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Diversity and symbiotic performance of peanut rhizobia from Southeast region of Brazil

Carlos Vergara Torres-Júnior¹, Jakson Leite¹, Carolina Etienne de Rosália e Silva Santos², Paulo Ivan Fernandes-Júnior³, Jerri Édson Zilli⁴, Norma Gouvêa Rumjanek⁴ and Gustavo Ribeiro Xavier^{4*}

¹Departamento de Solos, Instituto de Agronomia. Universidade Federal Rural do Rio de Janeiro, BR 465, Km 07, Seropédica, RJ, CEP 23890-000. Brazil.

²Departamento de Agronomia, Universidade Federal Rural de Pernambuco, Recife, PE, CEP 52171-900. Brazil.

³Embrapa Semiárido, BR 428, km 152, Zona Rural, CEP 56302-970 Petrolina, PE. Brazil.

⁴Embrapa Agrobiologia, BR 465, Km 07, Seropédica, RJ, CEP 23890-000. Brazil.

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The use of peanut as a trap-plant for obtaining rhizobia may result in a high diversity of efficient isolates. This study aimed to evaluate the diversity and symbiotic efficiency of peanut rhizobia from the Southeastern region of Brazil. The bacteria were isolated from nodules of two genotypes in soils from the States of São Paulo and Rio de Janeiro, Brazil. The bacteria were authenticated and evaluated regarding to their symbiotic capacity. The rhizobial diversity was evaluated through their culture characteristics on yeast mannitol agar (YMA) culture media and by polymerase chain reaction Box-PCR. Two selected isolates were characterized phenotypically regarding their capacity to solubilize phosphate, to grow on media with different concentrations of NaCl, pH's and incubation temperatures. For these the 16S rRNA gene sequencing and symbiotic efficiency using non-autoclaved soils were conducted. The majority of the obtained isolates showed rapid growth and acidified the culture medium. Analysis of the isolates through the Box-PCR revealed low similarity to the reference strain indicated for the culture and a large genetic variability of the obtained isolates. The isolates AM 01 and AM 07, which clustered with *Bradyrhizobium* and *Rhizobium* genus, respectively, showed a potential for evaluations and a means to recommend new rhizobia strains for peanut.

Key words: *Arachis hypogaea* L., inoculant, biological nitrogen fixation.

INTRODUCTION

The peanut (*Arachis hypogaea* L.) is a legume that belongs to Papilionoideae subfamily which originated from South-America (Hancock, 2004). This specie offers several uses and in Brazil, there are cultivated genotypes for consumption of grains *in natura*, roasted peanuts or for the production of oil. The main peanut production regions in Brazil are the Southeast and the Northeast where production increases annually, which, today, is about 300 thousand tons, placing Brazil as the world's fifth largest producer (Santos et al., 2012). In the technological package

used for peanut cultivation, the handling of fertilizing is very important because the species requires large amounts of diverse nutrients such as Ca, P and N. The supply of nitrogen can occur by means of biological nitrogen fixation (BNF) through symbiosis with efficient rhizobia dispensing or reducing the application of nitrogen fertilizers.

Nowadays, there is a rhizobial strain that has been authorized for the production of inoculants for peanut in Brazil (Brasil, 2011), but inoculation technology of seeds

with commercial inoculants is still poorly adopted by producers. The reason for this is that the peanut possesses the capacity to nodulate with a great variety of rhizobia native to the Brazilian soil, which frequently reduces positive answers of inoculation (Borges et al., 2007). In this sense, the obtainment of new bacteria with high efficiency and capable of associating themselves with peanut plants in field conditions seems an acceptable strategy to increase the interest of producers in using inoculation techniques.

Over the past few years, prospecting of efficient and competitive rhizobia for peanuts has been carried out systematically (Santos et al., 2007; Lyra et al., 2013), as has been the case for the selection of bacteria for other legumes that are important in Brazil, such as cowpea (Martins et al., 2003; Zilli et al., 2009; Fernandes Júnior et al., 2012) and the common bean (Hungria et al., 2000). Preliminary studies for peanuts that have been conducted in Brazil show genetic and phenotypic variability of the native bacteria (Borges et al., 2007; Santos et al., 2007; Lyra et al., 2013). However, there still is a lack of bacteria with increased efficiency.

The analysis of phenotypic, genetic and ecologic information of the rhizobia isolates is an important stage in the selection process of new isolates since this information contributes to the understanding of the symbiosis between the plant and the bacteria (Garcia-Fraile et al., 2007; Hunter et al., 2007). In addition, the pre-selection of efficient bacteria generates genetic resources for the implementation of new test programs under field conditions for the recommendation of new strains (Lima et al., 2012). In this sense, the objective of this study was to evaluate the phenotypic and genetic diversity, and the symbiotic efficiency of native rhizobia obtained in the southeast of Brazil for peanut crop.

MATERIALS AND METHODS

Obtainment of nodules and rhizobia isolation

To obtain the nodules and to isolate the bacteria, an experiment was carried out in pots using six soil samples and the peanut (*Arachis hypogaea* L.) genotypes BR 1 and BRS Havana as trap-plants. The soil samples were collected in the cities of Jaboticabal, Sertãozinho and Taquaritinga at São Paulo State and in an Integrated Agroecological Production System in the municipality of Seropédica in the State of Rio de Janeiro. The samples were collected from the superficial horizon of each area and each sample was composed of at least 6 sub-samples. The land use of the areas at the moment of soil sampling, the class of the soil, its location and fertility characteristics are summarized in Table 1.

The soil samples were broken up, sieved in a 5 mm sieve and added to polyethylene pots with a capacity of five liters with 4.5 kg of each soil sample per pot. The seeds were superficially disinfected for sowing with 70% alcohol for 30 s, with 5% sodium hypochlorite for 60 s and washed ten times with sterile distilled water (Vincent, 1970). In each pot three seeds were sown that were exposed to the outside air and 10 days after emergence, thinning was carried out, leaving one plant per pot. An entirely randomized design was used for the installation of the experiment, following a

factorial scheme of 6 (soil samples) × 2 (plant genotypes) with four replications. During the experiment, the soil humidity was maintained close to the field capacity, using drinking water, passed through an activated carbon filter. The plants were harvested 50 days after emergence. The roots were separated from the shoot, washed and the nodules were excised and stocked in jars with silica gel for later isolation of bacteria.

At least ten nodules were isolated for each replication (40 nodules per treatment), randomly chosen. The nodules were rehydrated in distilled autoclaved water for 40 min, disinfected superficially with 70% alcohol for 1 min, with 5% sodium hypochlorite for three minutes and washed ten times with sterilized distilled water. With a pincer, the disinfected nodules were pressed on a Petri dish containing yeast-mannitol-agar medium (YMA) (Vincent, 1970) with Congo red (Somasegaran and Hoben, 1994). The dishes were incubated at 28°C until the appearance of the bacterial colonies. To purify the isolates, the cultures were inoculated successive times to YMA media containing bromothymol blue as pH indicator to obtain pure cultures. The pure cultures were stored in YM media containing glycerol (1:1 v/v) at -80°C.

Evaluation of the diversity of the isolates

The following properties were observed for the phenotypic characterization of the isolates in YMA media with bromothymol blue (Vincent, 1970): growth time in days of the isolated colonies (fast: until three days; intermediate: four to five days and slow: more than six days), change of medium pH (acid, neutral and alkaline), size (<1 mm, between 1 and 2 mm or > 2 mm), transparency (translucent or opaque), elevation (plain and convex), color (white or yellow), shape (circular, oval or irregular) and border (smooth or irregular) of the colonies; appearance of the mucus and the colony (homogeneous or heterogeneous) and the type of mucus, which is classified as butyric and viscous mucus (Martins, 1996).

The isolates AM 01, AM 07 and the strain SEMIA 6144, were evaluated for their capacity to solubilize calcium phosphate "*in vitro*", for which the GL medium (Sylvester-Bradley et al., 1982) with two distinct carbon sources (mannitol and glucose) was used, as well as for their growth capacity on YMA media with different pH levels (4;5;7;9 and 11), incubation temperatures (39 and 45°C and supplemented with different concentrations of NaCl (0.17; 0.34 and 0.51 M).

To evaluate the capacity to solubilize calcium phosphate, the bacteria were previously grown on YM culture media during the necessary growth time for each isolate. For inoculation on GL medium, 10 µL of the culture broth was used at three equidistant points on the Petri dish. The solubilization of calcium phosphate was evaluated by measuring the diameter of the translucent halo around the bacterial colonies. For growth on media with different pH levels, salt concentration and incubation temperatures, the bacteria were grown on YM culture media as mentioned previously and inoculated on Petri dishes containing the modified YMA medium, whereby only one bacterium was inoculated per dish. All the evaluations were done in triplicate.

For the genotypic characterization of the bacteria, fingerprinting of the 37 isolates was carried out by means of the Box-PCR technique. For the PCR, the primer Box A1 (CTACGGCAAGCGACGCTGACG) was used (Versalovic et al., 1994). The PCR and electrophoresis conditions adopted by Hungria et al. (2008) were followed. After electrophoresis, the gel was colored with ethyl bromide (0.01% v/v) and visualized under a photo-documentation system with UV light. The digitized image of the gel was evaluated using BioNumerics software (Applied Maths, Kortrijk, Belgium).

The gene sequencing of the 16S rRNA gene was carried out by the isolates AM 01 and AM 07. To amplify the 16S rDNA gene, the PCR reaction was dimensioned for a final volume of 50 µL.

Table 1. Characteristics of the area where the soil samples were collected.

Soil	Characteristic of the area	Municipality	Coordinate	pH	Sorting complex				
					Al ⁺³	Ca ⁺²	Mg ⁺²	K ⁺¹	P
					cmolc dm ⁻³			mg dm ⁻³	
HP*	Dead tomato covering (<i>Lycopersicon esculentum</i> L.)	Seropédica-RJ	22°46'59" S, 43°40'45" W e 33 m	6.5	0	3.6	1.3	132	263
AVA1	Dead corn covering (<i>Zea mays</i> L.)	Seropédica-RJ	22°46'59" S, 43°40'45" W e 33 m	6.3	0	1.8	0.8	104	50
AVA2	Dead colonial grass covering (<i>Panicum maximum</i>) and coco-grass (<i>Cyperus rotundus</i> L.)	Seropédica-RJ	22°46'59" S, 43°40'45" W e 33 m	5.4	0	2.5	0.8	48	21
NV	Dead sugar cane straw covering (<i>Saccharum officinarum</i> L.)	Jaboticabal-SP	21°15'17" S, 48°19'20" W e 605 m	6.4	0	2.8	0.5	56	13
AV	Dead sugar cane straw covering (<i>Saccharum officinarum</i> L.)	Taquaritinga-SP	21° 24' 22" S; 48° 30' 17" W e 565 m	5.4	0.1	1.4	0.4	72	6.4
LV	Dead sugar cane straw covering (<i>Saccharum officinarum</i> L.)	Sertãozinho-SP	21° 08' 16" S 47° 59' 25" W e 579 m	6.2	0	3.4	0.9	300	17
Red Yellow Ultisol**		Seropédica-RJ	22°46'59" S, 43°40'45" W e 33 m	6.3	0.02	3.5	1.5	70	36.8
Haplic Planosol**		Seropédica-RJ	22°46'59" S, 43°40'45" W e 33 m	6.4	0.02	1.9	0.6	27	29.6

*Classification according the Brazilian Soil Classification System. Haplic Planosol (HX), Red Yellow Ultisol 1 (AVA1), Red Yellow Ultisol 2 (AVA2), Red Nitosol (NV), Red Ultisol (AV) and Red Oxisol (LV). **Chemical characteristics of non-autoclaved soils, used for the cultivation of two peanut varieties (BR 1 and BRS Havana) until 45 days after planting inoculated with the isolates AM 01 and AM 07.

(1X buffer, 1.75 mM of MgCl₂, 0.25 mM of each dNTP, 1 U of Taq DNA polymerase and 0.25 μM of each primer). The PCR conditions were performed conform to what was described by Leite et al. (2009) using the Y1 and Y3 primers (Young et al., 1991). The sequencing was conducted on the ABI 3730/xl platform (Applied Biosystems, Foster City, CA, USA) at Macrogene (Seoul, South Korea).

Symbiotic efficiency of the isolates in pots with sterilized substrate

Two experiments under greenhouse conditions were conducted to evaluate the nodulation capacity and the symbiotic efficiency of the isolates. Leonard jars (Vincent, 1970) containing sand and vermiculite (2:1 w/v) were used as sterile substrate. An experimental outline with a randomized block design with three replications was used. The first experiment was implemented using the genotype BR 1 and the second with the BRS Havana. For both experiments the treatment used was the inoculation of the isolated bacteria from nodules of the genotype itself. In addition, the following controls were included: a control inoculated with the SEMIA 6144 (*Bradyrhizobium* sp.) strain, which is used for the production of peanut inoculants in Brazil (Brasil, 2011), a nitrogen supplied control, which received 100 mg of N per jar per week in the form of ammonium nitrate and an absolute control without inoculation or N mineral supplementation.

For the sowing, the seeds were disinfected superficially as was previously described and for the inoculation, the bacteria were grown on YM media during the adequate growth period for each isolate. At the time of planting, 1 mL of the culture broth with approximately 10⁹ of viable cells was applied to each seed. From the second week of conduction onwards, a nitrogen free nutritive solution (Norris and T'Mannetje, 1964) was applied every seven

days. The plants were harvested 45 days after emergence and the following variables were analyzed: shoot dry weight (SDW), root dry weight (RDW), nodules dry weight (NDW), and the accumulation of total N in the shoots was also determined by the semi-micro Kjeldahl method (Liao, 1981), which is the content of N in the shoots obtained by the product between the concentration and the dry mass. Analysis of variance and a comparison of means were carried out using the Scott-Knot test (p < 0.05) with the aid of Sisvar software (Ferreira, 2008).

Symbiotic efficiency of the isolates in pots with non-sterilized soil

An experiment was conducted in a greenhouse using non-autoclaved soil as substrate. Soil samples were collected from the superficial horizon of a Haplic Planosol and a Red Yellow Ultisol of the Integrated Agroecological Production System at the main building of Embrapa Agrobiologia in Seropédica municipality, Rio de Janeiro State. The soil samples were processed as was previously described. An experimental randomized block design following a factorial scheme of 5 (inoculation treatments) × 2 (genotypes) was adopted for this experiment. The bacteria used for the inoculation treatments were the isolates AM 01 and AM 07 obtained from nodules of BR 1 and BRS Havana peanut genotypes respectively, and the strain SEMIA 6144 *Bradyrhizobium* sp. In addition to the inoculated treatments, the nitrogen supplied control treatment, with an application of 100 mg N per week per pot in the form of ammonium nitrate and the absolute control treatment, non-inoculated without N mineral supplementation, were evaluated.

The seeds were superficially disinfected for sowing as previously described. The bacteria were grown on YM media under constant shaking during the adequate growth period for each bacterium. The

Table 2. Number, growth time and pH reaction of obtained isolates of nodules from the peanut varieties (BR 1, BRS Havana) when used as bait plant in six soil samples collected in the state of Rio de Janeiro and São Paulo.

Solo	Isolate obtained		Growth time				pH reaction					
	BR-1	BRS-Havana	BR1		BRS-Havana		BR-1			BRS-Havana		
			R	L	R	L	AC	NE	AL	AC	NE	AL
HP	4	3	2	2	3	-	1	1	2	3	-	-
AVA1	0	4	-	-	4	-	-	-	-	4	-	-
AVA2	3	5	3	-	5	-	3	-	-	4	1	-
NV	4	4	3	1	4	-	1	2	1	2	2	-
AV	2	3	2	-	3	-	1	1	-	3	-	-
LV	3	2	3	-	2	-	3	-	-	2	-	-
Total	16	21	13	3	21	-	9	4	3	18	3	-

R: rapid growth; L: slow growth. AC: reaction of acid pH; NE: reaction of neutral pH; AL: reaction of alkaline pH.

inoculation was carried out at the moment of sowing, applying 1 mL of culture broth with approximately 10^9 colony forming unities. The soil humidity was maintained close to the field capacity, using drinkable water, passed through an activated carbon filter. The harvest was carried out after 45 days. The shoots were separated from the roots during harvesting. All the components were accommodated in a forced ventilation chamber at 65°C until the constant weight to determine the SDW, RDW and the NDW and the accumulation of total N of the aerial part as described above. The data were submitted for analysis of variance. The comparison of means test used was the t-test (LSD) at 5% of probability, using the SISVAR program (Ferreira, 2008).

RESULTS

Phenotypic and genetic variability of the isolates

From all bacteria obtained, 37 bacteria that re-nodulated the original host were obtained. Among the nodulating isolates of the genotype BR 1, four and three were obtained from the Planosol and from the Red Yellow Ultisol 2, respectively, soils that were collected in the state of Rio de Janeiro. Four, two and three of the soils that were collected in the state of São Paulo were obtained from plants grown in samples of Red Nitosol, Yellow Ultisol and Red Oxisol, respectively (Table 2). Among the 21 bacteria that nodulated, the genotype BRS Havana, three, four and five were isolated from plants grown in samples of Planosol, Red Yellow Ultisol 1, Red Yellow Ultisol 2, collected in the state of Rio de Janeiro. The bacteria isolated from plants grown in soil samples in the state of São Paulo, four, three and two were obtained from Red Nitosol, Red Ultisol and Red Oxisol, respectively. Among the 37 obtained isolates, 34 were classified as fast growing, and only three as slow growing. Analyzing the pH reaction of the culture media, 27 isolates showed the capacity to acidify the culture medium, seven did not alter the pH of the medium and three alkalized the YMA medium.

In the similarity dendrogram, based on fingerprinting

the isolates using the Box-PCR technique, it was possible to observe eight distinct clusters with 50% similarity among themselves (Figure 1). Cluster I gathered four isolates of which two came from the same soil in the state of São Paulo and the others were collected in distinct states. Clusters II, IV and V clustered only two isolates. Cluster III was formed only by one isolate. The two isolates of cluster IV were isolated from the same soil in the state of São Paulo while the two representatives of groups II and V were collected from different states. Two main clusters (VI and VII) can be observed in the dendrogram. Cluster VI included 13 isolates, which were two bacteria obtained from nodules of the genotype BRS Havana from the Haplic Planosol in the state of Rio de Janeiro and three from the same peanut genotype in Red Nitosol from the state of São Paulo. Among the other bacteria of this group, two isolates were collected from Red Oxisol and five from Red Nitosol, both from the state of São Paulo. Cluster VII joined eight isolates besides the SEMIA 6144 reference strain. In this group, 50% of the isolates were collected from each of the studied states. Cluster VIII included five isolates, four of which were isolated from soil samples in the state of Rio de Janeiro.

Symbiotic performance of the isolates in sterilized substrate

In the two trials that were carried out in sterilized substrate, a pre-selection was made based on the symbiotic efficiency of the isolates obtained in this study. Two isolates (AM 01 and AM 03) that were obtained from the cultivar BR 1 stood out compared to the others, once the plants inoculated with these bacteria showed nodule weight and dry roots similar to what was observed for the plants inoculated with the SEMIA 6144 strain (Table 4). The inoculation treatment with these isolates resulted in plants with a shoot dry weight which was larger than what was observed for plants that received mineral N

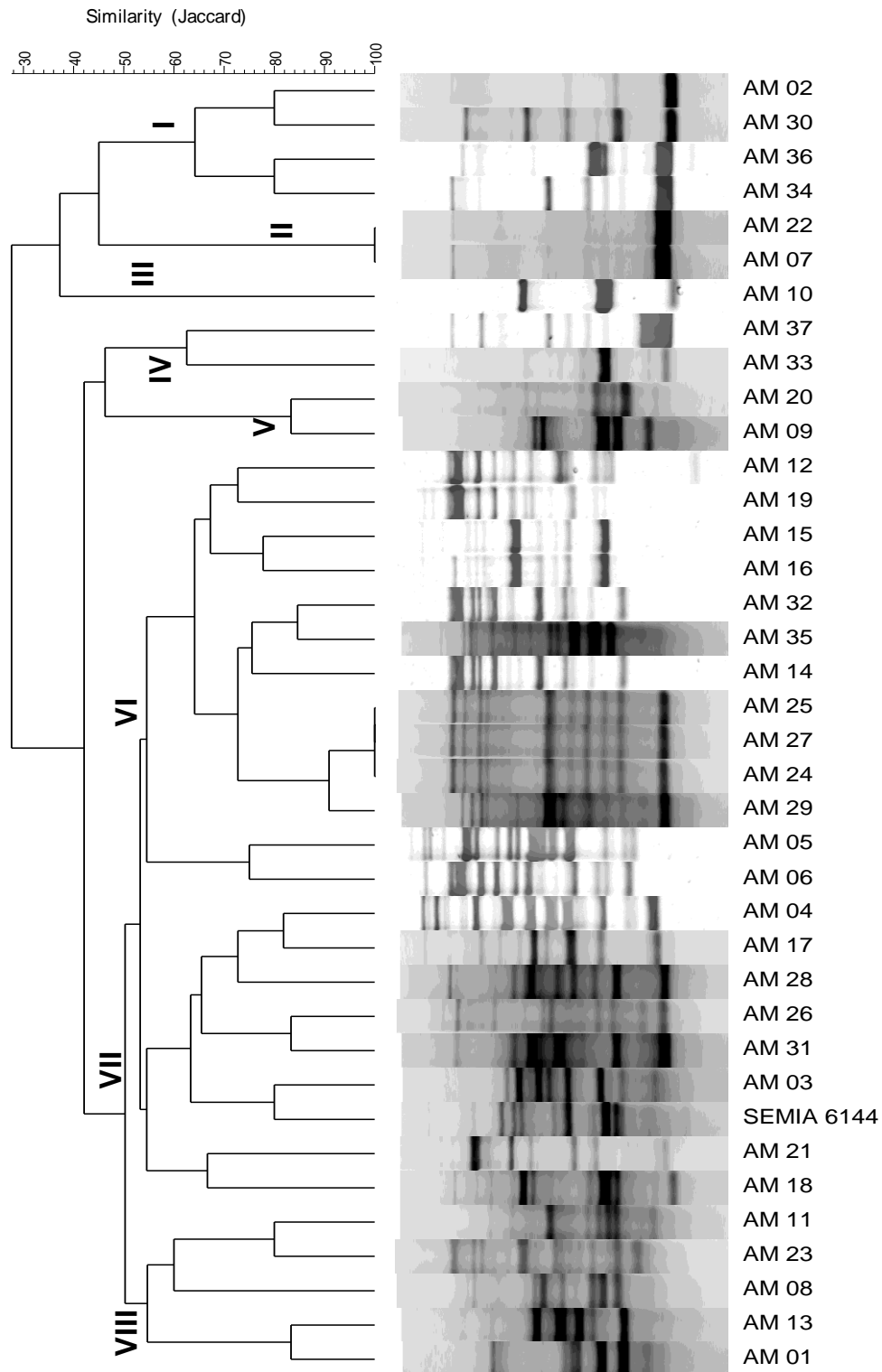


Figure 1. Similarity dendrogram among the bacterial isolates obtained from nodules of two peanut varieties (BR-1 and BRS Havana) and the officially recommended strain for the peanut (SEMIA 6144) in Brazil.

supplements, yet smaller than what was observed for plants inoculated with the SEMIA 6144 strain. The plants

inoculated with the isolate AM 01 showed a larger total N in the shoot than the treatment plants that were

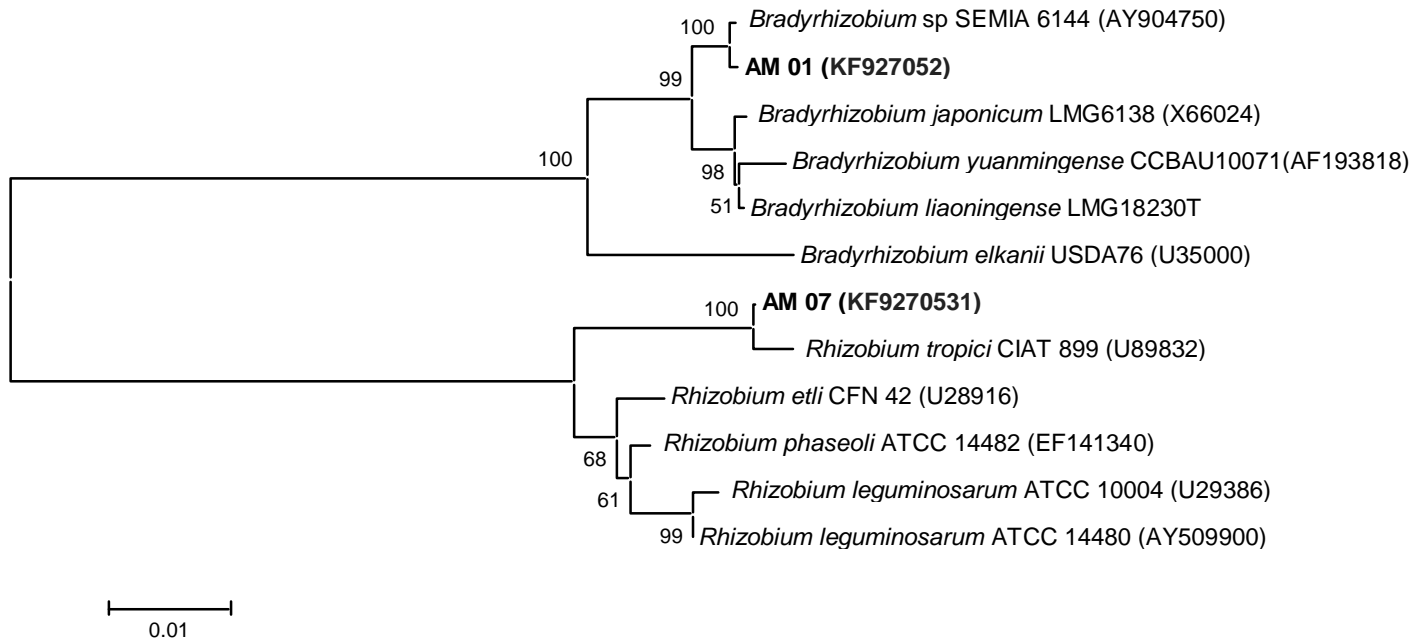


Figure 2. Phylogenetic tree reconstructed from the 16S rRNA gene sequences of the bacteria AM 01 and AM 07 which were isolated from nodules of the varieties BR 1 and BRS Havana, respectively, using the Neighbor-Joining method, with distances calculated by the Jukes-Cantor method. The values of each branch represent 1000 bootstrap replications.

supplemented with N mineral and it was the same for the plants inoculated with the SEMIA 6144 strain. The plants inoculated with the isolate AM 03 showed a higher total N than the N supplemented control and a lower total N than the plants inoculated with the SEMIA 6144 strain.

In the experiment using the genotype BRS Havana in sterilized substrate, it was possible to observe that the plants inoculated with AM 07, AM 11, AM 15, AM 24 and AM 32 showed a larger nodule dry weight when compared to the treatment inoculated with the SEMIA 6144 strain (Table 5). It was also possible to observe that plants of the variety BRS Havana inoculated with the isolates AM 07, AM 10, AM 15, AM 31, AM 32 e AM 36 showed a larger dry mass of the aerial part than the plants that received mineral nitrogen. However, in none of the inoculated treatments with the isolates obtained in this study did the plants show dry mass of the aerial part or total N equal to what was observed for plants inoculated with the SEMIA 6144 strain.

The 16S rRNA sequence analysis

The 16S rDNA sequence analysis of the isolates AM 01 and AM 07, isolates which show better symbiotic performance, made it possible to verify that the isolates showed similarities with distinct genera of rhizobia (Figure 2). The isolate AM 01, which shows slow growth and the capacity to alkalinize the medium culture (belonging to group eight according to the Box-PCR

technique) showed 99.85% of identity with the SEMIA 6144 strain of *Bradyrhizobium* sp. On the other hand, the fast growing isolate that acidify the culture medium, AM 07, was belonged to group 2 in the similarity dendrogram using the Box-PCR technique, showed an identity of 99.62% with the CIAT 899¹ of *Rhizobium tropici*. The sequences of these two isolates were deposited to the GenBank database with accession numbers KF927052 (AM01) and KF9270531 (AM07).

Solubilization phosphate and effect of pH, NaCl and temperature on AM 01 and AM 07

The isolates AM 01, AM 07 and the SEMIA 6144 strain grew on the YMA media under all tested pH levels (4; 5; 7, 9 e 11) and only isolate AM 07 was capable of solubilizing the calcium phosphate on GL media when glucose was used as a carbon source, in spite of being able to also grow on GL media with mannitol without showing the capacity to solubilize the phosphate (Table 3). As for growth at different incubation temperatures, the three tested bacteria grew until 39°C but were not able to grow when incubated at 45°C (Table 3). To evaluate the growth capacity of the bacteria on culture media supplemented with different concentrations of NaCl, the isolate AM 01 and the SEMIA 6144 strain showed growth only in the control treatment, without salt supplements. However, the isolate AM 07 was capable to grow on the media supplemented with up to 0.34 M de NaCl.

Table 3. Evaluation of growth of the isolates AM 01 and AM 07 in pH ranges, of temperature, salinity and its ability to solubilize calcium phosphate.

Bacteria	pH					Solubilization of calcium phosphate								Temperature			Salinity			
						Mannitol				Glucose				SI	25°C	39°C	45°C	1%	2%	3%
	4	5	7	9	11	3*	6	9	12	3	6	9	12							
SEMIA 6144	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
AM 01	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
AM 07	+	+	+	+	+	+	+	+	+	1.43	1.33	1.33	1.31	low	+	+	-	+	+	-

(+) Bacterial growth was observed. (-) Growth of the isolate was not observed. SI = Solubilization index. * Days after inoculation

Table 4. Dry mass of the aerial part, of the nodules, and accumulation of nitrogen in the aerial part of the peanut variety BR 1 inoculated with different rhizobial isolates, SEMIA 6144 reference strain, nitrogen supplied control (with N and without inoculation) and absolute control (without N and without inoculation).

Treatment	Dry shoot weight	Dry root weight	Dry nodule weight	Total N of the shoot
	g plant ⁻¹		mg plant ⁻¹	
AM 02	1.5 ^e	0.6 ^a	90 ^a	32.9 ^c
AM 04	1.7 ^e	0.5 ^a	100 ^a	39.2 ^c
AM 14	1.3 ^e	0.5 ^a	40 ^b	21.2 ^c
AM 12	1.6 ^e	0.8 ^a	90 ^a	22.3 ^c
AM 13	1.5 ^e	0.5 ^a	10 ^b	19.6 ^c
AM 20	1.3 ^e	0.3 ^b	70 ^a	22.9 ^c
AM 21	1.1 ^f	0.2 ^b	50 ^a	16.0 ^c
AM 22	1.1 ^f	0.5 ^a	100 ^a	14.0 ^c
AM 23	1.3 ^e	0.5 ^a	90 ^a	16.4 ^c
AM 28	1.0 ^f	0.7 ^a	70 ^a	11.3 ^c
AM 29	1.5 ^e	0.7 ^a	70 ^a	18.1 ^c
AM 33	1.5 ^e	0.4 ^b	90 ^a	28.3 ^c
AM 34	1.1 ^f	0.3 ^b	100 ^a	19.7 ^c
AM 35	0.8 ^f	0.1 ^b	70 ^a	25.5 ^c
AM 03	3.6 ^b	0.6 ^a	100 ^a	109.3 ^b
AM 01	3.1 ^c	0.5 ^a	100 ^a	144.3 ^a
SEMIA 6144	4.5 ^a	0.7 ^a	110 ^a	158.9 ^a
N supplied control	2.7 ^d	0.8 ^a	0 ^b	106.1 ^b
Absolute control	1.2 ^f	0.3 ^b	0 ^b	17.3 ^c
CV (%)	12.5	35.9	28.4	31.2

Values are means of three repetitions. Values followed by the same letter do not differ from the Scott-Knott test at 5%.

Symbiotic performance of the isolates in non-autoclaved soil substrate

For the experiment that was carried out using non-autoclaved soil as substrate, it was possible to observe that the inoculation of the three bacteria (AM 01, AM 07 - which showed better results in previous tests - and the SEMIA 6144 strain) stimulated the nodulation in both genotypes when cultivated in samples of Red Yellow Ultisol (Table 6). In this same soil, plants of the geno-

types BR 1, inoculated with the isolate AM 01, showed a higher dry mass of the aerial part than what was observed in the absolute control treatments and SEMIA 6144, and the values were the same as those observed in the N fertilized control treatments and AM 07. In the Haplic Planosol, plants of the variety BR1, inoculated with the isolates AM 01 and AM 07, showed a dry weight of the shoot equal to what was observed in the absolute control treatments and SEMIA 6144 where the mass of the plants inoculated with AM 01 was also equal to the

Table 5. Dry mass of the aerial part, of the root, of the nodules and nitrogen accumulation in the aerial part of de peanut variety BRS Havana, inoculated with different rhizobial isolates, SEMIA 6144 reference strain, nitrogenized control (with and without inoculation) and absolute control (without N and without inoculation).

Treatment	Dry shoot weight	Dry root weight	Dry nodule weight	Total N of the shoot
	g plant ⁻¹		mg plant ⁻¹	
AM 08	1.3 ^c	0.7 ^a	97 ^b	15.2 ^b
AM 06	1.2 ^c	0.6 ^a	100 ^b	11.1 ^b
AM 05	1.2 ^c	0.6 ^a	80 ^b	11.6 ^b
AM 37	0.8 ^c	0.3 ^b	96 ^b	13.2 ^b
AM 09	0.9 ^c	0.3 ^b	79 ^b	11.0 ^b
AM 11	1.2 ^c	0.6 ^a	103 ^a	14.9 ^b
AM 10	1.6 ^b	0.7 ^a	68 ^b	21.4 ^b
AM 15	1.7 ^b	0.7 ^a	117 ^a	27.2 ^b
AM 17	1.2 ^c	0.6 ^a	95 ^b	18.4 ^b
AM 18	1.0 ^c	0.4 ^b	80 ^b	15.6 ^b
AM 16	1.2 ^c	0.6 ^a	73 ^b	14.6 ^b
AM 19	1.4 ^c	0.8 ^a	52 ^c	28.3 ^b
AM 25	1.0 ^c	0.4 ^b	85 ^b	15.7 ^b
AM 24	1.5 ^c	0.6 ^a	140 ^a	20.4 ^b
AM 26	1.2 ^c	0.5 ^b	80 ^b	17.8 ^b
AM 27	0.9 ^c	0.3 ^b	75 ^b	12.2 ^b
AM 30	1.3 ^c	0.7 ^a	68 ^b	19.2 ^b
AM 31	1.9 ^b	0.6 ^a	100 ^b	26.2 ^b
AM 32	1.7 ^b	0.5 ^b	111 ^a	18.3 ^b
AM 36	1.8 ^b	0.8 ^a	92 ^b	21.5 ^b
AM 07	1.8 ^b	0.6 ^a	136 ^a	37.0 ^b
SEMIA 6144	2.9 ^a	0.4 ^b	82 ^b	106.0 ^a
N supplied control	0.7 ^c	0.4 ^b	0 ^d	24.2 ^b
Absolute control	1.1 ^c	0.8 ^a	0 ^d	13.0 ^b
CV (%)	17.9	28.1	29.9	25.2

Values are means of three repetitions. Values followed by the same letter do not differ from the Scott-Knott test at 5%.

nitrogenized control.

The inoculation of both isolated bacteria in this study in the variety BRS Havana did not positively influence the production of the aerial part, which was inferior to the N fertilized control treatment, however, equal to the treatments inoculated with SEMIA 6144 strain. In the Red Yellow Ultisol, which has better fertility characteristics than the Haplic Planosol, plants of the variety BR 1 inoculated with the isolates AM 01 and AM 07 showed a higher N content than what was observed in the absolute control treatment, SEMIA 6144 and N fertilized treatments.

In this same soil, plants of the genotype BRS Havana, inoculated with the same isolates, showed an N content equal to the absolute control treatments and SEMIA 6144 with an N content of the plants inoculated with the isolate AM 01 that was also similar to that of the N supplied control. In the Haplic Planosol, which has a fertility

condition lower to the red yellow ultisol, plants belonging to the variety BR 1, inoculated with isolates AM 01 and AM 07, showed an N content equal to that of the absolute control treatments and SEMIA 6144 with an N content of the plants inoculated with the isolate AM 01 that was also similar to the N supplemented control. In the same soil, plants of the genotype BRS Havana did not respond to any of the treatments. The isolate AM 01 stimulated the development of the root system of the variety BRS Havana in both soils used.

In the plants inoculated with the isolate AM 01, the dry root mass was similar to that of the N fertilized control treatments and inoculation with the SEMIA 6144 strain, to plants that grew in samples of the Red Yellow Ultisol and higher when the same variety was grown in Haplic Planosol. The same isolate still stimulated the development of the root system of the variety BR1, but only when the plants were cultivated in Haplic Planosol. The isolate

Table 6. Dry mass of the root, of the aerial part, of the nodules and nitrogen accumulation in the aerial part of the peanut varieties BR 1 and BRS Havana, grown in pots with two distinct types of soil and inoculated with rhizobial isolates or recommended strain in the absence or presence of mineral nitrogen.

Isolate	Red Yellow Ultisol		Planosol		Mean
	BR-1	BRS-Havana	BR-1	BRS-Havana	
Root dry weight (g planta⁻¹)					
Absolute control	1.8 ^{abA}	1.5 ^{bA}	3.8 ^{abA}	2.5 ^{abB}	2.4 ^c
N supplied control	2.5 ^{abA}	3.1 ^{aA}	4.2 ^{aA}	3.4 ^A	3.3 ^a
SEMIA 6144	2.1 ^{abA}	2.7 ^{aA}	2.9 ^{bcA}	2.1 ^{cA}	2.5 ^{bc}
AM 07	2.8 ^{aA}	1.4 ^{bB}	2.4 ^{cA}	2.8 ^{abA}	2.4 ^c
AM 01	1.7 ^{bA}	2.3 ^{abA}	3.0 ^{abcB}	4.9 ^A	3.0 ^{ab}
CV (%)			25.45		
Nodule dry weight (mg plant⁻¹)					
Absolute control	160	190	130	100	145 ^b
N supplied control	160	140	40	40	95 ^c
SEMIA 6144	250	200	150	120	180 ^{ab}
AM 07	290	210	160	110	193 ^a
AM 01	360	260	120	140	220 ^a
CV (%)			26.35		
Shoot dry weight (g plant⁻¹)					
Absolute control	14.7 ^{cA}	12.3 ^{cA}	10.2 ^{abA}	8.4 ^{aA}	11.4 ^c
N supplied control	21.0 ^{aA}	19.6 ^{aA}	11.6 ^{aA}	10.2 ^{aA}	15.6 ^a
SEMIA 6144	14.7 ^{cA}	17.7 ^{abA}	9.3 ^{abA}	8.9 ^{aA}	12.7 ^{bc}
AM 07	17.7 ^{bcA}	14.7 ^{bcA}	7.3 ^{bA}	9.5 ^{aA}	12.3 ^{bc}
AM 01	19.0 ^{abA}	14.5 ^{bcB}	8.5 ^{abA}	10.3 ^{aA}	13.1 ^b
CV (%)			14.16		
Total N of the shoot (mg plant⁻¹)					
Absolute control	408 ^{bA}	409 ^{abA}	265 ^{abA}	211 ^{aA}	323 ^b
N supplied control	473 ^{bA}	545 ^{aA}	354 ^{aA}	332 ^{aA}	426 ^a
SEMIA 6144	482 ^{bA}	534 ^{abA}	225 ^{abA}	236 ^{aA}	369 ^{ab}
AM 07	512 ^{abA}	400 ^{bA}	197 ^{bA}	246 ^{aA}	339 ^b
AM 01	628 ^{aA}	450 ^{abB}	214 ^{abA}	252 ^{aA}	384 ^{ab}
CV (%)			22.89		

The same letter, lower case in the column and upper case on the line (among genotypes on the same soil) do not differ statistically among themselves, by the t-test (LSD) at a 5% level of probability. Means without a letter were not significant by the F-test at 5% probability.

AM 07 did not positively influence the development of the root system of none of the varieties in both the soils.

DISCUSSION

Fast-growing bacteria able to acidify the culture medium have been isolated from peanut nodules (Santos et al., 2005; Borges et al., 2007; Lyra et al., 2013) and from other tropical legumes such as velvet beans (Lima et al., 2012), the cowpea (Leite et al., 2009), the pigeonpea (Fernandes Júnior et al., 2012) and the yam bean (Freitas et al., 2007) in studies carried out in Brazil. Apart

from nodulating abundantly with slow-growing bacteria, commonly called as cowpea miscellany or tropical *Bradyrhizobium* (Thies et al., 1991), the peanut is considered a species capable of forming nodules in association with a wide range rhizobia showing great diversity (Borges et al., 2007), including the strains of fast growth (Santos et al., 2005; Taurian et al., 2006; Lyra et al., 2013). In this study, the majority of the bacteria showed rapid growth and acidification of the culture media.

In this study, the non-solubilization of calcium phosphate by *Bradyrhizobium* sp. is possibly related to the increase of the pH, which is a characteristic of bacteria of this genus. In spite of there are bradyrhizobia with able to

solubilize calcium phosphate “*in vitro*” (Marra et al., 2011) a mechanism that better explains this characteristic is the exudation of organic acids (Marra et al., 2012), which would probably be rare for bacteria with a reaction to alkaline pH and does not occur in the strain AM 01 that showed a high similarity to *Bradyrhizobium* sp. In spite of a large quantity of rhizobial isolates being able to solubilize insoluble phosphates, the low efficiency of solubilization by this group of bacteria has been reported in the literature (Hara and Oliveira, 2005; Chagas Júnior, 2010; Marra et al., 2012).

Abiotic factors such as temperature, pH and salinity interfere positively or negatively with the growth of the rhizobia (Nóbrega et al., 2004; Xavier et al., 2007; Fernandes Júnior et al., 2012). The tolerance of the isolate AM 07 to saline stress was higher than what was observed for the other two evaluated bacteria, which may be related to the high production of mucus by this isolate, as available results in the literature report that, among other mechanisms, the production of exopolysaccharides may be related to the survival of rhizobial isolates and various potentially stressing environmental factors (Bushby and Marshall, 1977; Fernandes Júnior et al., 2010; Bomfeti et al., 2011).

The molecular analysis of the evaluated rhizobial isolates showed that there was a great genetic variability among the evaluated bacteria. It was found that many bacteria have less than 40% similarity. The slow growing strains also showed low similarity with the SEMIA 6144 strain, which indicates that, apart from sharing phenotypic characteristics, they are isolates that differs genetically.

The variability of rhizobia and other tropical rhizobacteria has been evaluated by the Box-PCR technique and the current results of this study corroborate the large genetic variability of the bacteria associated with the crop species (Kaschuk et al., 2006; Freitas et al., 2007; Stocco et al., 2008; Lyra et al., 2013) and native plant species (Fernandes Júnior et al., 2013). Recently, Lyra et al. (2013) applying the Box-PCR technique to assess the diversity of peanut rhizobia from Brazilian northeast region also showed less than 50% of similarity among the 22 bacteria evaluated. There are no available data in the literature where the genetic variability of peanut rhizobia from Brazilian southeast region was evaluated by the Box-PCR technique.

The almost complete 16S rDNA gene sequencing of the bacteria with better symbiotic efficiency in the original variety showed an increased proximity with *Bradyrhizobium* sp., the SEMIA 6144 strain and *Rhizobium tropici* CIAT 899^T. The genetic similarity of the isolate AM 01 with the recommended strain for peanut culture in Brazil may be related to the high symbiotic performance of this isolate. The two classic genera of rhizobia identified in this study are widely distributed in soils in South-America and their capacity to nodulate the peanut, including the isolate of the *Rhizobium* genus, has already been demonstrated (Taurian et al., 2006).

The nodulating bacteria of peanuts here studied show a large variability with respect to their symbiotic efficiency. One isolate from BR 1 genotype showed increased efficiency and did not differ statistically from SEMIA 6144 reference strain and was superior to the N supplied control in regarding the nitrogen accumulated in the aerial part and nodulation parameters. None of the isolates of the variety BRS Havana showed a performance equal to the SEMIA 6144 strain. The variability in symbiotic efficiency among isolates has been noted while evaluating rhizobia cultures of various species of tropical legumes (Florentino et al., 2010; Lima et al., 2012). The variability in the symbiotic capacity of the rhizobial culture collections has been in agreement with the biodiversity observed in these micro-organisms. Therefore, symbiotic efficiency of rhizobia is very dependent, among other factors, on various bacterial genes. It is expected that bacteria with a large genetic variability, such as the ones evaluated in this study, show a variable symbiotic efficiency.

The genotype BR 1 was more responsive to the inoculation of the rhizobial isolates in this study compared to the genotype BRS Havana, both in the trials in sterile substrate and in the soil. However, when analyzing the treatments inoculated with the reference strain, the effectiveness of inoculation was larger for the genotype BRS Havana. The variability in the response of these varieties to inoculation with the recommended SEMIA 6144 strain has already been demonstrated in a recent study where the variety BRS Havana showed a larger effectiveness when compared to the variety BR 1 when inoculated in a sandy loam soil (Melo, 2013). This way, strain selection studies can be conducted with a greater emphasis on inoculation of the variety BR 1 because the variety BRS Havana establishes a more efficient association with the currently recommended strain.

In the present study, a large genetic and phenotypic variability can be observed in rhizobia isolated from the soils in the states of Rio de Janeiro and São Paulo. The strains AM 01 and AM 07 showed potential for evaluation in network trials aiming the recommendation of new rhizobial strains for the inoculation of peanut.

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