility spectrum of cowpea.

Three strains were identified as *Methylobacterium*, and this genus belongs to Methylobacteriaceae, a large *Rhizobiales* family. *Methylobacterium nodulans*, unlike other members of the genus, can induce nodule formation and fix atmospheric nitrogen in *Crotalaria* and *Lotononis* (Green and Ardley 2018). Previously, Leite et al. (2009) found only one isolate in cowpea in soils of the São Francisco River Valley that had low similarity (36%) with strain BR 2006 of *Methylobacterium nodulans*.

Other genera identified were *Leifsonia*, *Cohnella* and *Paenibacillus* (Table 3). These bacteria are components of the nodule microbiome and can penetrate nodules through the infection cord, and the function of these bacteria is not fully known yet. Some non-rhizobial endophytic bacteria include plant growth promoting rhizobacteria, which can produce indole acetic acid, solubilize phosphate, siderophores producing, and it may exhibit pathogen antagonist activity (Velázquez et al. 2017). The non-rhizobial endophytic bacteria identified in the present work should be characterized as to possible plant growth promotion mechanisms, because they can be used for new inoculants development.

CONCLUSION

Legume-based silvopastoral systems did not influence cowpea rhizobial population density and diversity, after five years of system implementation.

Silvopastoral system soils harbor populations of rhizobia efficient in biological nitrogen fixation in cowpea, which can be recommended for agronomic efficiency assays.

Cowpea nodulated with a high diversity of nitrogen-fixing bacterial genotypes obtained from silvopastoral systems soils. Most of the identified genotypes belong to different species of *Bradyrhizobium*.

This is the first report of *Methylobacterium* strains with high similarity to *M. radiotolerans* and *M. longum* nodulating cowpea in Brazil.

AUTHORS' CONTRIBUTION

Conceptualization: Santos, A. B., Fracetto, G. G. M. and Lira Junior, M. A.; **Methodology:** Santos, A. B., Fracetto, G. G. M., Fracetto, F. J. C. and Lira Junior, M. A.; **Investigation:** Santos, A. B., Fracetto, G. G. M., Fracetto, F. J. C. and Lira Junior, M. A.; **Writing:** Santos, A. B., Fracetto, G. G. M., Fracetto, F. J. C. and Lira Junior, M. A.; **Funding Acquisition:** Lira Junior, M. A.; **Supervision:** Fracetto, G. G. M. and Lira Junior, M. A..

DATA AVAILABILITY STATEMENT

All dataset were generated and analyzed in the current study.

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