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Phenotypic and molecular fingerprinting of fast growing rhizobia of field-grown pigeonpea from the eastern edge of the Brazilian Pantanal

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ABSTRACT. The aim of this study was to evaluate the diversity of rhizobial isolates obtained from root nodules of pigeonpea plants grown at the eastern edge of the Brazilian Pantanal. The bacterial isolates were isolated from root nodules from field-growing pigeonpea grown in two rural settlements of the Aquidauana municipality. The bacterial isolates were characterized phenotypically by means of

cultural characterization, intrinsic antibiotic resistance (IAR), salt and high incubation temperature tolerance, and amylolytic and cellulolytic activities. The molecular characterization of the bacterial isolates was carried out using amplified ribosomal DNA restriction analysis (ARDRA) and Box-polymerase chain reaction (PCR) techniques. In addition, the symbiotic performance of selected rhizobial isolates was evaluated in a greenhouse experiment using sterile substrate. The phenotypic characterization revealed that the bacterial strains obtained from pigeonpea root nodules presented characteristics that are uncommon among rhizobial isolates, indicating the presence of new species nodulating the pigeonpea plants in the Brazilian Pantanal. The molecular fingerprinting of these bacterial isolates also showed a highly diverse collection, with both techniques revealing less than 25% similarity among bacterial isolates. The evaluation of symbiotic performance also indicated the presence of microorganisms with high potential to increase the growth and nitrogen content at the shoots of pigeonpea plants. The results obtained in this study indicate the presence of a highly diversified rhizobial community nodulating the pigeonpea at the eastern edge of the Brazilian Pantanal.

Key words: Biological nitrogen fixation; Diversity; Microbial ecology

INTRODUCTION

Biological nitrogen fixation (BNF) is a natural process carried out by diazotrophic bacteria, which reduces atmospheric nitrogen into ammonia through the action of the nitrogenase enzymatic complex. Among the diazotrophs, the rhizobia group can establish associations with legume plants forming root and/or stem nodules, where BNF occurs very efficiently (Sprent, 2007). The isolation and characterization of rhizobia from grain legumes such as soybean, cowpea, and common bean, have resulted in the identification of bacteria able to fix high amounts of N for use in improving crop nutrition and yield (Hungria et al., 2000; Martins et al., 2003). On the other hand, rhizobial isolation from other neglected crops, such as those used as green manure, is beginning in Brazil, and recent studies regarding the diversity and biotechnological applications of bacteria from green manure root nodules have shown great potential (Fernandes Jr. et al., 2010, 2011; Lima et al., 2012).

Pigeonpea (*Cajanus cajan* L Millsp.) is the only crop member of the Cajaninae tribe (Hancock, 2004). This species is a very rustic crop that can grow vigorously in soils with low fertility, and for this reason, is cultivated mainly in marginal lands in Brazil (Beltrame and Rodrigues, 2007). Indeed, pigeonpea is able to associate with a large diversity of indigenous rhizobia in soil, reaching more than 150 kg of fixed N per hectare per year (Peoples et al., 1995). To exploit the BNF potential of this crop, the selection and evaluation of new rhizobial strains from different areas where pigeonpea is cropped must be carried out.

The slow and fast growing pigeonpea rhizobia present great genetic and metabolic diversity and are likely to have new species among the culture collections worldwide (Ramsubhag et al., 2002; Fernandes Jr. et al., 2012). In addition to being efficient in fixing

nitrogen in field conditions, pigeonpea rhizobia also present other biotechnological applications, such as biopolymer production and enzymatic activity (Fernandes Jr. et al., 2010, 2011, 2012).

The Brazilian Pantanal is a large floodplain covering more than 140,000 km^2. Flooding is the most important ecological phenomenon and characterizes the Pantanal as a special ecological macrosystem (Abdon and Silva, 2006). Although the term "Pantanal" refers to a region with essentially hydrophyte vegetation that is common in swamps, xerophytic, and mesophytic species are also distributed in various Pantanal landscape units, with the floristic composition being influenced by the phytogeographic provinces of Cerrado, the Amazon rainforest, semi-deciduous forests, and Chaco (Damasceno-Junior et al., 2005; Oliveira, 2008). Flooding pulses can be caused by a large range of factors, such as fluctuations in the water level of the river and the local distribution of rainfall and groundwater. According to Junk and Silva (1999), the reason for flooding is very important for many flooded ecological areas, because it affects the diversity of organisms in and the nutritional status of the soil.

A non-floodable edge surrounds the floodable lands, where large municipalities, such as Corumbá and Aquidauana, are located. The phytophysiognomies of these provinces are mixed in the Pantanal wetlands, in floodable and flood-free areas, in edge areas, and in the plateaus (Scremin-Dias et al., 2011). Accordingly, evaluations of the diazotrophic bacteria associated with native species from the Pantanal have indicated the presence of a highly diverse and efficient diazotrophic community (James et al., 2001; Brasil et al., 2005), and that the flood regime influences the diversity of plant-associated diazotrophic bacteria (James et al., 2001).

Evaluations of the diversity of rhizobial strains using phenotypic characterization combined with molecular fingerprinting have revealed the great diversity in strains isolated from different Brazilian regions from cowpea (Zilli et al., 2004; Leite et al., 2009; Florentino et al., 2010), yam bean (Freitas et al., 2007), and common bean (Stocco et al., 2008). In the Brazilian Pantanal, studies evaluating the diversity of nodule-forming bacteria with phenotypic and molecular methods remain scarce in spite of the great diversity of the plant cover in the region. The aim of this study was to evaluate the phenotypic and molecular diversity of rhizobia isolated from pigeonpea root nodules from field-growing plants at the eastern edge of the Brazilian Pantanal.

MATERIAL AND METHODS

Bacterial isolation

To obtain the rhizobial isolates, field-growing pigeonpea (*Cajanus cajan* L. Millsp.) (cv. Caqui) was collected at two rural settlements located on the eastern edge of the Brazilian Pantanal in the Corumbá municipality, Mato Grosso do Sul State, Brazil. At the 1st collection site, the "Taquaral" settlement (W57°40'; S19°06'), the pigeonpea was cropped in a Chernozem soil, whereas at the "Mato Grande" settlement (W57°24'; S19°19'), the field-grown pigeonpea was collected at an Oxisol. Ten plants were collected at each site. The roots were separated from the shoots, packed in plastic bags, and transported to the laboratory.

The roots were vigorously washed with tap water, and the nodules were carefully detached and stored in glass pots with silica gel. For rhizobia isolation, the nodules were re-hydrated with sterile distilled water, disinfected superficially with 2% NaOCl (v/v), and washed 10 times with sterile distilled water (Vincent, 1970). The nodules were crushed in Petri dishes containing yeast mannitol agar (YMA) media with Congo Red (Vincent, 1970). The inocu-

lated dishes were maintained in a growth chamber at 28°C for 10 days. After the incubation period, typical rhizobial cultures were purified on standard YMA media and stored at -80°C.

Phenotypic characterization

The cultural characteristics of pure bacterial colonies on YMA media were evaluated according to Teixeira et al. (2010). The features evaluated were pH reaction (neutral, acid, or alkaline), colony size (mm), colony color, and mucus production (high or low production). After the cultural characterization of the colonies, the bacterial isolates were evaluated for their growth ability at different temperatures and under increased NaCl content of the culture medium.

For the temperature tolerance essay, the bacterial isolates were inoculated individually on Petri dishes containing YMA media and incubated in a growth chamber at 28°C (control treatment), 39°, 42°, and 45°C. For the salinity assay, the bacterial isolates were inoculated on Petri dishes containing YMA media as described above. The culture medium was supplemented with 0 M (control), 0.14 M, 0.27 M, and 0.47 M NaCl. The bacterial isolates were incubated in a growth chamber as described above. For both assays, the evaluation was carried out with three replications and the evaluation was performed after 3 days of incubation. The presence or absence of growth was evaluated after the incubation period, and only isolates presenting positive growth in all replications were considered to show positive growth. For isolates that presented different results among replicates, the assay was repeated to verify the characteristics.

Intrinsic antibiotic resistance (IAR) was evaluated using the double agar layer gradient method described by Bromfield et al. (1982), and modified by Xavier et al. (1998). Briefly, Petri dishes containing double YMA medium layers were prepared using low melting point agar. The bottom agar layer, supplemented with 500 mg/L kanamycin (kan), chloramphenicol (clo), or streptomycin (str), was distributed, and the culture media on the dishes were solidified on a surface with a 30° incline. After basal layer solidification, the upper layer, which did not have an antibiotic supplement, was added. The antibiotic diffusion from the bottom to upper layer gave rise to three sections of antibiotic concentration: 0 to 167 mg/L (1st section), 168 to 333 mg/L (2nd section), and 334 to 500 mg/L (3rd section).

For inoculation, the bacterial isolates were grown in 250 mL Erlenmeyer flasks containing 100 mL YM liquid medium and were stirred at 105 rpm for 3 days. One milliliter each culture broth was centrifuged (8000 x *g* for five minutes), the supernatant was discarded, and the pellet was dissolved in 1 mL sterile saline solution (0.15 M NaCl). Ten microliters each cell suspension were dropped on the 1st section of Petri dishes that were inclined to drain the cell suspension toward the end of the 3rd section. The dishes were incubated at 28°C for 3 days. A score of 0 was attributed to isolates that did not present any growth (no resistance), 1 to isolates that were able to grow only on the 1st section (low resistance), 2 to isolates that were able to grow on the 1st and 2nd sections of the dish, but were not able to grow on the 3rd section (intermediate resistance), and 3 to isolates that presented positive growth through all three zones (high resistance) (Xavier et al., 1998, Fernandes et al., 2003, Fernandes Jr. et al., 2012).

The proteolytic and amylolytic activities of all isolates were also assessed. The bacterial isolates were cultivated in YMA liquid media, and 10 µL culture broth was inoculated at three equidistant points of the dish containing the appropriate substrate. The proteolytic activity was evaluated in YM medium in which the mannitol was replaced with 10 g/L casein as the sole carbon source. The plates were incubated in a growth chamber for 4 days. To ob-

serve the degradation zone surrounding the colonies, the plates received 5 mL 0.08 M acetic acid. The amylolytic activity of the bacterial isolate was evaluated in YMA medium in which the mannitol was replaced with starch as the sole carbon source. The inoculated Petri dishes were incubated for 4 days and the starch degradation surrounding the colonies was observed after the addition of 5 mL commercial iodine solution diluted 10 times. On the basis of the enzymatic activity results, the enzymatic index resulting from the ratio between the degradation zone diameter and the colony diameter was calculated (Hankin and Anagnostakis, 1975). These assays were conducted with three replications.

The calcium phosphate solubilization assay was performed according to the procedure described by Sylvester-Bradley et al. (1982). The bacterial broth from YM medium (as described above) was inoculated in Petri dishes containing GELP medium. The plates were incubated for 6 days. For the enzymatic evaluations and the phosphate solubilization assays, the data were evaluated using analysis of variance, and means were compared by applying the Tukey test at the 5% significance level.

Molecular characterization

Molecular fingerprinting of the bacterial isolates was carried out using the polymerase chain reaction for box elements (Box-PCR) and amplified ribosomal DNA-restriction analysis (ARDRA) techniques. For the Box-PCR, the DNA amplification was performed in a final volume of 50 μL with the Box-A1 primer (TACGGCAAGGCGACGCTGACG) (Versalovic et al., 1994). The amplification was performed with an initial denaturation stage at 95°C for 5 min, followed by 35 cycles each of annealing (55°C for 1 min), extension (72°C for 3 min), and denaturation (95°C for 1 min) (Hungria et al., 2000). For the horizontal electrophoresis, a 20 x 25 cm cube was used, where the PCR products were applied on a 1.5% agarose gel (w/v) and submitted to 120 V for 8 h, using the 1 kb plus DNA Leader (Invitrogen, Carlsbad, CA, USA) on each side of the gel. The gel was stained with 0.05% ethidium bromide (v/v) and visualized in an automated UV chamber.

For the ARDRA, the PCR for amplification of 16S rDNA was dimensioned to a final volume of 35 μL following the recommendations of Leite et al. (2009). The universal primers used were Y1 and Y3 (Young et al., 1991). The amplification cycle consisted of an initial denaturation step of 93°C for 5 min; followed by 35 cycles each of 93°C for 1 min, 62°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 10 min

The restriction analysis was carried out using the restriction endonucleases *Dde*I, *Msp*I, and *Hin*fI, and 5 μL amplified DNA, according to manufacturer recommendations. The digested DNA was analyzed on 3% agarose gel (w/v) in 0.5% Tris-borate-EDTA (TBE) buffer for 3 h at a constant voltage of 75 V, using the molecular weight marker φX174 on both sides of the gel (Invitrogen).

As reference strains for Box-PCR and ARDRA, five fast growing rhizobial strains were used: *Ensifer meliloti* (LMG 85217), *Ensifer terangae* (USDA 4894), *Ensifer saheli* (USDA 4893), *Rhizobium etli* (CFN 42), and *Rhizobium leguminosarum* bv. *trifolii* (LMG 8820). These reference strains were obtained from the diazotrophic bacteria culture collection at Embrapa Agrobiologia. The gel images were analyzed with the BioNumerics 7.0 software (Applied Maths, Kortrijk, Belgium) followed by clustering analysis to generate similarity dendrograms with the unweighted pair group method with arithmetic mean (UPGMA) and the Jaccard index.

Symbiotic efficiency

The nodulation capacity of all bacterial isolates was assessed in a greenhouse experiment following the Koch protocol. The bacterial isolates were grown in YM liquid medium over the time required for each isolate. The pigeonpea seeds were superficially disinfected (as described for the nodules) and sown in autoclaved Leonard jars containing 2:1 sterile sand and vermiculite (w/v) as substrate. Three seeds were sown in each pot, and on the 15th day after emergence (DAE), two plants were removed, leaving only one plant per pot. The plants received water as necessary and nutritive Norris solution once a week (Norris and T'Manetje, 1964). The harvest was carried out at 60 DAE. The experiment was performed in a completely randomized design with three replications. The parameter evaluated was the presence or absence of nodules.

An experiment under greenhouse conditions was performed to evaluate the symbiotic efficiency of the selected isolates. In this assay, the bacterial isolates MFG 6, MFG 15, MFG 30, and MFG 9 from the Mato Grande settlement, and FG 9 from the Taquaral settlement were evaluated. The experiment was performed as described above. The experimental design adopted was a randomized block design with four replications. The parameters evaluated were shoot height and diameter, shoot dry matter, root dry matter, nodule number, and dry matter nitrogen accumulated at the shoot using the semi-micro method (Liao, 1981).

RESULTS

Phenotypic characterization and symbiotic efficiency

A total of 21 bacterial isolates showed the ability to nodulate the pigeonpea in the authentication experiment. Among these bacteria, the fast growing isolates presented a high diversity of cultural characteristics as shown in Table 1. Ten isolates showed the capacity to acidify the culture medium after the growth period. Among these bacteria, nine presented large colonies (above 3 mm) and a high mucus production, characteristics similar to those presented by bacteria belonging to the *Rhizobium* genus. The other nine isolates did not change the medium pH, showing a neutral pH reaction. Each of these nine bacteria also showed colonies with diameters ranging from 1 to 3 mm and low mucus production, features similar to those shown by the *Mesorhizobium* and *Methylobacterium* isolates. Only three bacteria showed the capacity to increase the medium pH, a feature that is relatively rare for fast growing rhizobial isolates.

When evaluating the complementary phenotypic characteristics, it was observed that all 21 rhizobial isolates evaluated were able to grow in YMA media with the pH adjusted to 9, while approximately 50% of these bacteria were also able to grow in a medium with the pH decreased to 4 (Table 2). Evaluating the tolerance to high NaCl concentrations in the medium, only one bacterial isolate did not show the ability to grow in the salt supplemented YMA medium. Among the bacteria that grew positively in the NaCl-added medium, three grew with 0.17 M NaCl in the medium, five grew in the 0.34 M NaCl, and surprisingly, 14 bacterial isolates showed positive growth in the YMA medium supplemented with 0.52 M NaCl. All evaluated bacterial isolates were able to grow in the YMA medium when an incubation temperature of 39°C was applied. A total of 10 isolates were

able to grow at an incubation temperature of 45°C, while the other nine bacterial isolates showed positive growth at 42°C

Table 1. Cultural characteristics of 21 pigeonpea rhizobial isolates from the eastern edge of the Brazilian Pantanal.

Rhizobial isolate	pH reaction	Colony size (mm)	Colony color	Mucus production
MFG3	Alkaline	1-3	Transparent	Dry
MFG4	Neutral	1-3	White	Low
MFG5	Acid	>3	Transparent	High
MFG6	Acid	>3	Creamy	High
MFG8	Neutral	1-3	White	Low
MFG9	Neutral	1-3	White	Low
MFG15	Alkaline	1-3	White	Low
MFG19	Neutral	1-3	Creamy	Low
MFG25	Neutral	1-3	Transparent	Low
MFG28	Acid	>3	Transparent	High
MFG30	Neutral	1-3	White	Low
MFG31	Acid	>3	Transparent	High
MFG34	Acid	>3	Transparent	High
MFG35	Acid	>3	Creamy	High
MFG36	Acid	>3	Creamy	High
MFG39	Neutral	1-3	Creamy	Low
FG1	Neutral	1-3	White	Low
FG4	Acid	1-3	Yellow	High
FG5	Acid	>3	Transparent	High
FG9	Neutral	1-3	Yellow	Low
FG11	Acid	>3	Transparent	High

Table 2. Intrinsic antibiotic resistance, acid and alkaline pH, NaCl concentrations and temperature tolerance of 22 pigeonpea rhizobial isolates from the eastern edge of the Brazilian Pantanal.

Rhizobial isolate	Intrinsic antibiotic resistance (IAR)[a]			pH[b]		[NaCl][c]	Temp.[d] (°C)
	Chl	Str	Kan	4	9		
MFG3	3	0	0	-	+	4	39
MFG4	3	1	1	+	+	2	42
MFG5	3	2	1	-	+	4	42
MFG6	2	1	0	+	+	4	45
MFG8	3	1	0	+	+	3	42
MFG9	3	3	3	-	+	4	45
MFG15	3	2	0	+	+	3	45
MFG19	1	0	0	-	+	4	42
MFG25	2	1	0	+	+	3	42
MFG28	3	3	0	+	+	4	39
MFG30	1	0	0	+	+	3	42
MFG31	2	1	1	-	+	2	42
MFG34	2	0	0	+	+	4	42
MFG35	3	2	1	+	+	4	45
MFG36	2	0	0	+	+	4	45
MFG39	1	0	0	-	+	4	45
FG1	3	0	0	+	+	2	45
FG4	2	3	0	-	+	4	42
FG5	2	2	1	-	+	4	45
FG9	0	0	0	-	+	1	39
FG11	3	3	0	-	+	4	45

[a]Maximum IAR presented by rhizobial strains to kanamycin (kan), streptomycin (str) and chloramphenicol (chl): 0-None IAR (no resistance); 1-Low IAR (resistant until 166 $mg.L^{-1}$); 2-Intermedite IAR (resistant until 333 $mg.L^{-1}$); 3-High IAR (resistant until 500 $mg.L^{-1}$); [b]Growth in YMA medium with pH 4 and pH 9: + = able to grow; - = unable to grow; [c]Maximum NaCl concentration in culture medium with positive grow: 1-growth only in the control without NaCl supplementation (1) or with NaCl 0,14 (1); 0,27 (2) and 0,42 M (3); [d]Maximum incubation temperature with positive growth.

Regarding the IAR, one rhizobial isolate presented high resistance to kanamycin. A total of zero, five, and 15 isolates presented intermediate, low, or no intrinsic resistance, respectively. Only four bacterial isolates presented high resistance to streptomycin, while intermediate, low, or no resistance was observed in four, six, and eight rhizobial isolates, respectively. The evaluation of IAR for chloramphenicol showed that 10 bacterial isolates presented high resistance. Seven other rhizobial isolates presented intermediate resistance, and three and two bacteria presented low or no resistance to chloramphenicol, respectively.

Among all bacterial isolates obtained in the present study, five and two presented amylolytic and proteolytic activities, respectively (Table 3). In the medium supplemented with starch or casein as the sole carbon source, all bacterial strains were able to grow but did not present the degradation zone around the colonies, indicating the inability of these isolates to produce extra-cellular proteolytic or amylolytic enzymes. Regarding the capacity for the solubilization of calcium phosphate, five rhizobia presented this capacity, which was observed based on the presence of the translucent zone surrounding the colonies on the GELP medium.

Table 3. Amylolytic and proteolytic activities and calcium phosphate solubilization of ten rhizobia isolated pigeonpea root nodules from the eastern edge of the Brazilian Pantanal.

Rhizobial isolate	Enzymatic index[1]		Solubilization index
	Starch[2]	Casein	Ca $(H_2PO_3)_2$[3]
MFG3	1.2[b]	-	-
MFG8	-	-	1.7[b]
MFG15	-	1.5[a]	3.1[a]
MFG19	2.3[a]	-	-
MFG25	-	2.0[a]	3.7[a]
MFG30	1.3[b]	-	-
MFG39	2.0[a]	-	-
MFG4	-	-	2.1[b]
FG1	-	-	1.7[b]
FG9	1.9[a]	-	-

[1]The enzymatic and solubilization indexes were calculated through the ration between the diameter of the degradation or solubilization zone surrounding the colony and the colony diameter (Hankin and Anagnostakis, 1975). [2]Amylolytic and proteolytic activities against corn starch and casein as substrates. [3]The substrate formed for the evaluation of phosphate solubilization on the GELP medium, according to Sylvester-Bradlay et al. (1982). Means in the column followed by the same letter do not differ by the Skott-Knott mean range test ($P < 0.05$)

Evaluating the symbiotic efficiency of the bacterial isolates, four among the five isolates tested showed some growth promotion on the pigeonpea plants. Pigeonpea plants inoculated with the bacterial isolates MFG 6, MFG 15, MFG 30, and FG 9 presented the same shoot weight and diameter observed in plants that received mineral nitrogen (Table 4). Plants inoculated with the rhizobial isolates MFG 6 and MFG 30 also presented the same shoot dry weight observed in the plants supplemented with mineral nitrogen. Surprisingly, the treatments for which the bacterial isolates MFG 15 and FG 9 were used showed higher shoot dry weight than that observed in plants that were supplied with nitrogen. Regarding the nodulation, the pigeonpea plants inoculated with the bacterial isolates MFG 15 and MFG 30 stood out with higher nodulation rates (nodule number and dry weight). In addition, the plants inoculated with the rhizobial isolate MFG 15 showed the same shoot nitrogen content as that observed in the other inoculated treatments as well as that of plants that received mineral nitrogen.

Table 4. Symbiotic efficiency of five selected rhizobia isolated from field growing pigeonpea at the eastern edge of the Brazilian Pantanal.

Rhizobial isolate	Shoot height	Shoot diameter	Shoot dry weight	Root dry weight	Nodule number	Nodule dry weight	Shoot N content
	cm	mm	g/pl		nod/pl	mg/pl	
MFG 6	31.82[a]	2.57[a]	1.22[b]	0.17[b]	18[b]	92.50[b]	37.45[b]
MFG 15	31.85[a]	2.67[a]	1.47[a]	0.14[c]	46[a]	165.00[a]	56.13[a]
MFG 30	32.43[a]	2.60[a]	1.32[b]	0.18[b]	42[a]	165.00[a]	32.86[b]
MFG 39	27.95[b]	1.87[b]	0.62[c]	0.10[c]	15[b]	40.00[c]	38.18[b]
FG 9	36.86[a]	2.70[a]	1.74[a]	0.21[b]	20[b]	100.00[b]	33.09[b]
Control with N	32.30[a]	2.80[a]	1.12[b]	0.27[a]	0[c]	0[c]	48.10[a]
Control without N	19.23[d]	1.65[b]	0.20[c]	0.05[b]	0[c]	0[c]	4.72[c]

Means in the column followed by the same letter do not differ by the Skott-Knott mean range test ($P < 0.05$).

Molecular characterization

Based on the Box-PCR profiles, all bacterial isolates and reference strains evaluated presented a similarity of only 23%, and the clustering method used identified only two isolates with 100% similarity (Figure 1). A total of 11 clusters were observed of those with 70% similarity. Cluster 1 presented 74% similarity and encompassed the *R. leguminosarum* reference strains together with five other isolates from pigeonpea root nodules, including two from the Mato Grande, and three from the Taquaral settlement. Cluster two also presented 74% similarity, and was formed by one rhizobial isolate from Taquaral and two others from Mato Grande. The 3rd, 5th, 9th, and 10th clusters were single clusters, formed by only one bacterial isolate. Cluster five had only the *E. saheli* reference strain, while the other three single clusters had rhizobia from the Mato Grande settlement. The 4th cluster was formed only by the reference strains of *E. fredii*, *E. terangae*, and *R. etli*. Cluster six encompassed two bacterial isolates from Mato Grande with 72% similarity. Isolates FG1, MG4, and MG8 were closely clustered with 93% similarity at the 8th cluster, and cluster 11 showed two 100% genetically similar isolates from Mato Grande.

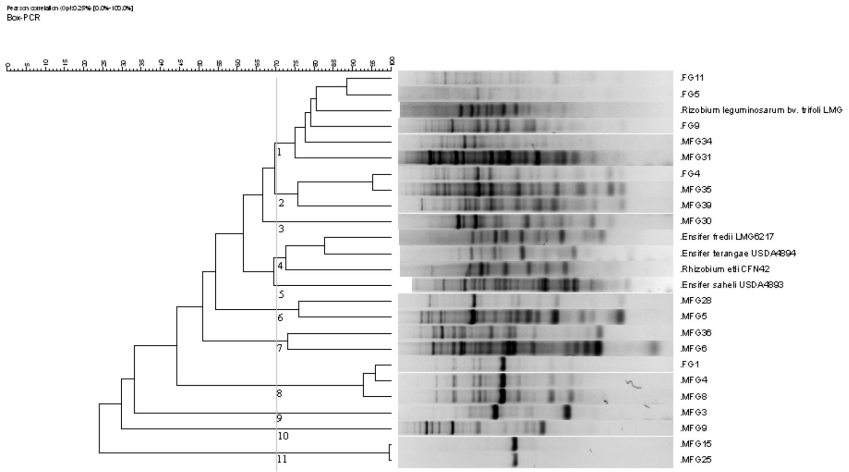

Figure 1. Genetic diversity of 21 rhizobia isolated from field growing pigeonpea at the eastern edge of the Brazilian Pantanal by the Box-PCR. Dendrogram built using the software Bionumerics and the UPGMA clustering method with the Pearson coefficient of similarity.

The genetic diversity evaluated using the ARDRA technique showed that all bacterial isolates presented 22% similarity (Figure 2). The clustering method at 70% similarity formed 12 clusters. In cluster one, two rhizobial isolates from the Mato Grande settlement showed 100% similarity. The 2nd, 4th, 7th, 8th, 9th, and 11th clusters were single clusters. Among the single clusters, only the cluster eight presented a bacterial isolate from the Taquaral settlement, and the other four single clusters showed bacteria from the Mato Grande settlement. The 3rd cluster was the largest one observed during fingerprinting with the ARDRA technique. In this cluster, 10 bacterial isolates with 71% similarity were encountered, including five isolates from Mato Grande, three from Taquaral, and both *Rhizobium* reference strains. Cluster five presented only the *Ensifer* reference strains with 89% similarity. Cluster six presented two isolates from the Mato Grande settlement and one from the Taquaral settlement with 100% similarity, while cluster ten presented two rhizobia from the Mato Grande settlement with 100% similarity.

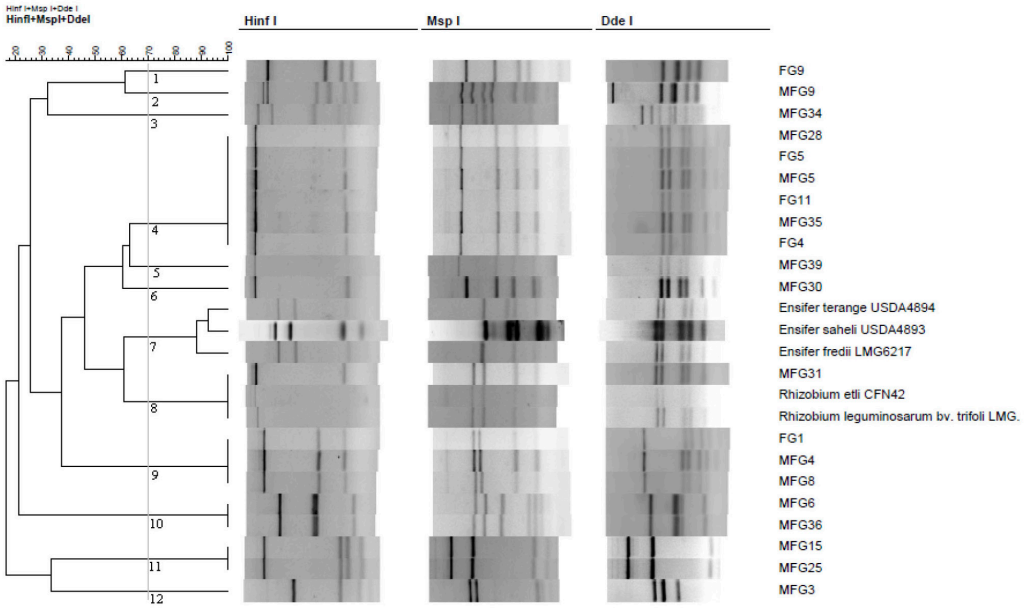

Figure 2. Genetic diversity of 21 rhizobia isolated from field growing pigeonpea at the eastern edge of the Brazilian Pantanal by ARDRA profiles using the restriction enzymes *Hin*fI, *Msp*I, and *Dde*I. Dendrogram built using the software Bionumerics and the UPGMA clustering method with the Jaccard coefficient of similarity.

DISCUSSION

Among the 21 fast growing rhizobial isolates obtained in this study, 10 bacterial isolates showed the ability to acidify the culture medium and nine did not change the medium pH. Two isolates showed an increase of the medium pH, which is an uncommon characteristic for fast growing rhizobial isolates. These features were also observed in some bacterial isolates obtained from tropical green manure such as pigeonpea (Fernandes Jr. et al., 2012), cowpea (Leite et al., 2009), velvet (Lima et al., 2012), and fava bean (Santos et al., 2011), which confirms the great diversity of rhizobial isolates, and most likely the existence of new taxonomic

groups nodulating tropical green manures (Sy et al., 2001; Trujillo et al., 2005). Despite the acidification of the culture medium and the fast growth of the isolates, some rhizobial isolates shared some features characteristic of slow growing bradyrhizobial strains, such as low mucus production and transparency of the mucus. These characteristics are not exclusive for slow growing rhizobia, but are not normally observed in fast growing bacteria such as those of the *Rhizobium* and *Ensifer* genera. Leite et al. (2009) observed similar results in an evaluation of the diversity of rhizobia isolated from cowpea.

The evaluation of the rhizobial IAR showed a very peculiar pattern. In spite of the great number of isolates that presented high or intermediate resistance to chloramphenicol (17 isolates in all), this is a very uncommon characteristic for pigeonpea nodulating rhizobia (Anand and Dogra, 1991; Fernandes et al., 2003; Fernandes Jr. et al., 2012). The isolates also showed a generally low IAR for kanamycin, which is a very common characteristic for tropical rhizobia. Previous studies evaluating bacterial isolates of pigeonpea reported a generally low or intermediate resistance to chloramphenicol and a high susceptibility to kanamycin (Anand and Dogra, 1991; Fernandes et al., 2003; Fernandes Jr. et al., 2012). Other studies also showed that cowpea and common bean rhizobia also presented intermediate to low resistance to kanamycin, as was observed in the present study; however, these isolates presented a low resistance to chloramphenicol (Xavier et al., 1998; Souza et al., 2003; Zilli et al., 2004; Florentino et al., 2010). Souza et al. (2003) reported that *Rhizobium tropici* and *E. fredii* showed low resistance to chloramphenicol and to kanamycin, indicating that the majority of the bacterial isolates obtained in the present study should not belong to these species or to related groups.

Another unexpected result for the pigeonpea rhizobia assessed in this study was the relatively low resistance to streptomycin. Studies regarding the antibiotic resistance of pigeonpea rhizobia found that the majority of these bacterial isolates presented an increased IAR to streptomycin (Fernandes et al., 2003; Fernandes Jr. et al., 2012). Rhizobia from other legume crops presented a variable IAR to streptomycin, ranging from low or no resistance (Zilli et al., 2004, Florentino et al., 2010) to high resistance (Xavier et al., 1998) for cowpea rhizobia, for example. Regarding the resistance of the three antibiotics evaluated, the isolate MFG 9 stood out because it presented a high resistance to all antibiotics, indicating a high capacity of these bacteria to survive in the soil and to compete with other soil bacteria, since these bacteria presented resistance to the antibiotics produced by the bacterial and fungal isolates inhabiting the soil.

The major part of the bacterial isolates evaluated showed an interesting characteristic with regard to their ability to grow in culture media with the pH adjusted to 4.0 or 9.0. This ability was also observed for rhizobial strains from cowpea (Mensah et al., 2006; Florentino et al., 2010) and white clover (Brose, 1994) and is desirable in the case of bacteria that can tolerate different pH values, especially those that are acidic, as it is more efficient for colonizing host plants in tropical soils (Choudhury et al., 2010). The same metabolic versatility was observed during the evaluation of the bacterial capacity to grow on the culture medium supplemented with different salt concentrations as the 13 bacterial isolates presented the capacity to grow on the YMA medium supplemented with 0.52 M NaCl. These results are in agreement with those found in previous studies evaluating the tolerance of tropical rhizobia from pigeonpea (Fernandes et al., 2003; Fernandes Jr. et al., 2012), cowpea (Nóbrega et al., 2004; Xavier et al., 2007, Florentino et al., 2010), and lentil (Jida and Assefa, 2011).

Regarding the high temperature tolerance of rhizobia, nine of the bacterial isolates were able to grow positively at 45°C incubation. Few studies have indicated the capacity of

rhizobial growth above 40°-42°C because evaluations are generally carried out below this temperature range (Florentino et al., 2010; Fernandes Jr. et al., 2012). The results found in this study point to a highly tolerant rhizobial community nodulating the pigeonpea in the Brazilian Pantanal. The evaluations of the *in vitro* bacterial tolerance simulated environment (salinity, high temperatures, antibiotic concentrations, etc.) are relatively cheap, require little space, are not time consuming, and can feasibly be conducted with a large number of bacterial isolates (Nóbrega et al., 2004, Xavier et al., 2007). Furthermore, the correlation between these results and field responses was recently demonstrated (Indrasumunar et al., 2012), indicating the relevance of these evaluations for rhizobial strains.

The amylolytic activity of the bacteria isolates showed that the bacterial isolates MFG 19, MFG 39, and FG 9 stood out with a higher enzymatic index. Only two rhizobial isolates showed proteolytic activity. These results indicate the presence of bacterial isolates with biotechnological potential other than inoculant production. Evaluations of the biotechnological potential of rhizobia have already been carried out in several studies (Oliveira et al., 2007; Fernandes Jr. et al., 2011, 2012). The capacity to produce enzymes of industrial interest under routine laboratory conditions is desirable to reduce the costs and offer an alternative for the inoculant industry in Brazil (Fernandes Jr. et al., 2012). Another interesting characteristic observed was the calcium phosphate solubilization results, which indicated that two bacteria (MFG 15 and MFG 25) had higher solubilization indices. This feature is normally observed in bacterial isolates from pigeonpea (Souchie and Abboud, 2007) and can be exploited through the inoculation of the bacterial isolate together with non-soluble P sources.

The symbiotic efficiency evaluation showed that the bacterial isolate MFG 15 was a very efficient bacterium, presenting the same shoot nitrogen content as pigeonpea plants that received mineral nitrogen supplement. Another important characteristic observed for green manure is the high biomass production. Two bacterial isolates evaluated caused an increase in pigeonpea biomass that was higher than that observed for the nitrogen-supplied plants. These results indicate a high efficiency of the isolates obtained in the present study. The selection of bacterial isolates for inoculation in green manures in Brazil has revealed bacterial strains with this characteristic (Lima et al., 2012), which shows the importance of continuous strain selection.

The genetic fingerprinting was performed using ARDRA and Box-PCR techniques. The dendrogram of similarity revealed 12 clusters with 70% similarity. Using the Box-PCR technique, the clustering provided 11 clusters with 70% similarity. Both techniques showed similar clustering patterns, with the majority of the bacterial isolates and reference strains belonging to the same clusters in both dendrograms. Stocco et al. (2008) observed similar results in an evaluation of the genetic diversity of common bean with both techniques. Both ARDRA and Box-PCR are feasible and inexpensive techniques that can be used to evaluate the fingerprinting of a diazotrophic bacterial collections (Zilli et al., 2004; Freitas et al., 2007; Stocco et al., 2008; Leite et al., 2009; Teixeira et al., 2010; Lima et al., 2012; Fernandes Jr. et al., 2013).

Evaluating the genetic diversity of the bacterial isolates, the same variability was observed using phenotypic characterization and molecular fingerprinting. The ARDRA and Box-PCR profiles showed that all bacterial strains evaluated had up to 25% similarity, corroborating the high genetic diversity of the bacterial isolates studied. The molecular fingerprinting of the rhizobia isolated from green manures revealed the existence of new bacteria nodulating the plants in the field (Sy et al., 2001). The results obtained in the present study also indicate the presence of new bacteria nodulating the pigeonpea in the Brazilian Pantanal.

Recent studies evaluating the diversity of rhizobial isolates have applied phenotypic and molecular approaches to obtain better understanding of rhizobial biology and efficiency (Florentino et al., 2010; Lima et al., 2012). This approach was adopted in the present study and relevant information on the phenotypic and genetic diversity of the bacterial isolates was obtained. Further evaluations are now being carried to gain a better understanding of the taxonomic positions and efficiency of the bacterial isolates.

The 21 bacterial isolates evaluated showed great phenotypic and genetic diversity, and some isolates presented potential for further evaluations of field efficiency and biotechnological potential.

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