Vol. 19(4), pp. 444-454, April, 2023
DOI: 10.5897/AJAR2023.16312
Article Number: AC9B92670633
ISSN: 1991-637X

http://www.academicjournals.org/AJAR

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African Journal of Agricultural Research

Full Length Research Paper

Efficiency and diversity of nitrogen fixing bacteria colonizing *Macroptilium lathyroides* (L.) Urb

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Received 6 January, 2023; Accepted 29 March, 2023

The objective of the present work was to study the efficiency and diversity of nitrogen-fixing bacteria that colonize the weed *Macroptilium lathyroides* (L.) Urb. M. lathyroides roots were collected in areas of natural infestation of the species in rural and periurban areas of the municipality of Alta Floresta-MT, Brazil. The roots that presented nodulation were washed and their nodules removed, disinfected and inoculated in culture medium 79 in Petri dishes. These were incubated in a germination chamber in the dark at 28°C for 10 days, after which time the colonies were purified by streaking. The authentication experiment was conducted in a greenhouse and showed that of the 39 isolates, 19 of them had potential to be conducted for the nitrogen fixation efficiency test. The sequencing of the 16S rDNA gene identified the following bacteria that colonize the roots of the species: *Mucilaginibacter gossypiicola*, *Novosphingobium arabidopsis*, *Bacillus haynesii*, *Bacillus xiamenensis*, *Bradyrhizobium namibiense* and *Flavobacterium anhuiense*. Isolates M004, M005 and M022 showed values of nitrogen content very close to the 'with N' treatment, being indicated for further studies with commercially important cultures.

Key words: Diazotrophic bacteria, isolates, nodulation, rice bean.

INTRODUCTION

The genus *Macroptilium*, from the Fabaceae family, has approximately 20 species in the Americas (Sousa et al., 2013).

M. lathyroides belongs to the same family of sirato (*Macroptilium atropurpureum*) and is used as forage in Brazil and in several regions of South America (Monks et al., 2006; Guerra et al., 2007; Vasconcelos et al., 2011). As a typical characteristic related to its genus (Leguminosa) and easily visually verified, when uprooting plants, the presence of nodules, reflects the possibility of

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this species being used as a possible nitrogen fixing plant, besides its current use as a forage, because they are grown for hay or silage production or even as a protein bank (Albuquerque, 2013).

However, the species has become a potentially weed plant in areas with corn and soybean cultivation and in renewal and pasture sites (Concenço et al., 2012). Thus, research to obtain information about its biology (Silva et al., 2018) and practices that allow its controlled use is deserving attention, since it can apparently fix nitrogen.

Biological nitrogen fixation is performed by several bacterial phylogenetic groups, called diazotrophic. These bacteria can live free in many different ecosystems, establish symbiosis or be associated with plants and be called associative (Moreira et al., 2010).

Rhizobium inoculation can replace the use of various chemical nitrogen fertilizers, such as soybean in Brazil, inoculated with *Bradyrhizobium* (Moreira et al., 2010). The use of inoculants from the 1960s onwards ensured international competitiveness for soybean cultivation, reflecting directly on the country's trade balance (Embrapa, 2016).

Due to their ability to fix nitrogen through symbiotic association with rhizobia, legumes are widely used as green manures and their importance is increasing, since their residues promote the gradual release of nitrogen, being absorbed by subsequent or associated crops. These have specific nutrient absorption, produce large amounts of green and dry phytomass and, due to their deep and well branched root system, allow for greater extraction and recycling of nutrients.

Thus, the use of species with this symbiotic capacity, even seen as weeds, such as M. lathyroides, may have potential for use as green manure in certain situations. And, in this way, even though they are seen as weeds, harmful, they can be used for benefits in soil quality. Grown in a manner and time appropriate to cropping systems, they would be a promising alternative from a technical and economic perspective (Amabile and Carvalho, 2006).

Therefore, the objective of the present work was to study the efficiency and diversity of nitrogen-fixing bacteria that colonize the leguminous species *Macroptilium lathyroides* (L.) Urb.

MATERIALS AND METHODS

The roots of *M. lathyroides* were collected in areas of natural infestation in the rural and periurban area of Alta Floresta - MT, at latitude 9°52'32"S, longitude 56°05'10" W and altitude 288 m (Inmet, 2018). The soil that was used for the study had a sandy-clay texture, had a pH of 5.6, base saturation of 70%, phosphorus of 9.7 mg/dm3, potassium of 222 mg/dm3, and absence of aluminum.

The root collection procedure was performed according to the methodology proposed by Hungria (1994). Random samples were collected from 20 plants. The roots were removed from the soil to keep the root system as intact as possible. These were packed in plastic bags, kept in a Styrofoam box and immediately taken to the laboratory.

Grouping of isolates by morphological similarity

After the cultural characterization of the isolates, with the data obtained with the morphology of bacterial colonies, a Euclidean distance similarity dendrogram and a biplot were constructed; this was used to group the isolates by morphological similarity, allowing verifying groups of similar individuals. The dendrogram was made with the aid of Statistica software (Statsoft, Inc., 2011).

Isolation of bacterial strains

The collected roots were washed and those with nodulation were wrapped in paper towels to remove excess water and, with the help of pruning shears, were fragmented into smaller particles to facilitate the removal of the inoculum from inside the nodule. Five active nodules were removed per root system.

For disinfection and purification of nodules, the methodology proposed by Hungria (1994) was adopted. The nodules were macerated and subsequently inoculated in culture medium 79 in Petri dishes (Vicent, 1970).

The plates were incubated in a germination chamber (BOD) in the dark at 28 °C for 10 days. Cultures were purified by the stretch mark depletion method as described by Tortora et al. (2012).

Isolation authentication

Nodulation capacity was verified following the methodology proposed by Everitt (1993). The experiment for this authentication was conducted in a greenhouse.

The seeds of *M. lathyroides* were subjected to dormancy break (Almeida et al., 1979) and then disinfected. Three seeds of the species were sown in an experimental unit represented by 355 cm3 tubes filled with sand and vermiculite in a 2: 1 ratio, previously sterilized. After emergence, thinning was performed maintaining only one plant per tube (this was chosen, seeking to maintain uniformity of size among all plants of the other experimental units).

The plants were irrigated with autoclaved nutrient solution according to Sarruge (1975) under two conditions: one with nitrogen (45%) and one with 10x less nitrogen.

From sowing up to 15 days of emergence, the plants were irrigated with a solution at 25% of the nutrient concentration and, after this date, until the experiment collection, a 50% of the solution concentration was used.

Each plant was inoculated with 1.0 mL of inoculum. The inoculum preparation consisted of the growth of the bacterial strains in liquid medium®79 under stirring at 150 rpm until the log phase of growth (Hungria, 1994).

The experiment was organized in a completely randomized design with 3 replications, consisting of 41 treatments, 39 bacterial strains, irrigated with nutrient solution containing 10 X less nitrogen than recommended, and two treatments without inoculation [one with nitrogen in concentrations recommended for the soybean crop (Embrapa, 2016) and another with 10 X less nitrogen than that recommendation].

Plant collection was performed 47 days after sowing, separating shoots, roots and nodules. The different parts of the plant were dried in a forced air circulation oven at 65 ° C for 72 h and then weighed. Relative efficiency (ER) was determined, which corresponds to the ratio between inoculated treatment yield and percentage nitrogen treatment of strains by the equation:

$$ER = \left(\frac{MSPA\ inoculated\ treatment}{MSPA\ treatment\ with\ N}\ x\ 100\right).$$

ER - Relative efficiency;

MSPA inoculated treatment - shoot dry mass of the inoculated treatment with the bacterial strain.

MSPA treatment with N - dry mass of the aerial part of the treatment without inoculation and with nitrogen.

The shoot dry mass (MSPA), root dry mass (MSR) and total dry mass data were transformed to Log (x) for data normality adjustment. Nodule dry mass (MSN) was transformed into Log (x + 10) and shoot and root ratio (Relation) into Log (x) + 0.5. Data were subjected to analysis of variance and, when significant, were compared by Scott-Knott test at 5% probability, with the aid of Sisvar 4.6 software (Ferreira, 2014).

Molecular identification of bacterial isolates

The bacteria selected through the authentication test, isolated M002, M004, M005, M014, M016, M019, M022, M024, M028, M033, M035 and M036, were subjected to molecular identification using partial 16S region sequencing. For molecular sequencing, bacterial colonies were transferred to 1.5 mL microtubes containing 200µL of autoclaved water, according to Ferreira (2008) protocol. This solution was used as a DNA source for PCR. The primers used were 1378R (5'CGGTGTGTACAAGGCCCGGGAACG-3') and PO27F (5'GAGAGTTTGATCCTGGCTCAG-3').

Reactions were performed containing 1µL of bacterial suspension, 39.30µL of autoclaved Milli-Q water, 10x buffer, 0.5µM of each deoxyribonucleotide triphosphate, 0.2 µL of each primer and 2.5 U of Taq DNA polymerase (SIGMA). ®).

A negative control, PCR reaction without bacterial material, was included in all reactions. Polymerase Chain Reaction - PCR - was performed in a T100 Thermal Cycler thermal cycler (BIO-RAD) programmed to perform initial denaturation at 94 ° C for 4 min, 35 cycles of 94 ° C for 30 s, 62.5 °C for 1 min, 72 ° C for 1 min and final extension at 72 ° C for 7 min. Confirmation of target DNA fragment amplification was performed by 1.5% (w / v) agarose gel electrophoresis run for 1 h at 60 volts, staining (GELRED) with observation of the expected size fragment (1400 bp) under UV light.

The PCR products were purified with 100% ice-cold isopropanol and centrifugation at 9,000 rpm for 15 min after supernatant discard, 200µL 70% alcohol and centrifugation at 9,000 for 5 min, followed by supernatant discard and bath evaporation dried at 35 ° C. After evaporation, the samples were resuspended in 20µL of autoclaved Milli-Q water and incubated in a refrigerator for 24 h.

Again, the samples were submitted to 2.0% agarose gel electrophoresis with low molecular weight marker for DNA quantification. After quantification the samples were diluted to 50ng / µL and 9.5µL aliquots were sent for partial sequencing of the 16S rDNA gene. The sequences obtained were compared by BLASTn against NCBI database. Only nucleotides with sequencing quality value greater than or equal to 500 were considered. Clustal X and MEGA X software were used to make the phylogenetic tree (Thompson et al., 1997; Kumar et al., 2018).

Biological nitrogen fixation test (FBN)

For this experiment, isolates that tested positive for nodule formation and higher shoot dry matter yield in the authentication experiment were studied.

The experiment was carried out in plastic pots with a capacity of 1.7 liters, with soil collected in a forest area, in the rural area of Alta Floresta - MT, Brazil, free of contamination. Five seeds previously submitted to dormancy break were sown and disinfected in 2.5% hypochlorite solution for 5 min. The inoculum consisted of LM broth (Vicent, 1970), with cell number determined and expressed in mL-1. The fertilization was performed according to chemical analysis of the soil, following the recommendation of Ribeiro et al. (1999), for soybean cultivation. The chemical characteristics of the soil, in the 0-0.20 m profile, are: pH (H_2O) of 5.6, P and K of 9.7 and 2220 mg dm^{-3} and Ca, Mg, Al and H contents 4.7; 1.1; 0.0; 1.7 $cmol_c$ dm^{-3} and organic matter of 26 g dm^{-3}.

The treatments consisted of 19 isolates, in addition to one treatment without nitrogen addition and another with the addition of N, according to soil analysis, totaling 21 treatments and a completely randomized experimental design with 4 replications. The experiment was conducted for 45 days.

At harvest, the aerial part of the plant was separated from the root system, placed in paper bags and oven-dried at 65 °C with forced ventilation for three days. The evaluations were: root length, root volume, root dry mass, shoot length, shoot dry mass, total dry mass, shoot and root ratio, number of nodules, nodule dry mass, green index, and nitrogen content according to the methodology described in Tedesco et al. (1995). Data were submitted to the Scott-Knott test at 5% probability using the Sisvar 4.6 software (Ferreira, 2014).

The factors root volume, number of nodules, shoot dry mass, nodule dry mass, total dry mass and nitrogen content were transformed into log (x + 2) of for data normality adjustment.

RESULTS AND DISCUSSION

Grouping of isolates

According to the similarity dendrogram, it is possible to observe the existence of 3 distinct groups of bacteria, formed according to the morphological characteristics at 15% similarity level for *Macroptilium lathyroides* isolates (Figure 1). From the obtained groups, it can be observed that group III had a large amount of isolates that is 20 in total (Figure 1).

Table 1 presents the results of the principal component analysis, with the eigenvalues and the correlation coefficients for the verification of the formation of the groups observed in the hierarchical cluster analysis. Results indicate that approximately 28% of total variability was explained by the first major component (CP1). In turn, the second main component (CP2) explained approximately 23% of the variability, with an accumulated value of approximately 51% of the variability contained in the original data. The third major component (CP3) explained approximately 11% of the total data variability.

Thus, the sum of the three main components accounts for approximately 62% of the variability contained in the original data set. The amount of variation was sufficient for the generation of three clusters, observed in the hierarchical clusters analysis.

The biplot graph allowed to verify the correlation of the variables with the main components (Figure 2), indicating the formation of the clusters. The discriminatory power of variables within a main component was measured by the linear correlation coefficients between each variable and its main component.

It can be inferred that for the main component CP1 and in order of importance, the attributes with the highest correlation coefficients were: pH (-0.84), mucus (0.81), growth time (-0.79) and color (0.52), approximate values

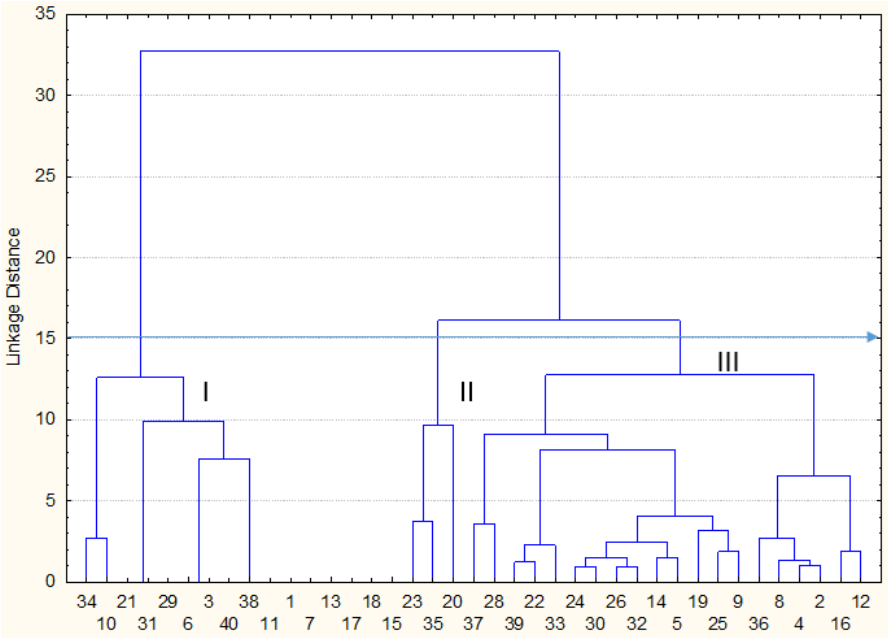

Figure 1. Dendrogram of rhizobium isolates obtained from Macroptilium lathyroides roots, by phenotypic characterization and formation of groups with similar characteristics, collected in Alta Floresta - MT.
Source: Authors

Table 1. Eigenvalues, amount of variation explained, correlation coefficients and eigenvectors among rhizobia isolates of *Macroptilium lathyroides* plants.

Components	**PC1**	**PC2**	**PC3**
Eigen value	3.103002	2.536786	1.246715
Cumulative	3.10300	5.63979	6.88650
% Total	28.20911	23.06169	11.33377
Cumulative	28.2091	51.2708	62.6046
Factor coordinates of the variables, based on correlations (*Macroptilium lathyroides*)			
Growth	-0.789961	-0.139977	-0.291305
Diameter	-0.408323	-0.534475	-0.129201
Color	0.516073	0.696599	-0.205930
Elevation	0.333336	-0.733610	0.318261
Edge	-0.463656	0.422065	0.545418
Transparency	-0.109680	0.605652	0.282816
Surface	0.437892	-0.650006	-0.229536
Mucus	0.808475	0.008860	-0.092064
Consistency	-0.198772	0.446279	-0.659025
Elasticity	-0.331166	-0.077426	-0.348701
pH	-0.844526	-0.188135	0.083259

Source: Authors

(Table 1 and Figure 2).

These components are interpreted as the main morphological characteristics that led to the grouping of the isolates. In general, the isolates contained in this group I have yellow coloration, fast growth, and abundant mucus and acidified the culture medium. The genera *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*, have the ability to modify the pH of culture medium 79, making it

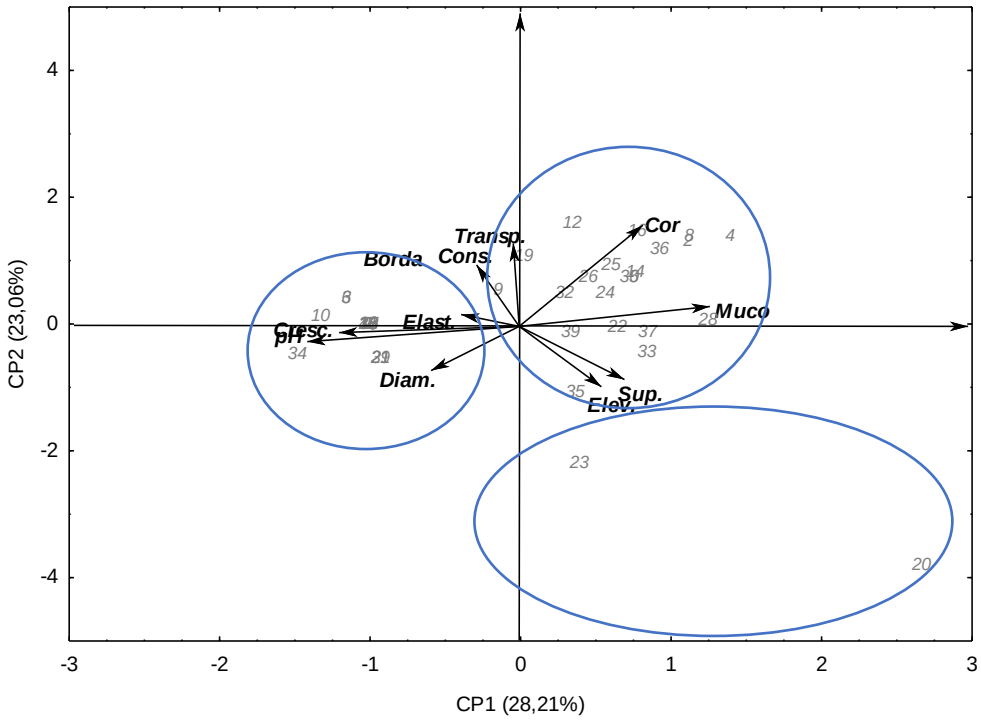

Figure 2. Biplot plot of principal components 1 and 2 of principal component analysis with morphological characteristics for growth, diameter, color, shape, edge, elevation, transparency, surface, mucus production, mucus consistency, elasticity, and alteration of pH of bacterial culture medium isolated from *Macroptilium lathyroides* roots.
Source: Authors

acidic (Coutinho et al., 2000). These data are indicative that group I may be composed of bacteria of the genus Rhizobium, as they were yellow in color, acidified the culture medium, had rapid growth and mucus production. These observed bacteria fit perfectly with what was suggested by Sprent (1994), the author reports that fast-growing bacteria, common in arid regions, are not good N2 fixators, because the priority of these organisms is survival, however, Martins et al. (1997) reports that there are several fast growing strains obtained in tropical regions, as is the case of the collection region of this study.

For the main component CP2, the indices with the highest correlation coefficients were: elevation (-0.73), color (0.70), surface (0.61), transparency (0.61) and diameter (0.53), approximate values (Table 1 and Figure 2), of these components the elevation of the bacterial colony was the main to group the isolates, the group II was composed by 3 isolates that presented the elevation type lens. Araújo and Gualter (2017) studying native bacteria from cerrado soils, reported having isolated 3 bacteria with lens-like colony elevation, the same number of isolates obtained in this research.

For the main component CP3, the indices with the highest correlation coefficients were: mucus consistency (-0.66) and edge (0.54) (Table 1 and Figure 2). These components are interpreted as the main morphological characteristics that led to the grouping of isolates. In general, the isolates contained in this group III are cream-to-white in color, and most had viscous mucus production and full border.

The genus *Bradyrhizobium* has the characteristic of having opaque, circular colonies, occasionally translucent or white, tending to alkalinize or maintain neutral pH (Bergey and Holt, 1994; Jordan, 1984; Araújo, 1994; Melloni et al., 2006). These data are indicative that the bacteria belonging to group III may fit into the genus Bradyrhizobium, as their morphological characteristics are similar.

Isolation authentication

According to Table 2, 19 isolates (M002, M004, M005, M008, M014, M016, M019, M022, M024, M025, M026, M028, M030, M032, M033, M036, M037 and M039) presented higher values, when compared to the other isolates, for the variables shoot dry mass (MSPA), nodule dry mass (MSN), total dry mass (MST) and shoot and root ratio (RELATION), without presenting significant difference with Nitrogen treatment.

Nineteen isolates showed a difference for the MSPA

Table 2. Shoot dry matter (MSPA), root dry matter (MSR), nodule dry matter (MSN), total dry matter (MST), shoot and root ratio (REL), of *Macroptilium lathyroides* plants inoculated with rhizobium isolates, values expressed in mg.

Isolate	MSPA	MSR	MSN	MST	REL
M001	146.13[b]	89.03[a]	4.13[b]	235.16[b]	1.67[a]
M002	215.86[a]	110.83[a]	16.40[a]	326.70[a]	1.89[a]
M003	110.03[b]	66.16[a]	3.56[b]	176.20[b]	1.64[a]
M004	270.36[a]	137.53[a]	10,10[a]	407.90[a]	1.97[a]
M005	264.50[a]	119.03[a]	11.50[a]	383.53[a]	2.25[a]
M006	68.06[b]	75.53[a]	0.50[b]	143.60[b]	0.89[b]
M007	64.76[b]	60.06[a]	8.90[a]	124.83[b]	1.08[b]
M008	267.36[a]	114.76[a]	11.06[a]	382.13[a]	2.30[a]
M009	124.90[b]	97.46[a]	3.76[b]	222.36[b]	1.27[b]
M010	177.46[b]	91.33[a]	7.36[a]	268.80[b]	1.72[b]
M011	68.20[b]	50.50[a]	0.86[b]	118.70[b]	1.43[b]
M012	163.20[b]	63.83[a]	8.30[a]	227.03[b]	3.86[a]
M013	106.80[b]	89.90[a]	4.83[b]	196.70[b]	1.14[b]
M014	213.63[a]	101.66[a]	8.00[a]	315.30[a]	2.24[a]
M015	101.40[b]	82.63[a]	2.10[b]	184.03[b]	1.21[b]
M016	193.26[a]	85.73[a]	7.40[a]	279.00[a]	2.37[a]
M017	163.23[b]	94.20[a]	4.13[b]	257.43[b]	1.67[a]
M018	99.53[b]	74.53[a]	5.73[b]	174.06[b]	1.33[a]
M019	244.96[a]	119.06[a]	11.23[a]	364.03[a]	2.05[a]
M020	141.80[b]	110.10[a]	6.93[b]	251.90[b]	1.14[b]
M021	73.50[b]	59.36[a]	3.60[b]	132.86[b]	1.25[b]
M022	270.73[a]	111.46[a]	12.76[a]	382.20[a]	2.29[a]
M023	132.00[b]	84.26[a]	5.00[b]	216.26[b]	1.64[a]
M024	269.10[a]	134.70[a]	14.6[a]	403.80[a]	2.01[a]
M025	255.56[a]	106.23[a]	6.30[a]	361.80[a]	2.42[a]
M026	240.13[a]	131.06[a]	13.60[a]	371.20[a]	1.79[a]
M028	239.56[a]	117.53[a]	8.23[a]	357.10[a]	2.13[a]
M029	136.43[b]	79.63[a]	1.86[b]	216.06[b]	1.54[b]
M030	209.33[a]	105.36[a]	10.00[a]	314.70[a]	1.97[a]
M031	90.30[b]	80.06[a]	2.23[b]	170.36[b]	1.12[b]
M032	192.20[a]	91.70[a]	7.50[a]	283.90[a]	2.20[a]
M033	274.33[a]	127.76[a]	11.36[a]	402.10[a]	2.14[a]
M034	130.06[b]	67.46[a]	1.10[b]	197.53[b]	1.85[a]
M035	265.06[a]	103.86[a]	14.26[a]	368.93[a]	2.46[a]
M036	285.13[a]	146.83[a]	13.00[a]	431.96[a]	1.96[a]
M037	246.30[a]	104.76[a]	8.23$_a$	351,06[a]	2.54[a]
M038	125.13[b]	74.56[a]	10.30[a]	199.70[b]	1.68[a]
M039	183.23[a]	88.46[a]	8.16[a]	271.70[a]	2.06[a]
M040	205.30[b]	106.46[a]	8.96[a]	311.76[b]	1.57[b]
Without N	99.23[b]	85.43[a]	0.00[b]	184.66[b]	1.15[b]
With N	256.23[a]	121.46[a]	0.00[b]	377.70[a]	2.22[a]
c.v. (%)	10.03	10.09	10.07	8.37	15.43

Averages followed by the same letter in the column do not differ by the Scott-Knott test at 5% probability.
Source: Authors

factor being higher than the other isolates and with the treatment without nitrogen supply (Table 2). These isolates were not different from nitrogen treatment. Vargas et al. (1990) and Vieira et al. (1994), working with

bean inoculated isolates also reported increase of MSPA in plants with N application. It is verified that these isolates have potential for nitrogen fixation, being indicated for efficiency testing in FBN. Antunes (2010) report similar results to this research, describing that of the isolates evaluated for MSPA, a large number of isolates were not different from the nitrogen treatment.

For the MSR factor, no difference was observed between the isolates and the treatments with and without nitrogen supply. Among the growth-stimulating effects that growth-promoting bacterial species can play in the relationships between rhizobia and legumin, one can cite the development of lateral roots and primary root elongation associated microbial species, reflecting directly on the amount of phythormonium available to the plant, affecting the promotion or inhibition of root growth (Patten and Glick, 1996). From this point of view, possibly the population level of bacteria in the rhizosphere of the studied plants was not sufficient to cause the greater root development of *M. lathyroides* plants. This result may be due to soil conditions that may not have affected this growth. Similarly, Antunes et al. (2011) and Souza (2015), studying rhizobia in fava beans and cowpea, respectively, found no difference for the MSR variable, corroborating the data presented here.

There are several mechanisms by which symbiont bacteria can alter root and nodule development via auxin synthesis by the microsymbiont or by altering the synthesis of this hormone in the host (Mathesius, 2008; Spaepen et al., 2007).

Auxin synthesis promotes the growth of these bacteria, such as cyanobacteria of the genus Frankia and Rhizobium itself, even in non-symbiont plants (Peret et al., 2007; Mathesius, 2008). In this way the exudation of compounds produced by plants can stimulate auxin synthesis in bacteria and thus they synthesize it through tryptophan exuded by plant roots (Kefford et al., 1960). However, in the present study, the nodule was possibly not efficient to assist in the development of MSR.

For MSN, most of the 19 isolates corresponded to the similarity in this variable, indicating a trend and relationship between root mass and nodule mass. Something already expected, since the volume of roots and consequent mass of these indicates the best development of this part of the plant, demonstrating that nodulation was efficient and used the larger volume of roots to increase the number of nodules and consequently greater mass produced.

Souza (2015) also reports values similar to those of this study for MSN. Fernandes et al. (2003) working with guandu beans, cowpea and pork beans, reported finding significant differences between rhizobial strains in their ability to promote vegetative growth (MSPA) and nodulation (MSN), as well as the results found here.

In rhizobial bacteria, auxin synthesis and alteration of the relationship between auxins and cytokines is directly related to the capacity of initiation and formation of nodules in the plant. Thus, the genetic mechanisms of auxin biosynthesis regulation by bacteria such as *Rhizobium*, *Bradyrhizobium* and *Azospirillum* are very similar and have functions that influence the physiology and development of the host plant (Costacurta and Vanderleyden, 1995).

Despite the predominance of fast-growing, yellow-colored isolates with acidification capacity of the culture medium, the most efficient isolates were white to cream, with intermediate to slow growth, with little mucus production and ability to alkalize the culture medium. These presented the highest averages for all analyzed variables, without presenting significant difference between them. Only one efficient isolate showed yellow coloration and fast growth, but capable of alkalizing the culture medium.

For the MST factor, 19 isolates did not differ significantly from the WITH N treatment (Table 2), but showed a significant difference from the WITHOUT N treatment, probably this result can be observed due to the lack of competitiveness with strains generally present in the soil, the fact that the substrate The autoclaving method used ensured the possibility of observing the effect of strains on plant development.

For the RELATIONSHIP factor, 27 isolates did not differ significantly from the WITH N treatment (Table 2), but differed from the WITHOUT N treatment, which shows that the isolates allowed the development of the shoot in relation to the root system in a similar way to the WITH N treatment. This means that the plant invested less in root development and more in leaf area showing greater nutritional balance compared to other isolates.

The relative efficiency (ER) of the isolates (Figure 3) was calculated and comparing with the WITH N treatment and without inoculation we found that 8 isolates obtained ER higher than the WITH N treatment (M004, M005, M008, M022, M024, M033, M035 and M036), demonstrating that the isolates had the capacity to supply higher amounts of nitrogen to the shoot than to the nitrogen treatment. Isolate M025 allowed growth similar to that observed with nitrogen supply.

Chagas Junior et al. (2010) obtained similar data working with cowpea isolates, the authors report obtaining isolates with high ER above treatment with N.

16S rDNA sequencing of *Macroptilium lathyroides* isolates

From the isolates selected for genetic identification by partial sequencing of the 16S rDNA gene, it was not possible to identify three of them (M014, M019 and M033). These samples did not show similarity, possibly due to the amount of base pairs contained in them and which were not sufficient for the identification of isolates.

The isolates M16, M22, M24 and M36 were white to cream in color, poorly produced mucus and alkalized the

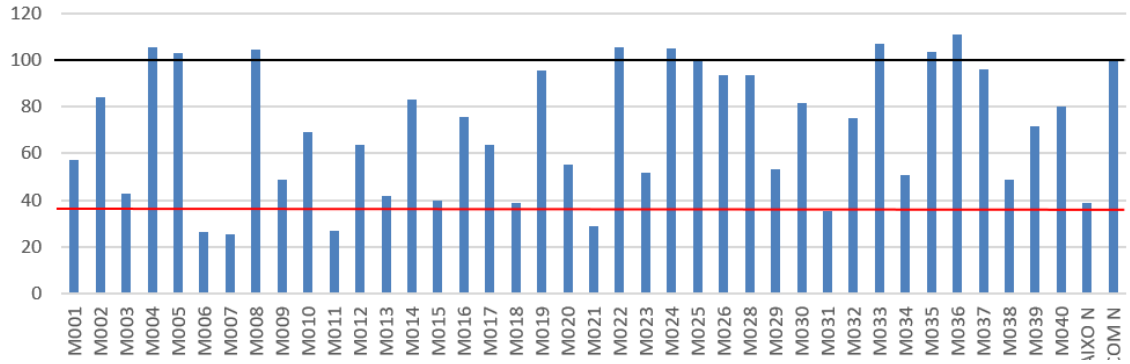

Figure 3. Relative efficiency (RE) of shoot dry mass production by Macroptilium lathyroides plants inoculated with rhizobia isolates obtained from *Macroptilium lathyroides* roots in soils of the periurban and rural region of Alta Floresta - MT. Brazil.
Source: Authors

Table 3. Genetic identification by partial sequencing of rDNA 16S gene from *Macroptilium lathyroides* root nodule isolates.

Isolate	Isolate Identification	General number	% of identification	Number of access
M002	*Mucilaginibacter gossypiicola*	1249	97	NR_116406.1
M004	*Novosphingobium arabidopsis*	625	84	NR_133799.1
M005	*Bacillus haynesii*	1332	99	NR_157609.1
M016	*Bradyrhizobium namibiense*	442	81	NR_159233.1
M022	*Bradyrhizobium namibiense*	326	82	NR_159233.1
M024	*Bradyrhizobium namibiense*	730	85	NR_159233.1
M028	*Flavobacterium anhuiense*	1375	100	NR_044388.1
M035	*Bacillus xiamenensis*	1129	95	NR_148244.1
M036	*Bradyrhizobium namibiense*	1048	92	NR_159233.1

Source: Authors

culture medium, as indicated by morphological characterization, similarity dendrogram and biplot. Sequence analysis of the 16S rDNA gene from the cited isolates classified them as *Bradryzobium namibiense* (Table 3), with pair sequence similarity and related gender lineage ranging from 81 to 92%. These results reinforce what was observed in the morphological analysis and grouping of isolates. *B. namibiense* was recently proposed as a new species by Grönemeyer et al. (2017) studying isolates in smelling beans.

The M002 isolate presented as white color, little mucus production and kept the pH of the culture medium neutral, as indicated by the morphological characterization, the similarity dendogram and the biplot. Sequence analysis of the 16S rDNA gene from the M002 isolate classified it into the *Mucilaginibacter genus* (Table 3) with 97% similarity in peer sequence and related gender lineage type.

The genus *Mucilaginibacter*, member of the family *Sphingobacteriaceae*, has as one of its characteristics the promotion of plant growth. Bacteria of this genus can promote this benefit directly or indirectly through the production of phytohormones or plant growth promoting enzymes, allowing increased nutrient absorption (Glick et al., 1999; Pankratov et al., 2007; Steyn et al., 1998; Whipps, 2001). According to Madhaiyan et al. (2010), the bacterium *Mucilaginicabter gossypiicola* produces a large amount of extracellular polysaccharides, indicating that this strain is probably responsible for promoting the growth of cotton plants.

Isolate M004 was characterized by white coloration, low mucus production changing the pH of the culture medium to alkaline, as indicated in the morphological characterization. Sequence analysis of the 16S rDNA gene from the isolate placed it in the *Novosphingobium* genus (Table 3) with 84% similarity in peer sequencing and gender lineage type. According to Lin et al. (2014), some bacteria of this genus found in the *Arabidopsis thaliana* (mustard) rhizosphere are resistant to DDT.

Isolate M005 was white in color, low in mucus production, and maintained neutral pH. This isolate was allocated to the genus Bacillus, more precisely as

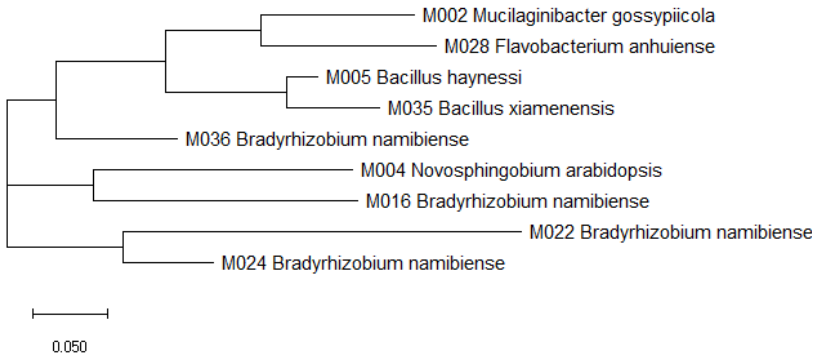

Figure 4. Phylogenetic tree based on 16S rDNA gene sequences from isolates obtained from *Macroptilium lathyroides* roots in soils of the periurban and rural region of Alta Floresta - MT. Brazil.
Source: Authors

Bacillus haynessi (Table 3) with 99% similarity. Dunlap et al. (2017) in a biodiversity survey of strains of the genus Bacillus, proposed the recognition of Bacillus haynessi as a new species, not finding recent studies on its characteristics and properties.

The M035 isolate had yellow coloration, abundant mucus production and acid pH. Gene sequence analysis classified it as Bacillus xiamenensis (Table 3) with 95% similarity. As for this species, it was not found in the literature reporting any interaction with plants.

In the phylogenetic analysis (Figure 4) it is possible to observe that isolates M024 and M022 were grouped as similar. Isolates M004 and M016 showed close genetic similarity, although the sequencing makes it clear that these are individuals of different genera. Already the isolates M002, M028, M005 and M035 presented similar characteristics in their sequencing that grouped them as similar, and these bacteria still have a degree of similarity with the isolate M036, belonging to the genus *Bradyrizobium*.

Studies on nitrogen-fixing bacteria using cowpea beans as bait have revealed a high incidence of *Bradyrhizobium* bacteria (Guimarães et al., 2012; Junior et al., 2010b; Pinheiro et al., 2014). This was similar to that found in this study, with a legume species from the same botanical family as cowpea.

Biological nitrogen fixation test (FBN)

Shoot length (CPA), shoot dry mass (MSPA) and total dry mass (MST) were not significant according to the f test, the root length (CR), Root volume (RV), root dry mass (MSR) and root shoot ratio (RELATION) did not differ significantly according to the Scott-Knott test at 5% probability.

According to the analysis of variance, the nitrogen content factor (WITH N) was not significant for the number of nodules (N° of nodules), dry mass of nodules (MSN), and nitrogen content (N) there was significance according to the f test, but MSN and IV factors did not show significant difference according to the Scott-Knott test at 5% probability.

For the nitrogen content factor, 4 samples showed no significant difference in relation to the WITH N treatment (Table 4), isolates M004, M005 and M022 obtained N contents close to the WITH N treatment. According to Malavolta et al. (1999), an adequate leaf nitrogen concentration would be 36 g per kg for soybean crop. It can be observed that the WITH N treatment presented equivalent nitrogen content for soybean crop, and the isolates mentioned presented very close to this value.

Conclusion

Thus, based on the results obtained from the study of rhizobia isolates of *M. lathyroides* roots collected from the periurban and rural zone of Alta Floresta - MT, Brazil, it is concluded that the isolates have great heterogeneity. There is a predominance of fast growing isolates, however only one fast growing isolate has efficiency in nitrogen fixation.

Intermediate and slow growth bacteria with low mucus production are more efficient in nitrogen fixation, compared with the other isolates of this study. M004, M005, M008, M022, M024, M024, M033, M35 and M036 isolates show higher ER than WITH N treatment and without inoculation, showing potential for further studies. The following bacteria were identified by 16S rDNA gene sequencing analysis: *Mucilaginibacter gossypiicola, Novosphingobium arabidopsis, Bacillus haynesii, Bacillus xiamenensis, Bradyrhizobium namibiense and Flavobacterium anhuiense*. Isolates M004, M005 and M022 have nitrogen content values very close to that of supplemental nitrogen treatment and are potentially indicated for further studies aiming at commercially important crops.

Table 4. Number of nodules (N°. of nodules) and nitrogen content in leaf analysis (N) of *Macroptilium lathyroides* plants inoculated with rhizobia isolates. Value expressed in g kg^{-1}.

Isolate	Number of nodules	Number
M002	80.17[a]	29.30[b]
M004	108.19[a]	31.40[a]
M005	133.92[a]	32.15[a]
M008	118.04[a]	29.17[b]
M014	94.69[a]	27.67[b]
M016	122.33[a]	28.77[b]
M019	108.46[a]	28.70[b]
M022	67.12[a]	30.45[a]
M024	122.23[a]	24.95[b]
M025	104.02[a]	26.42[b]
M026	83.83[a]	27.17[b]
M028	131.16[a]	27.72[b]
M030	100.00[a]	24.85[b]
M032	147.25[a]	28.72[b]
M033	95.60[a]	27.40[b]
M035	125.66[a]	26.80[b]
M036	130.42[a]	28.37[b]
M037	112.08[a]	25.07[b]
M039	104.17[a]	28.65[b]
SEM N	114.16[a]	28.02[b]
COM N	14.66[b]	36.10[a]
C.V.%	9.29	1.72

Means followed by the same letter in the column do not differ from each other by the Scott-Knott test at p> 0.05 probability.
Source: Authors

It is important to point out that collecting information on beneficial microorganisms for agriculture is beneficial for future food production. Research in regions of great biological diversity, such as the Amazon, can bring very important information in this area of knowledge.

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

REFERENCES

Albuquerque GP (2013). Avaliação de acessos de *Desmanthus* e *Macroptilium* no Semiárido Pernambucano [dissertation]. Serra Talhada: Universidade Federal Rural de Pernambuco.

Almeida LDA, Maeda JA, Falivene SMP (1979). Efeitos de métodos de escarificação na germinação de sementes de cinco leguminosas forrageiras. Bragantia 38(9):84-96.

Amabile RF, Carvalho AM (2006). Cerrado: Adubação Verde. Embrapa Cerrados. Planaltina – DF. 369 p.

Antunes JEL (2010). Diversidade genética e eficiência simbiótica de isolados de rizóbios nativos em feijão fava (*Phaseolus lunatos* L.) [dissertation]. Piauí: Universidade Federal do Piauí.

Antunes JEL, Gomes RLF, Lopes ACA, Araújo ASF, Lyra MCCP, Figueiredo MVB (2011). Symbiotic efficiency of rhizobia isolated from nodules of lima bean (*Phaseolus lunatus* L.). Revista Brasileira de Ciencia do Solo 35(3):751-757.

Araújo CL, Gualter RMR (2017). Morphophysiological characterization of bacteria native to Cerrado soils, isolated from cowpea nodules. Biotemas 30(1):25-35.

Araújo SR (1994). Caracterização morfológica, fisiológica e bioquímica do Rizóbio. In: Hungria M, Araújo SR. (Ed), Manual de métodos empregados em estudos de microbiologia agrícola. Brasília: Embrapa, pp. 7-29.

Bergey DH, Holt JG (1994). Bergey's manual of determinative bacteriology. Baltimore: Bergey 78-94pp.

Chagas Junior AF, Oliveira LA, Oliveira AN (2010). Phenotypic characterization of rhizobia strains isolated from Amazonian soils and symbiotic efficiency in cowpea. Acta Scientiarum Agronomy 32(1):161-169.

Concenço G, Correia IVT, Galon L (2012). Controle de *Macroptilium lathyroide*s com herbicidas aplicados em pós-emergência inicial. In: XXVII Congresso Brasileiro de Ciência das Plantas Daninhas. Proceedings. Campo Grande: SBCPD pp. 18-22.

Costacurta A, Vanderleyden J (1995). Synthesis of phytormones by pnat-associated bacteria. Critical Reviews of Microbiology 21(1):1-18.

Coutinho HLC, Oliveira VM, Moreira FMS (2000). Systematic of legume nodule nitrogen fixing: agronomic and ecological applications. Applied Microbial Systematics 1(5):107-134

Dunlap CA, Schisler DA, Perry EB,Connor N, Cohan FM, Rooney AP (2017). *Bacillus swezeyi* sp. nov. And *Bacillus haynesii* sp. nov., isolated from desert soil. International Journal of Systematic and Evolutionary Microbiology 67(8):2720-2725.

Embrapa (2016). Empresa Brasileira de Pesquisa Agropecuária. Página temática: Fixação biológica de nitrogênio. Brasília. Avaliable to:< https://www.embrapa.br/tema-fixacao-biologica-de-nitrogenio>.

Everitt BS (1993). Cluster analysis. New York: John Wiley pp. 1-70.

Fernandes MF, Fernandes RPM, Hungria M (2003). Seleção de rizóbios nativos para guandu, caupi e feijão de porco nos tabuleiros costeiros de Sergipe. Pesquisa Agropecuária Brasileira 38(7):835-842.

Ferreira A (2008). Interação entre bactérias endofíticas e do rizoplano com Eucaliptus [dissertation]. Piracicaba: Escola Superior de Agricultura "Luiz de Queiroz", USP.

Ferreira DF (2014). Sisvar: a Guide for its bootstrap procedures in multiple comparisons. Ciencia e Agrotecnologia 38(2):109-112.

Glick BR, Patten CL, Holguin GM (1999). Biochemical and genetic mechanisms used by plant growth promoting bacteria. London: Imperial College Press pp. 22-67.

Grönemeyer JL, Bünger W, Reinhold-Hurek B (2017). *Bradyrhizobium namibiense* sp. nov., a symbiotic nitrogen-fixing bacterium from root nodulesof lablab purpures, hyacinth bean, in Namibia. International Journal of Systematic and Evolutionary Microbiology 67(12):4884-4891.

Guerra JGM, Espíndola JAA, Perin A, Teixeira MG, Almeida DL, Assis RL (2007). Desempenho de leguminosas tropicais perenes como plantas de cobertura do solo. Seropédica: Embrapa, pp. 1-22.

Guimarães AA, Jaramillo PMD, Nóbrega RSA, Florention LA, Silva KB, Moreira FMS (2012). Genetic and symbiotic diversity of nitrogen fixing bacteria isolated from agricultural soils in the western Amazon by using cowpea as the trap plant. Applied Environmental Microbiology 78(18):6726-6733.

Hungria M (1994). Coleta de nódulos e isolamento de rizóbios. In: Hungria M, Araujo RS (Eds), Manual de métodos empregados em estudos de microbiologia agrícola. Brasília: Embrapa, pp. 45-59.

INMET (2018). Instituto Nacional de Meteorologia. Base de dados. Avaliable at: <http://inmet.gov.br/portal/>.

Jordan DC (1984). Family III Rhizobiaceae. In: Kriegd NR, Holt JG. (Eds.). Bergey's Manual os Systematic Bacteriology. Baltimore:Bergey pp. 234-256.

Junior AF, Rahmeier W, Fidelis RR, dos Santos GR, Chagas LF (2010b). Agronomic efficiency of rhizobium strains inoculated in cowpea in the Cerrado, Gurupi-TO. Ciência Agronomica 41(4):709-714.

Kefford NP, Brockwell J, Zwar JA (1960). The symbiotic synthesis of auxin by legumes and nodule bacteria and its role in nodule

development. Australian Journal of Biology Sciences 13(4):456-467.
Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018). Mega X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular and Biological Evolution 35(6):1547-1549.
Lin SY, Hameed A, Liu YC, Hsu YH, Lai WA, Huang H, Young CC (2014). *Novosphigobium arabidopsis* sp. Nov., a DDT-resistant bacterium isolated from the rhizosphere of *Arabidopsis thaliana*. International Journal of Systematic and Evolutionary Microbiology 64(2):594-598.
Madhaiyan M, Poonguzhali S, Lee JS, Senthilkumar, M, Lee KC, Sundaram C (2010). *Mucilaginibacter gossypii* sp. nov. and *Mucilaginibacter gossypiicola* sp. nov., plant-growth-promoting bacteria isolated from cotton rhizosphere soils. International Journal of Systematic and Evolutionary Microbiology 60(10):2451-2457.
Malavolta E, Oliveira SA, Wadt PGS (1999). Foliar diagnosis: the status of the art. In: Siqueira JO. (Eds.), Inter-relação fertilidade, biologia do solo e nutrição de plantas. Viçosa: SBCS. 205-242p.
Martins LMV, Neves MCP, Rumjanek NG (1997). Growth characteristics and symbiotic efficiency of rhozibia isolated from cowpea nodules of the north-east region of Brazil. Soil Biology and Biocience 29(5-6):1005-1010.
Mathesius U (2008). Auxin: at the root of nodule development? Functional Plant Biology 35(8):51-68.
Melloni R, Moreira FMS, Nóbrega RSA, Siqueira JO (2006). Efficiency and phenotypic diversity among nitrogen-fixing bacteria that nodulate cowpea [*Vigna unguiculata* (L.) Walp] and common bean (*Phaseolus vulgaris* L.) in bauxite-mined soils under rehabilitation. Revista Brasileira de Ciencia do Solo 30(2):235-246.
Monks PL, Ferreira OGL, Polo EA, Silva JB (2006). Produção e qualidade de sementes de *Macroptilium lathyroides* (L.) Urb. sob diferentes espaçamentos e épocas de colheita. Pesquisa Agropecuária Brasileira 36(2):107-112.
Moreira FMS, Silva K, Nobrega RSA, Carvalho F (2010). Bactérias diazotróficas associativas: diversidade, ecologia e potencial de aplicações. Comunicata Scientiae 1(2):74-99.
Pankratov TA, Tindall BJ, Liesack W, Dedysh SN (2007). *Mucilaginibacter paludis* gen. nov. sp. Nov. and *Mucilaginibacter grasilis* sp. Nov., pectin, xylan and laminarin degrading members of the family Sphingobacteriaceae from acidic Sphangum peat bog International Journal of Systematic and Evolutionary Microbiology 57(10):2349-2354.
Patten CL, Glick BR (1996). Bacterial biosynthesis os índole-3-acetic acid. Canadian Journal of Microbiology 42(3):207-220.
Peret B, Swarup R, Jansen L, Devos G, Auguy F, Collin M, Santi C, Hocher V, Franche C, Bogusz D, Bennett M, Laplaze L (2007). Auxin influx activity is associated with Frankia infection during actinorhizal nodule formation in Casuarina glauca. Plant Physiology 144(4):1852-1862.
Pinheiro MS, Sousa JB, Bertini CHCM, Martins SCS, Martins CM (2014). Isolamento e seleção de estirpes de rizóbios nativas do semiárido tolerantes a estresses ambientais. Enciclopedia Biosfera 10(18):2071-2082.
Ribeiro AC, Guimarães PTG, Alvarez VHV (1999) Recomendação para uso de corretivos e fertilizantes em Minas Gerais. 5ª Aproximação. Viçosa: SBCS 120-360p.
Sarruge JR (1975). Soluções nutritivas. Summa Phytopatologica 1(3):231-233.
Silva MSA,Yamashita OM, Rossi AAB, Concenço G, Carvalho MAC, Felito RA (2018). Cytotoxic and genotoxic effects of *Macroptilium lathyroides*. Planta Daninha 36(10):e018166574.
Sousa LMG, Gois RWS, Lemos TLG, Arriaga AMC, Andrade-Neto M, Santiago GMP, Braz-Filho R, Costa JGM, Rodrigues FGF (2013). Constituintes químicos e avaliação da atividade antibacteriana de *Macroptilium lathyroides* (L.) Urb. (Fabaceae). Química Nova 36(9):1370-1374.
Souza ACM (2015). Seleção de rizóbios e compatibilidade em diferentes variedades de feijão caupi (*Vigna unguiculata* (L.) Walp) [dissertation]. Recife: Universidade Federal Rural de Pernambuco.
Spaepen S, Vanderleyden J, Remans R (2007). Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiology Reviews 31(4):425-448.
Sprent J (1994). Evolution and diversity in the legume-rhizobium symbiosis: chaos theory? Plant and Soil 161(1):1-10.
Statsoft, Inc (2011). Statistica data analysis software system, version 10.
Steyn PL, Segers P, Vancanneyt M, Sandra P, Kersters K, Joubert JJ (1998). Classification of heparinolytic bacteria into a new genus, *Pedobacter*, comparising four species: *Pedobacter heparinus* comb. nov., *Pedobacter piscium* comb. nov., *Pedobacter africanus* sp. nov. and *Pedobacter saltans* sp. nov. Proposal of the family *Sphingobacteriaceae*. International Journal of Systematic Bacteriology 48(1):165-177.
Tedesco MJ, Volkweiss SJ, Bohmen H (1995). Análise de solo, plantas e outros materiais. 2 ed. Porto Alegre: UFRS pp. 100-174.
Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The Clustal X Windows interdace: flexuble startegies for multiple sequence alignment ideal by quality tool. Nucleic Acids Research 25(24):4876-4884.
Tortora GJ, Funke BR, Case CL (2012). Microbiologia. Porto Alegre: Artmed pp. 167-170.
Vargas AAT, Athayde JT, Silveira JSM (1990). Inoculação com rizóbios, adubação com macro e micronutrientes e parcelamento do N-mineral aplicados no feijoeiro (*Phaseolus vulgaris* L.). Bioscience Journal 11(1):3-14.
Vasconcelos WA, Santos EM, Andrade AP, Bruno RLA, Edvan RL (2011). Germination of Seeds and development of figo de pombo plants (*Macroptilium lathyroides*). Tropica 5(1):3-11.
Vicent JM (1970). A manual for the practical study of root-nodule bacteria. Oxford: Blackwell pp. 120-164.
Vieira RF, Salgado LT, Vieira C (1994). Rizóbio, molibdênio e cobalto na cultura do feijão no Alto Paranaíba e Noroeste de Minas Gerais. Ceres 41(238):688-694.
Whipps JM (2001). Microbial interactions and biocontrol in the rhizosphere. Journal of Experimental Botany 52(1):487-511.