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POLYPHASIC CHARACTERIZATION OF ENDOPHYTIC BACTERIA OF SORGHUM GROWN ON CERRADO SOIL OF THE GOIÁS STATE – BRAZIL

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ABSTRACT

Sorghum is a cereal of great commercial importance adapted to the tropical and subtropical regions. Isolation of plant-associated bacteria of agronomic interest has been the subject of several research works, however, there are few studies on bacteria isolating from sorghum. The objective of this work was to characterize as plant growth promoters endophytic bacteria isolated from sorghum roots. A total of 24 isolates were selected using the NFb-lactate, LGI-P, and YM semi-selective medium. The characterization was based on the resistance to antibiotics, use of carbon sources, enzyme activity and 16S-23S intergenic analysis. The isolates were more sensitive to the antibiotic streptomycin and showed a higher capacity to produce lipases and cellulases. Carbon sources of acid nature were more restrictive to the bacterial growth. Most of the isolates (95.8%) showed catalase and urease activity, besides to be able to solubilize phosphate. Intergenic analysis (16-23S rDNA) revealed a great diversity among the isolates, with eight groups with about 60% of similarity. In this study, it was possible to identify great physiological and genetic diversity among the bacteria isolated from sorghum roots. Some of these isolates can be considered promising plant growth-promoting rhizobacteria or sources of enzymes for biotechnological use.

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INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench) is the fifth most cultivated cereal in the world and can be compared to corn in relation to its agronomic and nutritional value. Highly adapted to extreme environments, sorghum can be grown in regions of low precipitation besides to be an interesting alternative for corn substitution in animal feed (Neto *et al*., 2014). Despite the economic advantages, sorghum production still depends on the use of industrialized fertilizers, which increase production costs and can cause environmental damage (Teixeira Filho *et al*, 2010). In order to mitigate the environmental impacts caused by the use of chemical fertilizers and, at the same time ensure high crop productivity, the inoculation with plant growth-promoting rhizobacteria (PGPR) has proven to be an efficient way of guaranteeing the production and sustainability of the crop (Moreira *et al*., 2010). In this aspect, the interaction between bacteria and plants of agronomic interest has been widely studied, mainly due to the growing interest in the prospection of microorganisms as an alternative source for new bioactive products that can benefit the plants and the soil, increasing productivity and reducing costs with inputs (Moreira *et al*., 2010, Glick, 2012). Among those plant-associative bacteria, the endophytic bacteria are of most interest because they are able to colonize inter and/or intracellular spaces in plants maintaining biochemically active interactions with their host (Brader *et al*., 2014). This group of bacteria can present a series of beneficial activities for the plants as: production of phytohormones, antagonism against phytopathogens, production of siderophores and biological

fixation of nitrogen been known as Plant Growth-Promoting Rhizobacteria - PGPR (Pohjanen *et al*., 2014). PGPRs are also associated with nutrient solubilization, such as phosphate (P) solubilization through various mechanisms that convert inorganic phosphates to organic phosphates, making it available to plants (Liu *et al*., 2014). The production of hydrolytic enzymes by PGPR has also been investigated because they are related to mechanisms of antagonism against phytopathogens, being the most studied: proteases, cellulases, amylases, and pectinases (Dinesh *et al*., 2015). All bacterial activities involve the participation of enzymes, and several of these enzymes have already been identified in PGPR and can be exploited in the formulation of bioproducts such as inoculants and biocontrol agents (Dinesh *et al*., 2015; Adrio & Demain, 2014). Rhizobia, which encompasses most of the genera of the family *Rhizobiaceae* as *Rhizobium* and *Bradyrhizobium*, is the most important bacterial group among the PGPRs already described. Other well-characterized PGPR genera are *Azospirillum*, *Herbaspirillum*, *Bacillus,* and *Gluconacetobacter*. Bacteria of these genera have already been described associated with the roots of plants such as rice, maize, and sugar cane (Kuss *et al*, 2007; Ikeda *et al*., 2013; Mbai *et al*., 2013, Muthukumarasamy *et al*., 2017).

The use of phenotypic and genotypic tools can help on the determination of metabolic, genetic and ecologic diversity of PGPR bacteria of varied environments (Reis Júnior *et al*. 2004; Dingman, 2012); however, studies related to the isolation and characterizations of PGPR associated to sorghum cultivated in the Cerrado of Goiás are scarce. In this way, this work aimed at the biochemical and molecular characterization of endophytic bacteria associated with roots of sorghum plants.

MATERIALS AND METHODS

Selected isolates and standard strains

Initially, 300 isolates were obtained from sorghum roots cultivated in Cerrado soil, according to Ferreira *et al* (2014). From these isolates the selection of those to be used in this study was carried out based on the growth capacity in three culture medium: two medium without nitrogen, the semi-selective NFb-lactate medium for isolation of bacteria of the genus *Azospirillum* and, the semi-selective LGI-P medium for purification and characterization of *Gluconacetobacter spp* (Baldani *et al*., 2014). Also, the semi-selective YM medium for isolation of bacteria of the family *Rhizobiaceae* (Hungria & Araújo, 1994). From each medium were selected 8 isolates, totaling 24 bacterial isolates used in this work. In addition, it was used as standard strains for all assays *Azospirillum brasilense* (strain FP2), *Rhizobium tropici* (strain BR 322) and *Gluconacetobacter diazotroficus* (strain Pal5).

Resistance to antibiotics

In the evaluation of antibiotics resistance, the 24 isolates and the standard strains (FP2- *A. brasilense*, BR 322- *R. tropici* and, Pal5- *G. diazotroficus*) were inoculated in solid medium NFb-lactate, YM and LGI-P containing different concentrations (30 μg mL^{-1}, 50 μg mL^{-1}, 100 μg mL^{-1} and, 200 μg mL^{-1}) of ampicillin (AMP), chloramphenicol (CLR), streptomycin (STR) and, tetracycline (TET). The samples were incubated (32 ºC; 7 days) on the shade to avoid photodegradation of the antibiotics (Hungria & Araújo, 1994).

Use of carbon source

In order to evaluate the ability of bacterial isolates to metabolize different carbon sources, the 24 isolates and the standard strains (FP2- *A. brasilense*, BR 322- *R. tropici* and, Pal5- *G. diazotroficus*) were grown in minimal broth (Hungria & Araújo, 1994), added separately with 14 Carbon sources: maleic acid, malic acid, succinic acid, arabinose, glycerol, glucose, fructose, inositol, mannitol, mannose, sucrose, sorbitol, and trehalose. Carbon sources were used at the concentration of 10 mM L^{-1}. The assays were performed on deepwell plates containing 1 ml of the medium under agitation (28 °C, 72 h). After incubation, 200 μL of each culture was transferred to Elisa plates to verify bacterial growth through optical density at 600 nm, using a Microplate Spectrophotometer model Epoch (BioTek Instruments, Inc.). Also, for the determination of the capacity of using sodium citrate as a carbon source, the isolates and the standard strains were inoculated in Simmons citrate solid medium according to Teixeira *et al*. (2008).

Enzymatic assays

The activity of the catalase enzyme was evaluated according to MacFaddin (1980) for the isolates and standard strains in semi-selective solid medium (NFb, YM, and LGI-P). For the activity of the urease enzyme, the solid urea medium (Christensen, 1946) was used following the methodology proposed by Teixeira *et al*. (2008). The activity of the nitrate reductase enzyme was performed using the methodology proposed by Neyra *et al*. (1977). The presence of the enzyme nitrogenase was detected by the qualitative method according to Kuss *et al*. (2007). The detection of the production capacity of extracellular enzyme (lipases, cellulases, and proteases) was performed according to Cappuccino & Sherman (1992)

Three broth media were used for the evaluation of the phosphate solubilization capacity: NBRIP medium (Nautiyal, 1999), Pikovskaya-PVK medium (Pikovskaya, 1948), and GL medium (Sylvester-Bradley *et al*., 1982). The broth media were modified by the addition of 5 g of $Ca_5(PO_4)$ 3OH in the GL media (pH 6.5), in the PVK medium (pH 7.0) and in the NBRIP medium to evaluate the calcium phosphate (P-Ca) solubilization (pH 7.0). The ability to solubilize aluminum phosphate (P-Al) was evaluated using modified GL medium containing 5.3 g of $AlCl_3$ at pH 4.5, according to Hara and Oliveira (2004). The ability to form Fe^{3+} chelating compounds was carried out using the universal CAS method (Schwyn & Neiland, 1987)

DNA Extraction and 16-23S rRNA intergenic analysis

For DNA extraction the isolates selected in NFb and YM medium were grown for 24 h, and the isolates selected in LGI-P medium were grown for 72 h in the respective media and performed DNA extraction (Reis Júnior *et al*. 2004). For the PCR of the intergenic region (16S-23S rRNA), the primers were used: pHr (reverse) (5'-TGC GGC TGG ATC ACC TCC TT-3') e p23S (5'- GGC TGC TTC T AA GCC AAC-3') (Massol-Deya *et al*., 1995). The PCR conditions were performed as recommended by Reis Júnior *et al*. (2004) and, modified according to Oliveira (2016).

Data analysis

The biochemical data for the enzymes indexes were analyzed by descriptive statistics whose results were expressed in percentage using the software Microsoft Excel® Office suite

2016. For the evaluation of the enzymatic activity and phosphate solubilization, the enzymatic index (EI) and the solubilization index (SI), respectively, were used, which are based on semiquantitative measurements. The EI and SI were determined by the ratio between the diameter of the degradation halo and the diameter of the colony (Hankin & Anagnostakis, 1975). Isolates that presented EI and SI values <1.0 were considered as non-extracellular enzyme producers, whereas those with EI and SI values ≥ 1.0 were considered positive for enzyme production. In addition, isolates with EI and SI values higher than 1.5 were considered to be good producers of extracellular enzymes. The biochemical and molecular data were also submitted separately to cluster analysis. Data were used to generate a binary matrix (presence/absence) to evaluate the similarity between the 24 isolates and the 3 standard strains. The similarity indices were estimated using the Jaccard's similarity coefficient (J) and the grouping was performed using the UPGMA (Unweighted Pair Group Method With Arithmetic Mean) method using the NTSYSpc 2.02i Applied Biostatistics software (Ikeda *et al*. 2013).

RESULTS

Resistance to antibiotics

The resistance evaluation to different antibiotics and its concentrations by the isolates revealed clear effects of both, antibiotics and concentrations (Table 1). Considering the growth capacity of the isolates across the different antibiotics and concentrations (%GIAC), the isolates NS14, NS23, NS48, LS6, LS29 and, LS82 besides the standard strains Pal5 and BR 322 were able to grow in all possible conditions, representing 100% of growth. On the other hand, the isolates YS10, YS25, YS26 and YS27 grew in less than 50% of the possible conditions, while the isolates YS54 and YS59 did not grow in any condition. All the isolates and standard strains were able to grow in chloramphenicol at 30 µg mL^{-1}. Based on the average of the isolates growth in each concentration of each antibiotic (%GIC), the greater inhibition was observed for streptomycin, with about 53% of growth. In addition, regardless of the antibiotic, the dose that promoted the greatest restriction to bacteria growth was 200 µg mL^{-1} (Table 1). Based on the results of Table 1, the antibiotic dose with greatest restriction to the bacterial growth (200 µg mL^{-1}) was chosen to perform a Venn diagram analysis (Figure 1). The cluster 1 is composed by six isolates and by the standard strains Pal5 and BR 322, which are resistant to high doses of the four antibiotics. Also, seven isolates (clusters 3, 6 and 7) are resistant to high doses of at least 3 different antibiotics (Figure 1).

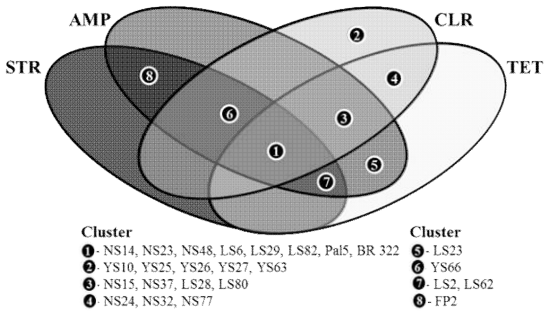

Figure 1. Venn diagram for the resistance of bacterial isolates to streptomycin (STR), ampicillin (AMP), tetracycline (TET) and, chloramphenicol (CHLO) at the concentration of 200 µg mL^{-1}.

Utilization of carbon sources

The evaluation of the use of different carbon sources revealed that the isolates obtained from sorghum were able to use most of the 13 different carbon sources (Table 2).

Table 1. Resistance evaluation of the isolates and standard strains to concentrations (30, 50, 100 and, 200 µg mL^{-1}) of different antibiotics

Bacteria	Ampicillin				Chloramphenicol				Streptomycin				Tetracycline				%GIAC[1]
	30	50	100	200	30	50	100	200	30	50	100	200	30	50	100	200	
NS14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100.0
NS15	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	75.0
NS23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100.0
NS24	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+	50.0
NS32	+	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+	56.3
NS37	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	75.0
NS48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100.0
NS77	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+	50.0
FP2	+	+	+	+	+	-	-	-	+	+	+	+	-	-	-	-	56.3
LS2	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	93.8
LS6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100.0
LS23	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+	81.3
LS28	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	75.0
LS29	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100.0
LS62	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	87.5
LS80	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	81.3
LS82	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100.0
Pal5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100.0
YS10	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	31.3
YS25	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	25.0
YS26	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	31.3
YS27	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	37.5
YS54	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	12.5
YS59	-	-	-	-	+	-	-	-	+	+	+	-	-	-	-	-	25.0
YS63	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	81.3
YS66	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	93.8
BR 322	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100.0
%GIC	81.5	70.4	66.7	63.0	100.0	92.6	85.2	77.8	59.3	55.6	51.9	44.4	74.1	74.1	74.1	66.7	

[1] %GIAC - Percent growth of the isolates across the different antibiotics and concentrations. [2] %GIC - Percent growth of the isolates in each concentration of each antibiotic.

Table 2. Qualitative evaluation of the carbon sources use by bacterial isolates obtained from sorghum roots (*S. bicolor*, L. Moench) and by the standard strains

Bacteria	Carbon sources[1]													
	MA	MaA	SCA	ARA	FTS	GCS	GCR	INT	MNT	MNS	SCS	SBT	TLS	%GIACS[2]
NS14	+	-	+	-	-	+	+	-	+	-	+	+	+	61.5
NS15	+	-	+	-	+	+	-	-	+	+	+	+	+	69.2
NS23	+	-	+	-	+	+	-	-	+	+	+	+	+	69.2
NS24	+	+	+	+	+	+	-	-	+	+	+	+	+	84.6
NS32	+	-	+	+	+	+	+	+	+	+	+	+	+	92.3
NS37	+	-	+	+	+	+	+	+	+	+	+	+	+	92.3
NS48	+	-	+	-	+	-	-	+	-	+	+	+	+	61.5
NS77	+	-	+	-	-	+	-	+	+	+	+	+	+	69.2
LS2	+	-	+	-	+	+	-	-	+	+	+	+	+	69.2
LS6	+	-	+	-	+	+	-	-	+	+	+	+	+	69.2
LS23	-	-	+	-	+	+	-	-	+	-	+	+	+	53.8
LS28	+	-	+	-	+	+	-	-	+	+	+	+	+	69.2
LS29	+	-	+	-	-	+	-	+	+	+	+	+	+	69.2
LS62	+	-	+	-	+	+	-	-	+	+	+	+	+	69.2
LS80	-	-	+	-	-	+	-	-	+	+	+	+	+	53.8
LS82	+	-	+	-	-	+	-	-	+	+	+	+	+	61.5
YS10	+	-	+	+	+	+	+	+	+	+	+	+	+	92.3
YS25	+	-	+	-	+	-	-	-	+	+	+	+	+	61.5
YS26	+	+	+	-	+	+	-	-	+	+	+	+	+	76.9
YS27	+	-	-	-	+	+	-	-	+	+	+	-	+	53.8
YS54	+	-	+	-	+	+	-	+	+	+	+	+	+	76.9
YS59	+	-	-	-	-	+	+	+	+	+	+	-	+	61.5
YS63	+	-	+	-	+	+	-	-	+	+	+	+	+	69.2
YS66	+	-	+	+	-	+	-	+	+	+	+	+	+	76.9
Pal5	-	-	+	+	+	+	+	-	+	+	+	+	+	76.9
FP2	+	-	+	-	-	-	+	-	+	+	+	+	+	61.5
BR322	+	-	+	-	-	+	+	+	+	+	+	+	+	76.9
%GIECS	88.9	7.4	92.6	22.2	66.7	92.6	25.9	37.0	96.3	92.6	100.0	92.6	100.0	

[1] MA= malic acid, MaA= maleic acid, SCA= succinic acid, ARA= arabinose, FTS= fructose, GCS= glucose, GCR= glycerol, INT= inositol, MNT= mannitol, MNS= mannose, SCS= sucrose, SBT= sorbitol and TLS= trehalose. [2] %GIACS - Percent growth of the isolates across the different carbon sources. [3] %GIECS - Percent growth of the isolates in each carbon source.

Table 3. Enzymatic activity and enzymatic index of bacterial isolates obtained from sorghum roots (*S. bicolor*, L. Moench) and by the standard strains

Bacteria	Enzyme activity						Enzymatic index[3]	
	CTT[1]	CLS	NTT	URS	NTG	%EAI[2]	Lipase	Cellulase
NS14	+	+	+	+	+	100	1.5 ± 0.2	1.8 ± 0.1
NS15	+	+	+	+	+	100	2.0 ± 0.2	0.00
NS23	+	-	+	+	+	80	0.00	0.00
NS24	+	+	+	+	+	100	2.2 ± 0.2	1.9 ± 0.2
NS32	+	+	+	+	+	100	0.00	0.00
NS37	+	+	+	+	+	100	0.00	0.00
NS48	+	+	+	+	+	100	1.4 ± 0.1	0.00
NS77	+	+	+	+	+	100	1.4 ± 0.2	0.00
LS2	+	+	+	+	+	100	2.1 ± 0.1	0.00
LS6	+	+	-	+	+	80	1.4 ± 0.1	0.00
LS23	+	+	+	+	+	100	1.9 ± 0.3	1.6 ± 0.1
LS28	+	+	+	+	+	100	1.5 ± 0.3	0.00
LS29	+	+	+	+	+	100	1.5 ± 0.1	1.5 ± 0.1
LS62	+	+	+	-	+	80	1.7 ± 0.3	1.4 ± 0.2
LS80	+	+	+	+	+	100	1.8 ± 0.3	1.4 ± 0.2
LS82	+	+	+	+	+	100	1.4 ± 0.1	1.4 ± 0.1
YS10	+	+	+	+	-	80	1.5 ± 0.3	0.00
YS25	+	+	+	+	+	100	n.d.	1.3 ± 0.1
YS26	+	+	+	+	+	100	1.0 ± 0.2	1.4 ± 0.1
YS27	+	+	+	+	+	100	1.5 ± 0.3	1.3 ± 0.1
YS54	+	+	+	+	-	80	0.00	2.0 ± 0.2
YS59	+	+	+	+	+	100	0.00	1.5 ± 0.2
YS63	+	+	+	+	+	100	1.5 ± 0.3	0.00
YS66	+	+	+	+	+	100	0.00	0.00
Pal5	+	+	+	-	+	80	1.3 ± 0.1	0.00
FP2	+	+	+	+	+	100	0.00	0.00
BR322	+	+	+	+	-	80	0.00	0.00
%IEEA[4]	100	96,3	96,3	92,6	88,9			

[1] CTT= citrate, CLS= catalase, NTT= nitrate reductase, URS= urease, NTG= nitrogenase. [2] %EAI – Percent of enzyme activity by isolate. [3] Values and standard deviation for the enzymatic indexes. [4] %IEEA – Percent of isolates by each enzyme activity. n.d. = not determined.

The Percent growth of the isolates across the different carbon sources (%GIACS) shows that the isolates NS24, NS32 and NS37 were able to use more than 85% of the carbon sources, while the isolates LS3, LS80, and YS27 just used about 53% of the evaluated carbon sources.

By the Percent growth of the isolates in each carbon source (%GIECS) results, sucrose and trehalose were used as carbon source by all the isolates and standard strains, whereas succinic acid, glucose, mannitol, and mannose were used by

more than 90% of the isolates and standard strains. Low capacity of use as carbon source was verified for maleic acid, arabinose, glycerol and inositol, which were used by 7.4%, 22.2%, 25.9% and 37%, respectively, of the isolates (Table 2).

Enzymatic assays

On the enzymatic activity assay, the results of the enzyme activity by isolate (%EAI) revealed that about 74% of the isolates and standard strains showed activity for the five evaluated enzymes. The other isolates and standard strains, at least for of the five evaluated enzymes had positive activity (Table 3). All the isolates, as well as the standard strains, showed positive activity for citrate, while catalase and nitrate activity was observed in about 96% of the isolates and standard strains. For the other enzyme activities (urease and nitrogenase), about 93% and 89%, respectively, of the isolates and standard strains showed activity for these enzymes (Table 3). Results for the production of extracellular enzymes assays revealed that 17 and 12 isolates showed activity for lipase and cellulase, respectively. Just one isolate was positive for and protease (results not shown).

Lipase activity was also observed for the standard strain Pal5 (Table 3). The enzymatic index (EI) for lipase ranged from 1.0 to 2.2 with this highest values been observed for the isolate NS24. All the isolates, except NS23; NS32, NS37, YS25, YS26, YS54, Y59, are good lipase producers, showing IE $\geq$ 1.4. The activity of cellulase was not observed among the standard strains; however, 50% of the isolates showed activity for this enzyme, with EI raging from 1.3 to 2. Among the twelve isolates showing cellulase activity, 6 are good producers of cellulase with IE $\geq$ 1.5. (Table 3). The ability to solubilize phosphate was identified in 95.8% of the isolates in both NBRI-P and PVK medium (Table 4).

Table 4. Solubilization index (SI) of 24 isolated bacteria of sorgho (*S. bicolor*, L. Moench) and standard strains FP2 (*A. brasilense*), BR322 (*R. tropici*) e Pal5 (*G. diazotrophicus*)

Bacteria	SI[1]	
	NBRI-P	PVK
NS14	1.8 ± 0.1	1.4 ± 0.1
NS15	1.8 ± 0.2	1.6 ± 0.2
NS23	0.00	0.00
NS24	1.7 ± 0.3	1.5 ± 0.2
NS32	1.4 ± 0.1	1.8 ± 0.2
NS37	1.3 ± 0.2	1.7 ± 0.2
NS48	1.6 ± 0.1	1.3 ± 0.1
NS77	1.7 ± 0.1	1.4 ± 0.2
LS2	1.8 ± 0.2	1.4 ± 0.1
LS6	2.0 ± 0.2	1.8 ± 0.2
LS23	2.0 ± 0.1	1.4 ± 0.1
LS28	2.0 ± 0.1	1.4 ± 0.1
LS29	2.0 ± 0.2	1.5 ± 0.1
LS62	1.8 ± 0.3	1.6 ± 0.2
LS80	2.1 ± 0.3	1.7 ± 0.1
LS82	1.9 ± 0.1	1.6 ± 0.1
YS10	1.6 ± 0.1	1.3 ± 0.1
YS25	1.9 ± 0.1	1.6 ± 0.1
YS26	1.8 ± 0.2	1.6 ± 0.2
YS27	1.7 ± 0.1	1.4 ± 0.2
YS54	2.1 ± 0.1	2.1 ± 0.3
YS59	1.7 ± 0.2	1.8 ± 0.2
YS63	1.8 ± 0.2	1.5 ± 0.1
YS66	1.9 ± 0.1	1.5 ± 0.2
Pal5	1.6 ± 0.1	1.4 ± 0.2
FP2	0.0	0.00
BR322	1.3 ± 0.1	1.7 ± 0.1
Mean	1.77	1.56

[1] Values and standard deviation for the solubilization indexes.

On general the solubilization index (IS) were greater in the NBRI-P medium with a mean value of about 1.77, while for PVK medium was 1.56. The highest IS value (2.07) was observed for the isolate YS54 in the NBRI-P medium. Solubilization of calcium phosphate in GL (Ca-P) medium was detected only for the isolates YS54 (SI 1.89) and YS59 (SI 2.16) and, for the standard strain Pal5 with SI 2.33.

Clustering analysis based on physiological data

The similarity analysis using the physiological data allowed identifying seven groups with about 75% of similarity (Figure 2). The group with the greatest number of isolates (G1) is formed by eleven isolates, followed by the groups G2 and G3 with 5 and 4 isolates, respectively. Within the group G1 the isolates NS15 and LS28 showed 100% similarity between them. About 72% of the isolates in the G1 group were obtained in LGI-P semi-selective medium for *Gluconacetobacter* spp., whereas about 80% of the isolates in the G2 group were obtained in YMA semi-selective medium for *Rhizobium* spp. Although the isolates show at least 62% similarity to the standard strains, they did not cluster with the isolates obtained in the semi-selective culture media for the respective species (Figure 2).

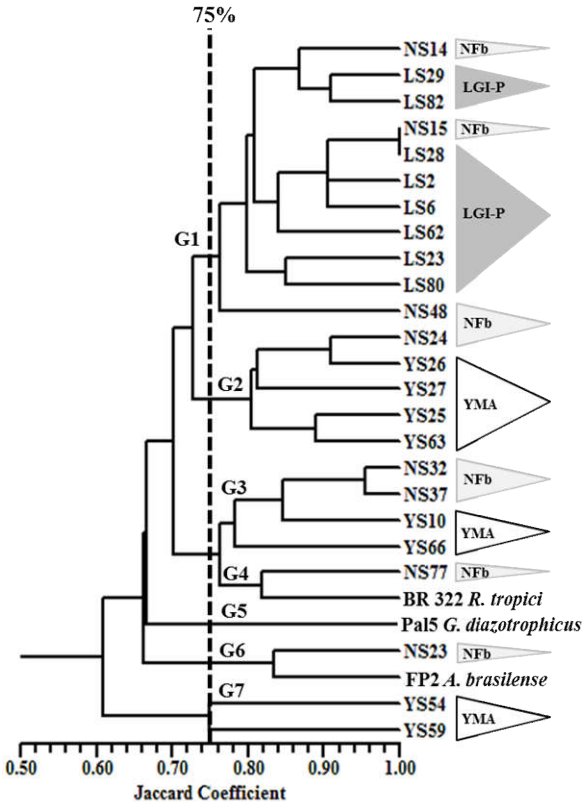

Figure 2. Consensus dendrogram obtained by combining resistance to antibiotics, use of carbon sources and enzyme activity data of 24 isolates obtained from sorghum roots and the standard strains. The similarity matrix was determined using the Jaccard coefficient and the dendrogram was generated using the UPGMA algorithm

The standard strain Pal 5 of *G. diazotrophicus* formed alone the G5 group, whereas the standard strain BR 322 of *R. tropici* grouped with the isolate NS77 in the G4 group. The standard strain FP2 of *A. brasilense* showed similarity above 80% with the isolate NS23, forming the G6 group.

Isolates YS54 and YS59 formed the G7 group, presenting the lowest similarity (62%) when compared to the other groups. Also, a higher grouping tendency was observed with greater similarity for the isolates of the G1 group obtained in LGI-P medium. For the other isolates, there were no groups associated with the isolation medium (Figure 2).

Genotypic similarity analysis

The similarity analysis based on the 16S-23S rRNA intergenic region showed a great diversity among the evaluated bacteria, with the clustering of the isolates and standard strains in eigth groups with about 60% of similarity (Figure 3).

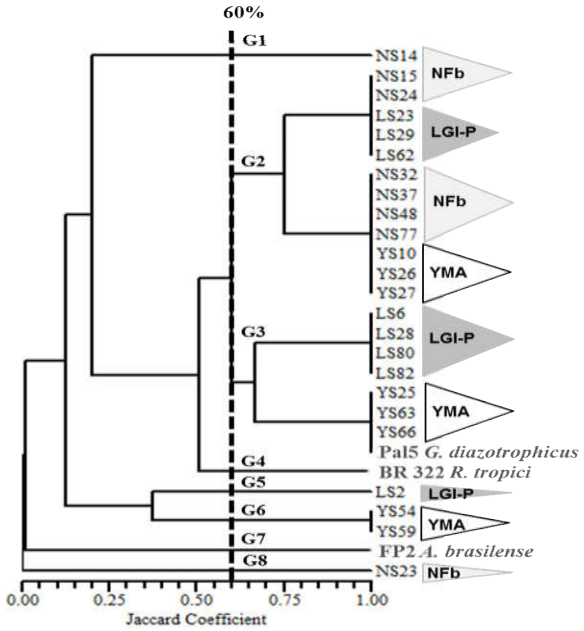

Figure 3. Clustering analysis based on the intergenic region 16-23S rDNA data of 24 isolates obtained from sorghum roots and the standard strains. The similarity matrix was determined using the Jaccard coefficient and the dendrogram was generated using the UPGMA algorithm

The largest group (G2) was composed by 12 isolates divided into two sub-groups with 100% similarity among five isolates into the first sub-group and, among seven isolates into the second one. The G3 group also showed two sub-groups showing 100% similarity among 4 isolates obtained in LGI-P medium into the first sub-group and, among three isolates obtained in YMA medium and the standard strain Pal5 of *G. diazotrophicus* into the second one (Figure 3). The isolates (NS15, NS24, LS6 LS23 and LS80) of the first sub-group in the G1 group were similar regarding the PCR fragments, showing two fragments corresponding to two regions of different sizes. The same situation was also observed for the standard strains Pal5 and BR 322. The formation of groups with more than one isolate (G1, G2, and G3) and groups containing isolates with less than 60% similarity suggests great genetic variability among the isolates of sorghum. Besides, there was no tendency of isolates grouping by the isolation medium (Figure 3).

DISCUSSION

The focus of the scientific research on the isolation and characterization of plant growth promoting rhizobacteria (PGPR) for use as low impact agricultural technologies has increased worldwide.

Currently, sorghum is cultivated with low input of biological products and the selection of PGPR bacteria that can be used in the formulation of inoculants can contribute to the sustainability of the crop, as well as bring economic benefits. Another contribution of these studies is associated with the knowledge of the microbiota from the ecological point of view and its relationship with other factors that affect the production system. Therefore, the demand for microorganisms with great metabolic flexibility, allowing them to adapt well to different conditions of the production systems is a key factor to ensure their survival. The presence of variability influences the adaptive efficiency of microorganisms that can respond to selection pressure, triggering resistance to antibiotics as a survival strategy or adaptation and competitiveness habitat (Levy, 1997; Brader *et al*., 2014).

Among these features, the resistance to antibiotics can contribute tremendously to a better establishment of the PGPR to the environment. Most of the isolates evaluated in our work showed high resistance to different doses of ampicillin, chloramphenicol, streptomycin, and tetracycline (Table 1) and, 50% of the isolates are resistant to a high dose (200 µg mL^{-1}) of at least three antibiotics (Figure 1). These findings corroborate the results of Mareque *et al*. (2015), which found that 89% and 81% of the isolates obtained from sorghum were resistant to streptomycin and tetracycline, respectively. However, Ikeda *et al*. (2013) reported that 87% of isolates obtained from maize were sensitive to tetracycline at a dose of 50 µg mL^{-1}, while in our work only 26% of the isolates were sensitive to this antibiotic at this dose (Table 1).
The lowest resistance to antibiotics was observed for the isolates obtained in the YMA medium, which is a semi-selective medium for bacteria of the family *Rhizobiaceae*. However, this family of bacteria is known to have intrinsic resistance to a wide range of antibiotics (Hungria *et al*, 1994), as presented by the standard strain BR 322. Therefore, this group of isolates may not belong to the rhizobia group. The use of carbon sources is another important tool for the adapting process of the microorganisms to the environment. Sorghum isolates tested for the ability to use different carbon sources showed a higher metabolizing capacity of compounds; however, on general eight of the thirteen evaluated carbon sources were used by more than 90% of the isolates. Bacteria with the ability to use a large variability of carbon sources was also verified by Ikeda *et al*. (2013) and Mareque *et al*. (2015) in works studying isolates of maize and sorghum, respectively. However, according to Teixeira *et al*. (2008), bacteria isolated from maize are more efficient in the use of citrate as carbon source, as compared to isolates obtained from sugarcane and other grasses.

Nineteen of the 24 isolates obtained from sorghum roots showed the activity of the enzymes citrate, catalase, nitrate reductase, urease and nitrogenase on the qualitative evaluations. This demonstrates that sorghum isolates can contribute to sorghum growth through indirect effects, as expected for PGPR bacteria. Indirect effects of microorganisms on plant growth are associated with environmental adaptability and colonization, such as resistance to phytopathogens as mediated by lytic enzymes and the relationship of catalase with the tolerance to environmental stresses (Glick 2012). About 96% of the isolates and standard strains were positive for catalase and nitrate reductase activities. Bacterial isolates showing these enzymes can be considered promising PGPR.

The presence of catalase enzyme may be potentially advantageous for bacterial strains, as bacteria showing this enzyme tend to be highly resistant to environmental, mechanical and chemical stresses (Kumar, *et al*., 2012). On the other hand, nitrate reductase enzyme is a component of the denitrification process, which produces nitrous oxide and releases N_2. This reaction is part of the nitrogen cycle and metabolism of several bacteria associated with grasses such as *Pseudomonas* and *Azospirillum* (Mbai *et al*, 2013; Ikeda *et al* 2013). Thus, isolates showing activity for this enzyme can effectively participate in the nitrogen cycle. The presence of other N cycle-related enzymes in microorganisms, such as urease and nitrogenase is a desirable feature for PGPR because microorganisms able to supply this compound can provide advantages to the plants. This is an interesting alternative to reduce the use of N-fertilizers since urea is the main component of the N-fertilizers used in the agriculture (Teixeira *et al*., 2008). Among the isolates evaluated in this work, about 93% of them were positive for urease. Our results differ from those reported by Ikeda *et al*. (2013) and Teixeira *et al*. (2008), whose did not observe urease activity in the majority of the bacteria isolated from Cerrado soils. Besides, about 89% of the isolates presented an aerotractic film, indicating activity of the enzyme nitrogenase.

Thus, these isolates are nitrogen-fixing bacteria that can produce direct effects on the development of plants. This activity was also observed in bacteria isolated from sorghum in Uruguay (Mareque *et al*, 2015). Also, among the isolates 17 and 12 of them showed the activity of lipase and cellulose, respectively; however, considering as good producer only the isolates showing enzymatic index above 1.5, just 6 and 4 isolates are considered good producers of lipase and cellulose, respectively. Lipase and cellulose are lytics enzymes related to the indirect effects of the PGPR, involved in resistance to phytopathogens (Glick, 2012). Mareque *et al*. (2014) observed among sorghum isolates that only 2% were positive for cellulase. However, Silva *et al*. (2015) verified that 75% of the isolates from the soil of the Brazilian semi-arid region were positive for cellulase with an enzymatic index of 6.9. The ability to secrete cellulolytic enzymes gives to the microorganism the ability to convert complex substrates, such as cell wall of both plants and phytopathogenic fungi, which may be a strategy for colonization of environments or to act as biocontrol agents (Silva *et al*., 2015). In this way, six isolates obtained from sorghum roots (NS14, NS24, LS23, LS29, YS54, and YS59) may be promising sources for the production of cellulolytic enzymes.

In our study, the sorghum isolates also showed P-solubilization ability. About 95% of the isolates were able to solubilize phosphate in somegrade (IS >1). This is a clear indication that these isolates have a great potential to be used as PGPR since the P-solubilization can directly affect the plant growth by supplying P for the plants. Similarly, Muthukumarasamy *et al*. (2017) observed high P-solubilization in the NBRI-P medium by bacterial isolates obtained from sugarcane. Other authors reported that 57% of the bacterial isolates obtained from several plants had P-solubilization capacity irrespectively of the phosphate compost, aluminum or calcium phosphate (Hara and Oliveira, 2005). Knowledge on the capacities and abilities of the microorganisms to promote plant growth through different pathways is an important factor for their selection as plant growth promoters. Additionally, clustering analysis based on physiological and genotypic characteristics helps in this selection process, since the use of metadata, as analyzes based on physiological and genetic characteristics, can promote a broader understanding of the diversity of microorganisms associated with plants, such as Sorghum (Moreira *et al*., 2010, Ikeda *et al*., 2013). In our study, the clustering analysis based on the physiological data revealed that the isolates tended to group by the semisselective medium of isolation; however, with a low occurrence of clusters at 100% similarity, evidencing a great physiological variability among the evaluated isolates. This tendency of grouping by semisselective medium was not observed on the clustering analysis based on the genotypic data. Intergenic 16S-23S rRNA data also revealed genetic variability among the isolates; however, many 100% similarity clusters were found. This may occur because the PCR analysis of the 16S-23S rRNA intergenic region may vary in number and size, depending on the genus and species of the microorganism; however, this analysis can provide a specie-specific discrimination, being considered an extremely useful tool in the taxonomic study of bacteria (Dingman, 2012).

Conclusion

Isolates obtained from sorghum roots showed high resistance to antibiotics and ability to use different carbon sources, indicating high metabolic flexibility. In addition, the isolates showed activity for many enzymes and ability to solubilize phosphate. The cluster analysis revealed high physiological and genetic diversity among isolates. In general, many isolates showed more than one mechanism related to plant growth promotion, especially the isolates NS24, NS32, and NS37, indicating their potential for biotechnological application.

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