academicJournals

Vol. 12(17), pp. 2147-2156, 24 April, 2013
DOI: 10.5897/AJB11.1574
ISSN 1684–5315 © 2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Phenotypic and molecular characteristics of rhizobia isolated from nodules of peanut (*Arachis hypogaea* L.) grown in Brazilian Spodosols

Maria do Carmo Catanho Pereira de Lyra[1], Ana Dolores Santiago de Freitas[2]*, Taciana Amorim Silva[3] and Carolina Etienne de Rosália e Silva Santos[2]

[1]Instituto Agronômico de Pernambuco (IPA), Laboratório de Genômica. Av. Gal. San Martin, 1371, Bongi. Recife, PE, CEP 50761-000, Brasil.
[2]Universidade Federal Rural de Pernambuco (UFRPE), Departamento de Agronomia, Laboratório de Microbiologia do Solo. Rua Dom Manoel de Medeiros, s/n, Dois Irmãos, Recife, PE, CEP 52171-900, Brasil.
[3]Universidade Federal do Amazonas (UFAM), Instituto de Ciências Biológicas. Rua Gen. Rodrigo Octávio Jordão Ramos, 3000, setor sul, Bloco D, Sala 08 Coroado I, Manaus, AM, CEP:69077-000, Brasil.

Accepted 17 April, 2013

Peanut (*Arachis hypogaea* L.) is an important crop that can fix nitrogen through symbiosis with rhizobia. Rhizobia populations with diverse characteristics than those traditionally described as peanut microsymbionts have been found in tropical soils. With the objective of studying the diversity and phylogeny of these rhizobia, 22 bacterial strains were isolated from nodules of seven peanut varieties grown in Spodosols of Pernambuco State, Brazil. The isolates were examined in culture medium by means of some of their phenotypic characteristics and tested for intrinsic antibiotic resistance (IAR). DNA profiles were determined with the BOX-PCR and compared with 19 reference strains. All isolates showed rapid growth, and most of them acidified the culture medium. In general, high antibiotic resistance was observed to penicillin G, chloramphenicol and tetracycline and susceptibility was observed to neomycin, erythromycin and rifampicin. The analysis of their phenotypic characteristics, that is, colony morphology and IAR, provided little information about the phylogeny of the isolates. However, using compilation of phenotypic and molecular characteristics, we were able to observe a great diversity of these rhizobia and to reveal the presence of new species.

Key words: Antibiotic resistance, *Arachis hypogaea* L., BOX-PCR, colony morphology, diversity.

INTRODUCTION

Due to the importance of nitrogen for crop productivity and economic and environmental problems associated with the use of chemical fertilizers, nitrogen-fixing microorganisms have attracted significant attention, especially the ones capable of forming symbiosis with Leguminosae, generically known as rhizobia. To date, current taxonomy of these bacteria includes species belonging to several species of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, *Azorhizobium* (Liu et al., 2007), *Methylobacterium* (Kato et al., 2008), *Devosia* (Verma et al., 2009), *Burkholderia* (Bontemps et al., 2010), *Cupriavidus* (Liu et al., 2012), *Shinella* (Lin et al., 2008) and others. Using phenotypic and molecular

*Corresponding author. E-mail: ana.freitas@depa.ufrpe.br. Tel: 55-81-33206237.

Table 1. Characteristics of the soils under peanut cultivation in Goiana municipalitiy, State of Pernambuco, Brazil.

Characteristic	Soil 1	Soil 2
Soil type	Spodosol	Spodosol
pH (H_2O)	4.5	5.0
P (mg dm^{-3})	5	3
Sand (g kg^{-1})	750	850
Silt (g kg^{-1})	100	150
Clay (g kg^{-1})	150	100
Ca^{2+}($cmol_c dm^{-3}$)	2.0	2.5
Al^{3+}($cmol_c dm^{-3}$)	1.5	1.0
H^{+}($cmol_c dm^{-3}$)	3.0	3.0
Mg^{2+}($cmol_c dm^{-3}$)	1.8	2.0
Na^{+}($cmol_c dm^{-3}$)	0	0
K^{+}($cmol_c dm^{-3}$)	86	86

methods, the presence of rhizobial populations with quite diverse characteristics has been verified in tropical soils (Martins et al.,1997; Lima et al., 2009), indicating the possibility of the existence of species that have not yet been described.

Traditionally, rhizobia isolated from peanut nodules (*Arachis hypogaea* L.) were categorized into the "cowpea miscellany" group (Allen and Allen, 1981), a very heterogeneous mixture comprising poorly characterized slow-growing strains that alkalinize the culture medium; these strains are known to belong to the genus *Bradyrhizobium* (Thies et al., 1991). However, Santos et al. (2007) reported the predominance of fast-growing bacteria that acidify the medium forming symbiosis with peanut grown in the soils of Northeastern Brazil. Previously, Taurian et al. (2006) also found that peanut forms symbiosis with fast-growing bacteria closely related to *Rhizobium giardini* and *Rhizobium tropici*.

Peanut is an important oilseed crop playing a significant role in the economy of various countries around the world. In the northeast region of Brazil, peanut is in great demand, and the cultivation of this legume is usually carried out on small farms with limited financial and technological resources, resulting in yields below the culture potential (EMBRAPA, 2006). Peanut nodulation by indigenous bacteria is considered sufficient, and inoculation is rarely performed. Studying the diversity of these bacteria is important in several aspects, both in terms of culture management, with the objective of facilitating the survival of more efficient and specific populations, and regarding the acquisition of adapted and efficient strains recommended for peanut inoculation.

Considering the economic potential of this agronomic crop and the lack of studies on native populations of peanut rhizobia in the soils of Northeastern Brazil, we isolated and characterized bacteria from peanut nodules grown in soils of the state of Pernambuco (07°33'38 S and 35°00'09 W, 13 m asl). The objective of this work was to study the diversity of these rhizobia and to assess the application of phenotypic and molecular methods in the characterization of these bacteria for future work on biological nitrogen fixation.

MATERIALS AND METHODS

Twenty-two (22) rhizobial isolates were obtained from root nodules of seven peanut varieties (L7, BR 1, Caipó, Jumbo, Tatu, IAC 8112 and Nativa) and cultured for 45 days in pots containing soil under peanut cultivation, collected from the layer of 0 to 20 cm in two sites of the municipality of Goiana (07°33'38 S and 35°00'09 W, 13 m asl), in the Zona da Mata physiographic zone of the state of Pernambuco, Brazil. The physical and chemical characteristics of the soils, determined according to the (EMBRAPA, 1997) methodology, were quite similar (Table 1). The isolation of rhizobia was performed according to the Vincent (1970) standard methodology on yeast mannitol agar medium (YMA) with 25 $mgkg^{-1}$ (w/v) Congo red. The isolates were preserved at -80°C in peptone-glycerol medium. The isolates and reference strains are listed in Table 2.

For morphological characterization (Vincent, 1970), the isolates were transferred to YMA medium and grown at 28°C. The following culture parameters were considered: regarding growth period, pH alteration of growth medium, colony morphology (shape, size, border, transparency and surface) and amount of extracellular polysaccharides (EPS) (Xavier et al., 1998).

The isolates were tested for intrinsic antibiotic resistance (IAR) using six antibiotics at three different concentrations: chloramphenicol (2.5, 5 and 10 µg mL^{-1}), erythromycin (100, 200 and 400 µg mL^{-1}), penicillin G (100, 200 and 400 µg mL^{-1}), rifampicin (25, 50 and100 µg mL^{-1}), neomycin (100, 200 and 400 µg mL^{-1}) and tetracycline (1, 2 and 4 µg mL^{-1}). The test was performed in Petri dishes plated with solid tryptone yeast extract (TY) medium.

For molecular characterization, the isolates, along with the 19 reference strains listed in Table 2, were grown in YM medium (YMA without agar) for 48 h. Subsequently, DNA extraction was performed using the methodology described by Lyra et al. (2006). The amplification of genomic DNA was performed by PCR using the primer BOXA1R (Versalovic et al., 1994). The amplification was performed with an initial denaturation step at 95°C for 7 min followed by 30 amplification cycles, each cycle consisting of a 30 s phase at 90°C, a 1 min phase at 52°C and an 8 min phase at 65°C.

Table 2. Identification of rhizobia isolates from seven varieties of peanut (*Arachis hypogaea* L.) grown in two Spodosols in Goiana municipality, State of Pernambuco, Brazil, and type strains.

Isolate	Peanut variety	Type-strain	
		Strain	Species
LG32	L7	BR 29 (SEMIA 5019)	*Bradyrhizobium elkani*
LG29	BR 1	BR 11 (ATCC 10324)	*B. japonicum*
LG30	BR 1	BR 112 (USDA 205)	*Sinorhizobum fredii*
LG11	Caiapó	BR 7411(ATCC 9930)	*S. meliloti*
LG12	Caiapó	BR 525 (USDA 1037)	*S. medicae*
LG16	Caiapó	BR 526(USDA 4893)	*S. saheli*
LG40	Jumbo	BR 527 (USDA 4894)	*S. terangae*
LG7	TATU	BR 2406 (NGR 234)	*Rhizobium sp*.
LG8	TATU	BR 10016 (CFN 299)	*R. tropici* tipo IIA
LG41	IAC 8112	BR 322 (CIAT 899)	*R. tropici*.tipo IIB
LG42	Native	BR 10026 (CFN 42)	*R. etli*
LG39	L7	BR 10052 (ATCC 14482)	*R. leguminosarum* bv. p*haseoli*
LG34	BR 1	BR 7605 (LGM 6119)	*R. leguminosarum* bv. t*rifolii*
LG35	BR 1	BR 10055 (HAMBI 540)	*R. galegae*
LG23	Caipó	BR 7801 (ATCC 33669)	*Mesorhizobium loti*
LG24	Caipó	BR 521 (USDA 3383)	*M. ciceri*
LG33	Caipó	BR 522 (USDA 3392)	*M. mediterraneum*
LG31	Jumbo	BR 524 (USDA 4779)	*M. huakuii*
LG37A	TATU	BR 5410 (ORS 571)	*Azorhizobium caulinodans*
LG37B	TATU		
LG43	IAC 112		
LG38	Native		

For the final step, an extra 16 min extension was performed at 65°C. The reagents and their final concentrations used in the reaction with a final volume of 10 µl were as follows: genomic DNA (10 to 40 ng), dNTP mix (0.3 mM), Taq DNA polymerase (1 U), $MgCl_2$ (2 mM), the BOX primer (2 mM), reaction buffer 10X (10%) and ultra pure water. The amplified products were stained with ethidium bromide, visualized using a UV transilluminator and photographed for further analysis for constructing dendrogram using bioinformatics tools.

From the phenotypic (culture and IAR) and molecular characteristics of the bacteria, matrices were constructed using the program NTSYS pc version 2.01 (Rohlf, 1973), and cluster analysis dendrograms were generated. Genetic distances were calculated by the Simple Matching coefficient (SM) using the unweighted pair-group method with arithmetic average (UPGMA) clustering method (Hart, 1983), and dendrograms were constructed using the SAHN method (sequential agglomerative hierarchical nested cluster analysis).

RESULTS

The 22 strains showed rapid growth (82% growing out in 24 h and 18% in 48 h) and most (91%) acidified the culture medium. Regarding colony characteristics, 18% of the isolates showed a yellowish color; 82% were beige; 9% were transparent; 36% were punctuate.

According to morphological characteristics, isolates were grouped into two main clusters, with the LG37B separated from the others due to its distinguished culture characteristics (Figure 1). In the first group, nine isolates (LG31, LG32, LG43, LG33, LG34, LG39, LG38, LG35 and LG37A) displayed 100% similarity, differing from other isolates of the same cluster by approximately 20%. The isolates LG40 and LG41 are with 100% DNA profile identity according to the morphological analysis. In cluster II, the isolates LG29 and LG30 are with 100% DNA profile identity and 60% similar to LG23. The dendrogram analysis did not show much variability among different isolates.

All 22 isolates were resistant to penicillin G and chloramphenicol at all tested concentrations (100, 200 and 400 µg mL^{-1} for penicillin; 2.5, 5 and 10 µg mL^{-1} for chloramphenicol). In the presence of tetracycline at a concentration of 1 µg mL^{-1}, 91% of the isolates grew on solid medium; this number fell to 73% at the concentrations of 2 and 4 µg mL^{-1}. In contrast, the isolates were susceptible to neomycin at all three tested concentrations (100, 200 and 400 µg mL^{-1}), with an overall index of 91% non-resistance. Only 14% of the isolates were resistant to the antibiotic erythromycin at the tested concentrations, with no change of the resistance index with increasing concentrations, and resistant to rifampicin at the concentration of 25 µg mL^{-1}. This resistance to rifampicin declined to 9% at the

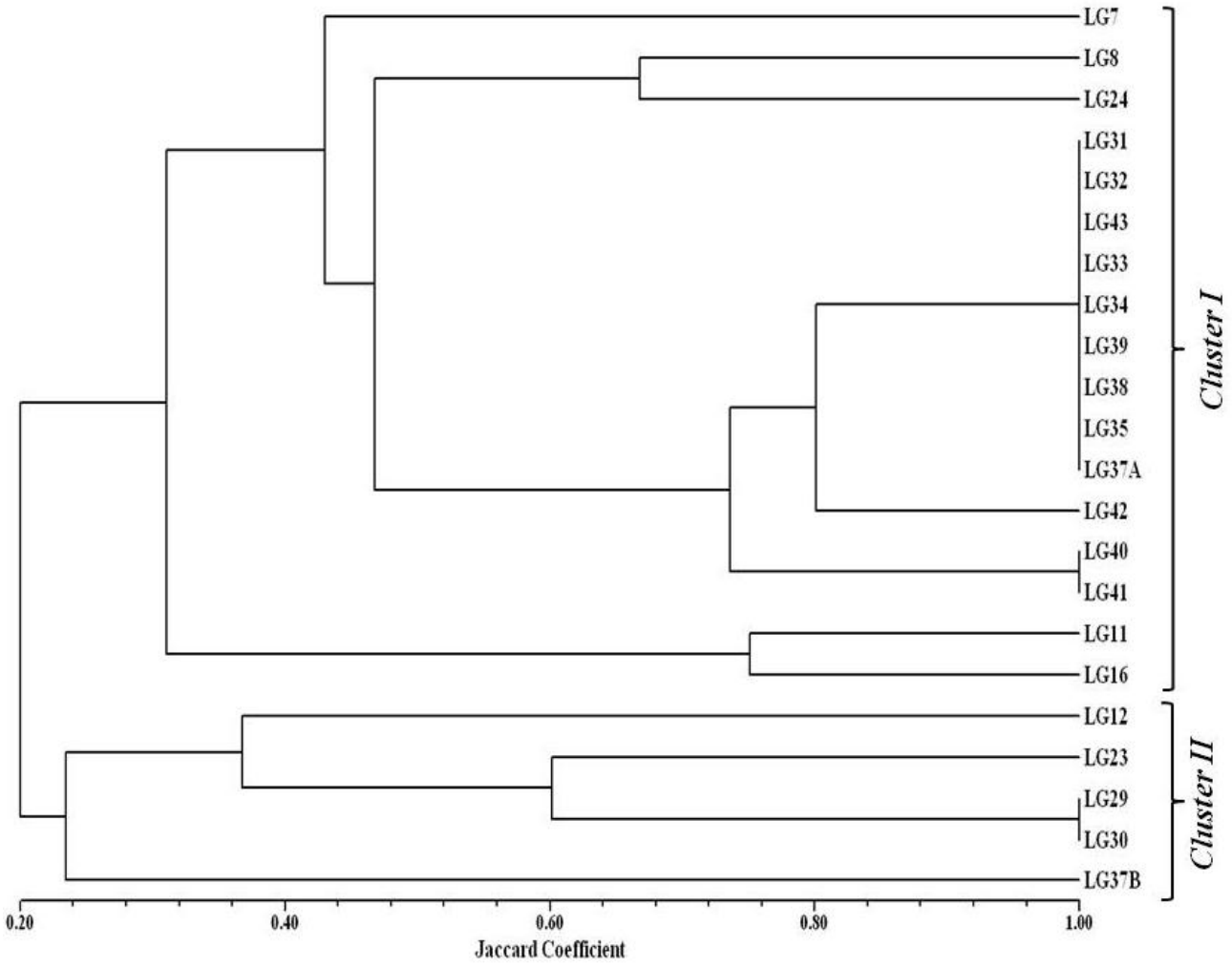

Figure 1. Similarity dendrogram constructed from the cultural characteristics of rhizobia isolated from peanut grown in two Spodosols in Goiana municipalitiy, State of Pernambuco, Brazil.

subsequent concentrations (50 and 100 µg mL^{-1}). The dendrogram constructed using the IAR data (Figure 2) was not effective in distinguishing classifications and grouping a large number of rhizobia within the same branch with maximum similarity indices.

Genetic characterization using the primer BOXA1R generated significant amounts of amplicons that are highly variable in size and number, both from the studied isolates (Figures 3 and 4) and from the reference strains (Figure 5). Most of the amplified fragments were shown as a band of 2000 to 200 bp. With this BOX marker, rhizobia (isolates and reference strains) were first grouped into four clusters (Figure 6). *Azorhizobium caulinodans* strain form external branch, with a genetic distance of 48% in relation to other strains and isolates.

Cluster I formed two major sub-clusters. In the first sub-cluster, all the strains of the genus *Sinorhizobium* were clustered, except *R. tropici* type IIA and type IIB, Rhizobium *etli* and the slow-growing bacteria *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum* that remained in the same branch. In the second sub-cluster, the isolates LG23, LG30 and LG34, as well as the BR52 strain of *Mesorhizobium ciceri*, were not successfully amplified with the BOX element under the amplification conditions performed in this study. The other reference strains (*Rhizobium* sp. BR 2406, *Mesorhizobium ciceri* BR 521, *Rhizobium leguminosarum* bv. *Phaseoli* BR 10052, *Rhizobium galegae* BR 10055 and *Mesorhizobium mediterraneum* BR 524) and the isolates LG 40, LG 41, LG 37 B, LG 42, LG 43, LG 29, LG 35, LG 39, LG 37 and LG 38 showed high variability. Cluster II grouped the isolates LG 8 and LG 12, which appear with a genetic identity of 100%, and LG 7, together with the *Mesorhizobium huakuii* BR 524 strain. In cluster III, the bacteria *R. leguminosarum* bv. *trifolii* and *Mesorhizobium loti* appear with 100% DNA profile

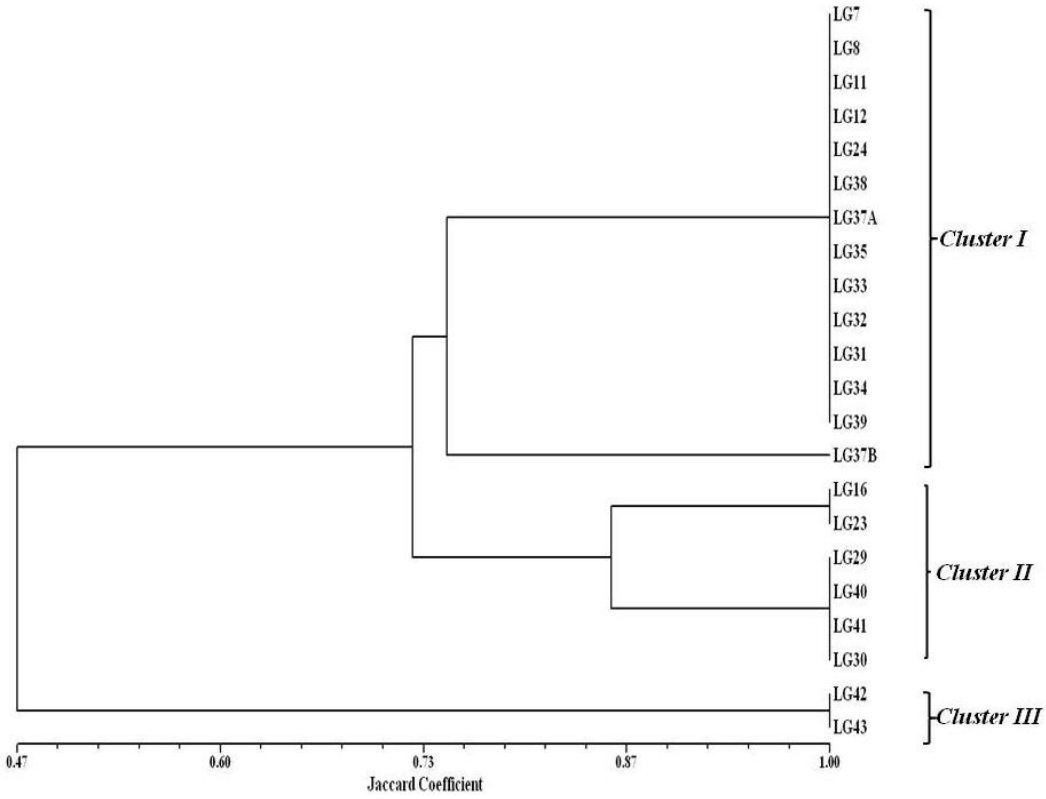

Figure 2. Similarity dendrogram constructed from the characteristics of antibiotic resistance of rhizobia isolated from peanut grown in two Spodosols in Goiana municipalitiy, State of Pernambuco, Brazil.

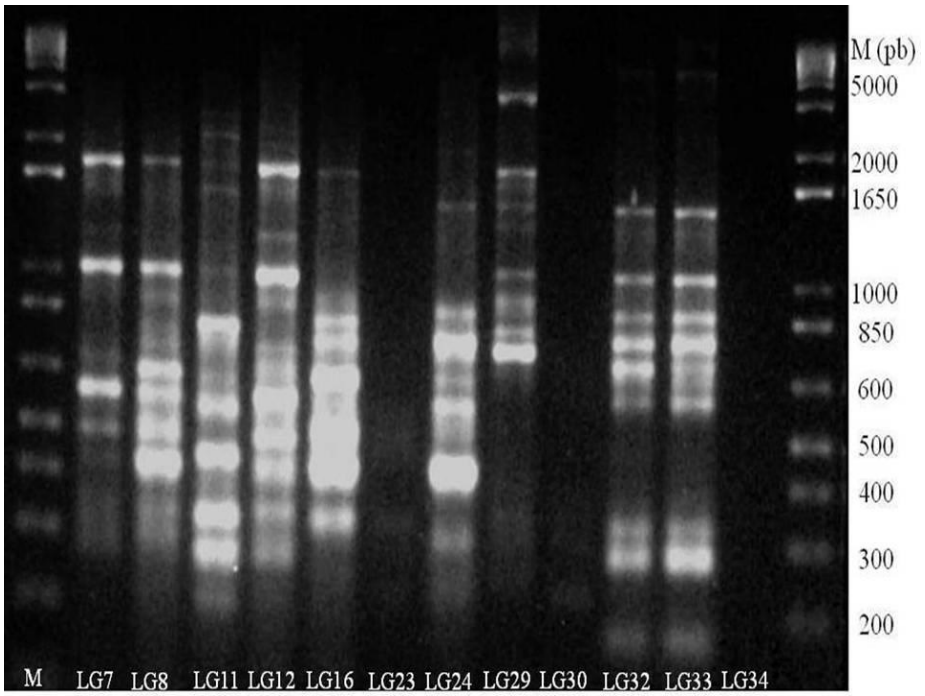

Figure 3. Profile of molecular DNA isolated LG 7, LG 8, LG 11, LG 12, LG 16, LG 23, LG 24, LG 29, LG 30, LG 32, LG 33 and LG 34, amplified with the primer BOXA1R in agarose gel 1%.

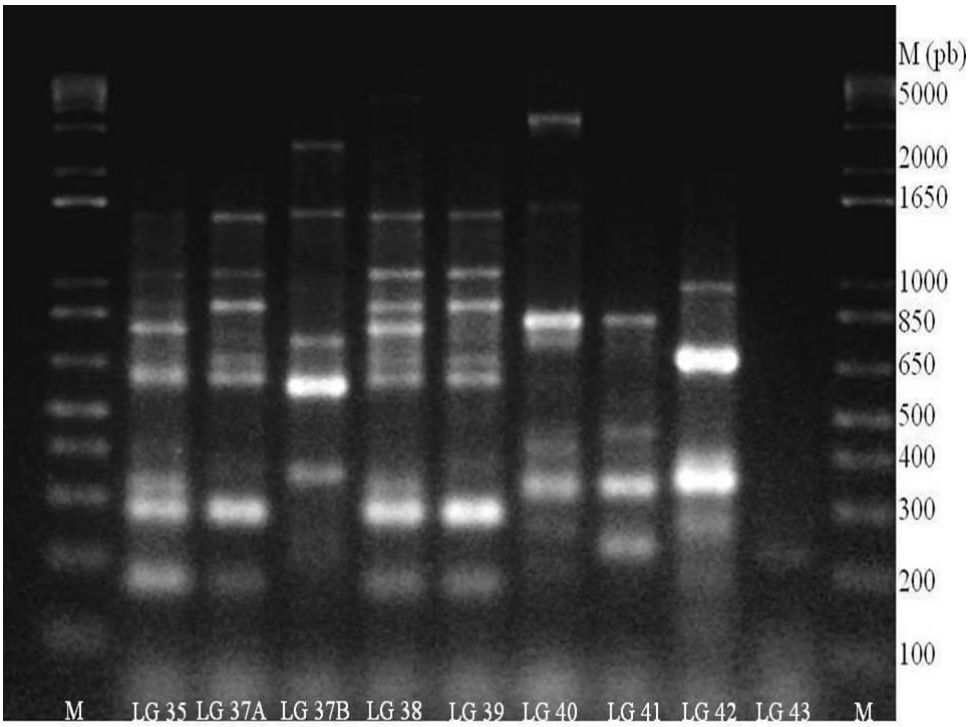

Figure 4. Profile of molecular DNA isolated from LG 35, LG 37 A, B LG 37, LG 38, LG 39, LG 40, LG 41, LG 42 and LG 43, amplified with the primer BOXA1R in agarose gel 1%.

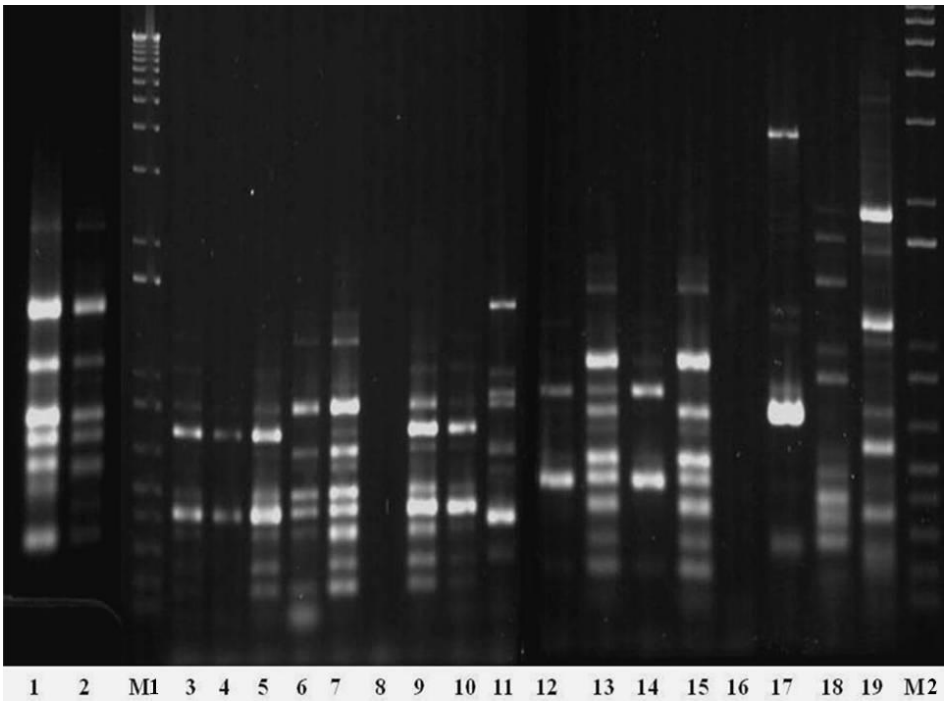

Figure 5. Profile of molecular DNA of reference strains amplified with the primer BOXA1R in agarose gel 1%. Legend: 1) BR29; 2) BR111; M1) Ladder 100 pb Invitrogen; 3) BR112; 4) BR7411; 5) BR525; 6) BR526; 7) BR527; 8) BR2406; 9) BR10016; 10) BR322; 11) BR10026; 12) BR10052; 13) BR7605; 14) BR10055; 15) BR7801; 16) BR521; 17) BR522; 18) BR524; 19) BR5410; M2) 1 Kb Plus Invitrogen.

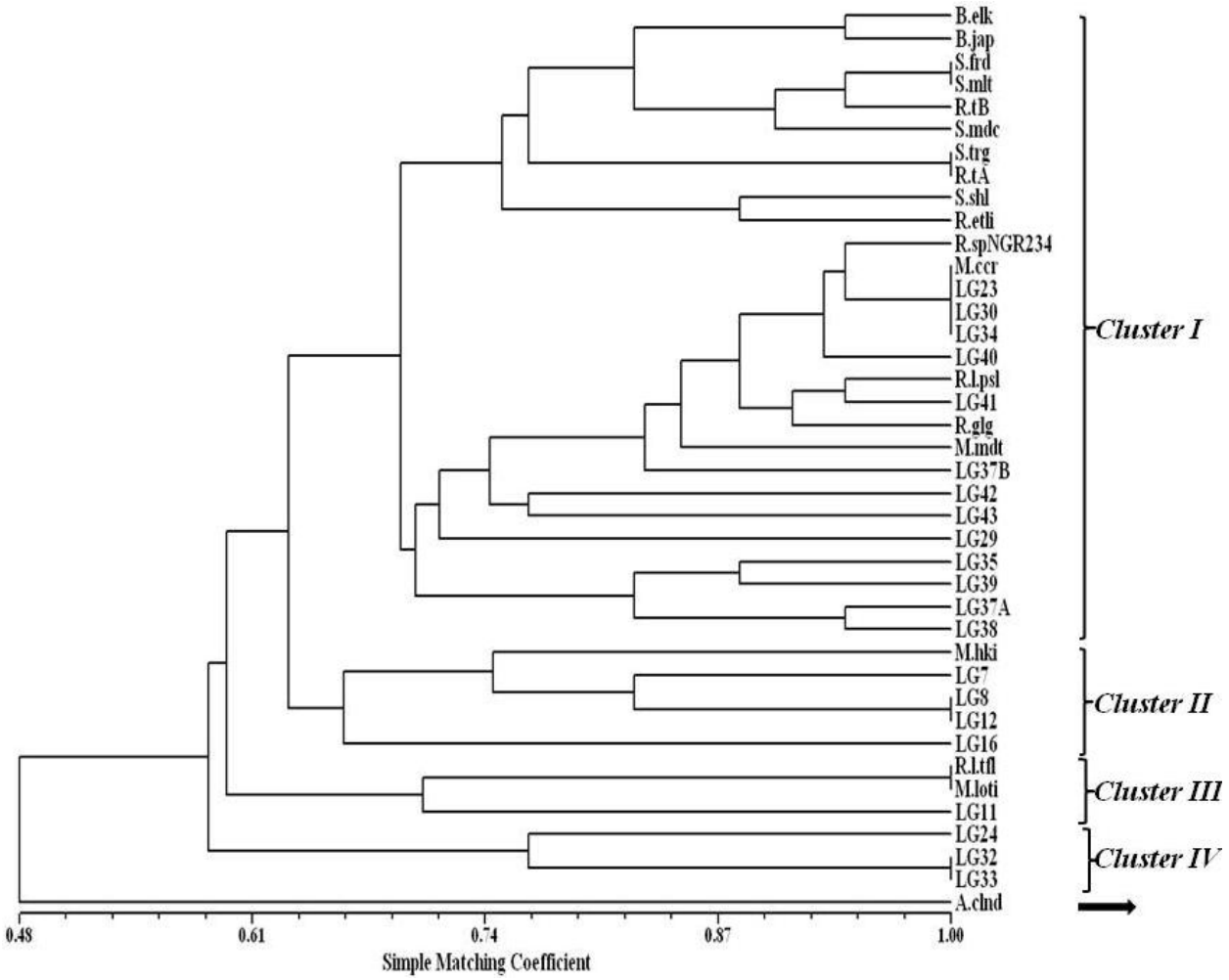

Figure 6. Dendrogram of similarity of molecular characteristics (profile of DNA amplified with primer BOXAR1 in agarose gel 1%) of reference strains and rhizobia isolated from nodules of peanut grown in two Spodosols in Goiana municipalitiy, State of Pernambuco, Brazil.

identity. In cluster IV, the isolates LG32 and LG33, appearing with 100% DNA profile identity, were grouped together with LG24, which showed a similarity of 77% to the previous two. Of the 22 isolates, 18 (85.71%) showed no similarity, a coefficient equal to 100%, to any of the reference strains used in this experiment.

The combination of the morphological, IAR and molecular data of these isolates (Figure 7) presented three clusters and two isolates with a monophyletic branch (LG16 and LG37B). Only the isolates LG32 and LG33 are with 100% DNA profile identity. In contrast, LG37B always appeared to be the most different.

DISCUSSION

According to morphological characteristics, isolates did not show much variability among different isolates. The 22 strains showed rapid growth according to the classification of Jordan (1984), with 82% growing out in 24 h and the remaining (18%) in 48 h. As expected for fast-growing rhizobia, most studied isolates (91%) acidified the culture medium. The pH changes generated by rhizobia in the culture medium are due to the preferential use of sugars by rapid-growth strains (Tan and Broughton, 1981), followed by excretion of organic acids and nitrogen compounds by slow-growing strains (Taurian et al., 2006). Other authors also isolated rhizobia with these growth characteristics in culture medium, in peanut growing in Brazilian (Santos et al., 2007) and Argentinean (Angelini et al., 2011; Nievas et al., 2012) soils. However, this finding is not in agreement with the current knowledge that the peanut crop is usually nodulated by slow-growing rhizobia (*Bradyrhizobium*). Pinto et al. (2004) confirmed the predominance of slow-growing bacteria in *Arachis pintoi* grown in the soils of the

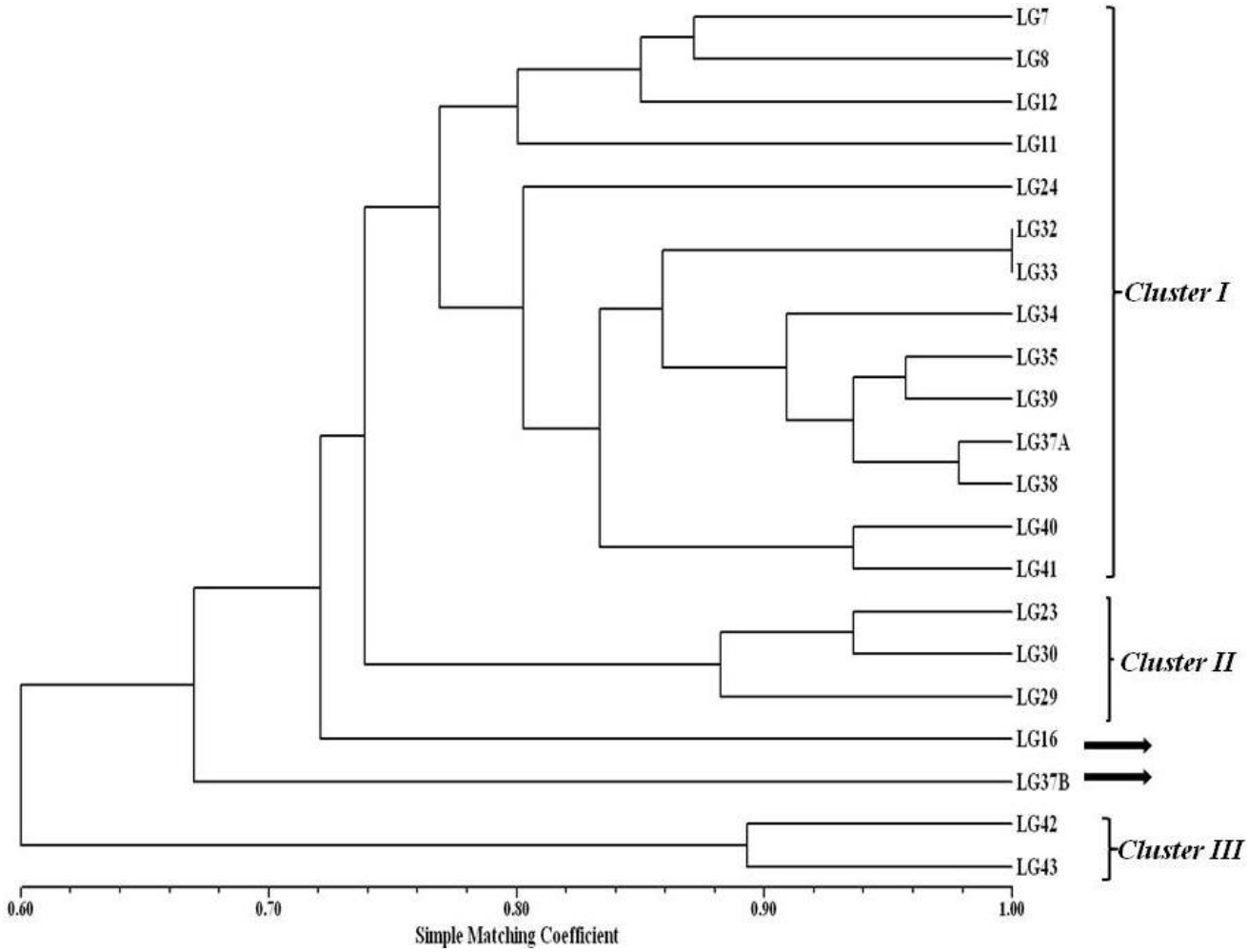

Figure 7. Similarity dendrogram resulting from the compilation of cultural characteristics, antibiotic resistance and molecular (DNA profile, amplified with primer BOXAR1 in agarose gel 1%) rhizobia from nodules of peanut grown in two Spodosols in Goiana municipalitiy, State of Pernambuco, Brazil.

Brazilian Cerrado (savannah). Taken together, these results indicate that peanut can form symbiosis with bacteria harboring diverse culture characteristics. As hypothesized by Angelini et al. (2011), in the field, peanut nodules are occupied by competitive strains. Thus, strains to be used for inoculation of peanuts in the Zona da Mata physiographic zone of the state of Pernambuco should be fast growing, to compete with the native strains.

The production of polysaccharide is a marked characteristic of rhizobia. In general, fast-growing strains produce more polysaccharides than the slow-growing ones (Teixeira et al., 2010). The strains that do not produce polysaccharides are called dry and are more common in slow-growing rhizobia (Eaglesham et al., 1990). In this study, nearly all isolates produced polysaccharide, except for the one that is classified as a dry colony. Polysaccharide can act as a protective barrier against biotic and abiotic factors, enabling the survival of the host organism in the soil (Teixeira et al., 2010; Xavier et al., 1998).

In general, the isolates demonstrated high resistance to the antibiotics penicillin G, chloramphenicol and tetracycline. Antibiotic resistance is a widely used parameter for rhizobium classification; it also serves as an indicator for the presence of actinomycetes in the soil from which rhizobia have been isolated (Martins et al., 1997). There is also a close correlation between antibiotic resistance and polysaccharide production (Fernandes Jr et al., 2012). Intrinsic antibiotic resistance is also one of the characteristics that can distinguish between strains of rhizobia and the pattern of IAR is useful in the strain identification (Rasul et al., 2012). However, the dendrogram constructed using the IAR data (Figure 2) was not effective in distinguishing classifications and grouping a large number of rhizobia within the same

branch with maximum similarity indices. Similar observations have been made by Silva et al. (2002), stating that IAR is not a useful parameter for taxonomic purposes.

Genetic characterization using the primer BOXA1R generated significant amounts of amplicons that are highly variable in size and number, both from the studied isolates (Figures 3 and 4) and from the reference strains (Figure 5). It is possible that some isolates grouped in cluster I represent new rhizobia species capable of nodulating peanut. In cluster III, the bacteria *R. leguminosarum* bv. *trifolii* and *Mesorhizobium loti* had 100% DNA profile identity. However, these species are quite different in their genetic organization. In *R. leguminosarum* bv. *trifolii*, nodulation genes are located in the symbiotic plasmid pSym, while in *M. loti*, these genes are in the chromosome (Spaink et al., 1998). This result can be attributed to the fact that the BOX element is located in a very similar region in the two strains, despite the fact that they have distinctive genetic organization. Of the 22 isolates, 18 (85.71%) showed no similarity, a coefficient equal to 100%, to any of the reference strains used in this experiment, despite the fact that these rhizobia were isolated from plants of the same species and native from the soils of the same physiographic region. This observation further emphasizes the high degree of variability of rhizobia present in the tropics.

Comparing the dendrograms constructed according to the culture, IAR and molecular characteristics, we observed that the first two parameters were not effective in demonstrating the variability among the isolates. The clustering of a large number of isolates in the same branch obtained in the IAR dendrogram showed a high discrepancy with the results obtained from the morphological and molecular analyses. An example of this discrepancy is that the strain LG11, appearing alongside the isolates LG7, LG8, LG12, LG24, LG38, LG37A, LG35, LG33, LG31, LG32, LG34 and LG39 in the same branch, was shown far apart from them in the subsequent dendrograms; it was clustered with the isolate LG16 in the morphological dendrogram and was completely isolated in the molecular dendrogram.

The dendrogram resulting from the compilation of cultural characteristics, antibiotic resistance and molecular data of the isolates (Figure 7) presented three clusters and two isolates with an external branch (LG16 and LG37B). LG37B appeared to be the most different isolate and may be a novel species because it presents high diversity in morphology, IAR and molecular profiles as compared to other studied isolates. By combining the data together, the high variability among the isolates was even more striking.

According to the results shown in the constructed dendrograms, the number of taxonomic groups generated from the compilation of phenotypic and molecular characteristics (20 groups) was larger than the number generated from molecular data (18 groups), culture data (12 groups) and from the IAR data (5 groups). These results were expected because the compilation of several characteristics decreases the possibility of interpretation errors as compared to the analysis based on only one cellular character (Tan and Broughton, 1981).

Taking into account the definition of species (Stackebrandt and Goebel, 1994), one can consider that the isolates LG32 and LG33 belong to the same taxonomic group based on the constructed dendrograms because they showed maximum similarity in all dendrograms. Regarding environmental impact and morphological polymorphism, a relationship between the origin and the presented similarity of the isolates was observed. For example, in the first cluster of the morphological dendrogram, the isolates LG31, LG32, LG43, LG33, LG34, LG39, LG38, LG35 and LG37A appeared in the same branch, and all, except LG32, were native to the same soil in a peanut plantation. The same correlation was observed in the isolates LG40 and LG41, as well as in LG29 and LG30, which were native to the soil of the Umbú mill in Goiana, PE. The similarity between rhizobial isolates sharing the same microclimate and edaphoclimatic environment can be explained by the fact that bacteria develop similar adaptation strategies in a similar environment. It is unlikely to identify changes incorporated into the bacterial genome solely based on the morphological characterization. Morphological changes are more relevant to abiotic factors than to genetic factors (Straliotto and Rumjanek, 1999). Therefore, the rhizobia populations in our study may be recently established and may have not been completely adapted yet.

Bacteria from nodules of peanut plants cultivated in soils of the Zona da Mata physiographic zone of the state of Pernambuco have different characteristics from those described for the cowpea miscellaneous group. In these soils, the predominant bacteria are fast growing, produce acid in YMA medium and have attributes related to survival strategies (abundant exopolysaccharides and resistance to antibiotics). However, phenotypical characteristics provide little information on the diversity and phylogeny of these bacteria, while molecular analyses reveal large genetic diversity and differences among strain types, which indicates the possible occurrence of new species.

REFERENCES

Allen ON, Allen EK (1981). The Leguminosae: a source book of characteristics, uses, and nodulation. The University of Wiscosin Press, Madison, USA.

Angelini J, Ibáñez F, Taurian T, Tonelli ML, Valetti L, Fabra A (2011). A study on the prevalence of bacteria that occupy nodules within single peanut plants. Curr. Microbiol. 62:1752-1759.

Bontemps C, Elliott GN, Simon MF, Reis Jr FB, Gross E, Lawton RC, Elias Neto N, Loureiro MF, Faria SM, Sprent JI, James EK, Young JPW (2010). *Burkholderia* species are ancient symbionts of legumes. Mol. Ecol. 19:44-52.

Eaglesham ARJ, Ellis JM, Evans R, Fleischam DE, Hungria M, Hardy RWF (1990). The first photosynthetic nitrogen-fixing *Rhizobium*: characteristics. In: *Nitrogen fixation: achievements and objectives* (Gresshoff PM, Roth LE, Stacey G, Newton WE ed.). Chapman, New York, pp. 805-811.

EMBRAPA. Centro Nacional de Pesquisa de Solos (1997). Manual de métodos de análise de solos. EMBRAPA ,Rio de Janeiro, Brazil.

EMBRAPA. Centro Nacional de Pesquisa do Algodão (2006). Cultivo do Amendoim. http://sistemasdeproducao.cnptia.embrapa.br/FontesHTML/Amendoim/CultivodoAmendoim/index.html

Fernandes Júnior PI; Lima AA, Passos SR, Gava CAT, Oliveira PJ, Rumjanek NG, Xavier GR (2012). Phenotypic diversity and amylolytic activity of fast growing rhizobia from pigeonpea [*Cajanus cajan* (L.) MILLSP.]. Braz. J. Microbiol. 43:1604-1612.

Hart G (1983). The occurrence of multiple UPGMA phenograms. In: *Numerical Taxonomy* (Felsenstein J ed.). Springer-Verlag, New York, pp. 254-258.

Jordan DC (1984). Family III. Rhizobiaceae. In: *Bergey's Manual of Systematic Bacteriology* (Krieg NR, Holt JG ed.). Williams and Wilkins, Baltimore, pp. 234-254.

Kato Y, Asahara M, Goto K, Kasai H, Yokota A (2008). *Methylobacterium persicinum* sp. nov., *Methylobacterium komagatae* sp. nov., *Methylobacterium brachiatum* sp. nov., *Methylobacterium tardum* sp. nov. and *Methylobacterium gregans* sp. nov., isolated from freshwater. Int. J. Syst. Evol. Microbiol. 58:1134-1141.

Lima AS, Nóbrega RSA, Barberi A, Silva K, Ferreira DF, Moreira FMS (2009). Nitrogen-fixing bacteria communities occurring in soils under different uses in the Western Amazon Region as indicated by nodulation of siratro (*Macroptilium atropurpureum*). Plant Soil 20:1-19.

Lin DX, Wang ET, Tang H, Han TX, He YR, Guan SH, Chen WX (2008). *Shinella kummerowiae* sp. nov., a novel symbiotic bacterium isolated from root nodule of the herbal legume *Kummerowia stipulacea*. Int. J. Syst. Evol. Microbiol. 58:1409-1413.

Liu XY, Wei1 S, Wang F, James EK, Guo XY, Zagar C, Xia LG, Dong X, Wang YP (2012). *Burkholderia* and *Cupriavidus* spp. are the preferred symbionts of *Mimosa* spp. in Southern China. FEMS Microbiol. Ecol. 80:417-426.

Lyra MCC, López-Baena FJ, Madinabeitia N, Vinardell JM, Espuny MR, Cubo MT, Bellogín RA, Ruiz-Sainz JE, Ollero FJ (2006). Inactivation of the *Sinorhizobium fredii* HH103 *rhcJ* gene abolishes nodulation outer proteins (Nops) secretion and decreases the symbiotic capacity with soybean. Int. Microbiol. 9:125-133.

Martins LMV, Neves MCP, Rumjanek NG (1997). Growth characteristics and symbiotic efficiency of rhizobia isolated from cowpea nodules of the north-east region of Brazil. Soil Biol. Biochem. 29:1005-1010.

Nievas F, Bogino P, Nocelli N, Giordano W (2012). Genotypic analysis of isolated peanut-nodulating rhizobial strains reveals differences among populations obtained from soils with different cropping histories. Appl. Soil Ecol. 53:74-82.

Pinto PP, Paiva E, Purcino H, Passos RVM, Sá NMH (2004). Characterization of rhizobia that nodulate *Arachis pintoi* by RAPD analysis. Braz. J. Microbiol. 35:219-223.

Rasul A, Amalraj Eld, Kumar Gp, Grover M., Venkateswarlu B. (2012). Characterization of rhizobial isolates nodulating *Millettia pinnata* in India. FEMS Microbiol Lett. 336:148-158.

Rohlf FJ (1973). Algorithm 76. Hierarchical clustering using the minimum spanning tree. Comput. J. 16:93-95.

Santos CERS, Stamford NP, Borges WL, Neves MCP, Rumjanek NG, Nascimento LRS, Freitas ADS, Vieira IMMB, Bezerra RV (2007). Faixa hospedeira de rizóbios isolados das espécies *Arachis hypogaea*, *Stylosanthes guyanensis e Aeschynomene americana*. Rev. Bras. Cienc. Agrárias. 2:20-27.

Silva MLRB, Burtiy HA, Figueiredo MVB, Freitas NSA, Mergulhão ACES, Lyra MCCP (2002). Caracterización via RAPD (Random Amplified Polymorphic DNA) de aislamiento de rizobios de suelos ácidos y alcalinos en la región semi árida de Pernambuco. Rev. Argent. Microbiol. 34:186-192.

Spaink HP, Kondorosi A, Hooykaas PJJ (1998). The rhizobiaceae: molecular biology of model plant-associated bacteria. Kluwer, Dordrecht, Germany.

Stackebrandt E, Goebel BM (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44:846-849.

Straliotto R, Rumjanek NG (1999). Aplicação e evolução dos métodos moleculares para o estudo da biodiversidade do rizóbio. EMBRAPA-CNPAB, Seropédica, Brazil.

Tan IKP, Broughton WJ (1981). Rhizobia in tropical legumes. XIII Biochemical basis of acid and alkali reations. Rev. Argent. Microbiol. 34:186-192.

Taurian T, Ibañez F, Fabra A, Aguilar OM (2006). Genetic diversity of rhizobia nodulating *Arachis hypogaea* L. in Central Argentina soils. Plant Soil 282:41-52.

Teixeira FCP, Borges, WL Xavier GR, Rumjanek NG (2010). Characterization of indigenous rhizobia from caatinga. Braz. J. Microbiol. 41:201-208.

Thies JE, Bohlool BB, Singleton PW (1991). Subgroups of cowpea miscellany: symbiotic specificity within *Bradyrhizobium* spp. for *Vigna unguiculata*, *Phaseolus lunatus*, *Arachis hypogaea* and *Macroptilum atropurpureum*. Appl. Environ. Microbiol. 57:1540-1545.

Verma M, Kumar M, Dadhwal M, Kaur J, Lal R (2009) Devosia albogilva sp. nov. and Devosia crocina sp. nov., isolated from a hexachlorocyclohexane dump site. Int. J. Syst. Evol. Microbiol. 59:795-799.

Versalovic J, Schneider M, De Bruijn FJ, Lupski JR (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Method. Mol. Cell Biol. 5:25-40.

Vincent JM (1970). A Manual for the Practical Study of Root Nodule Bacteria. IBP Handbook No 15. Blackwell, Oxford, England.

Xavier GR, Martins LMV, Neves MCP, Runjanek NG (1998). Edaphic factors as determinants for distribution of intrinsic antibiotic resistance in a cowpea rhizobia population. Biol. Fertil. Soils 27:386-392.