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RESEARCH PAPER

Diazotrophic bacteria isolated from *Brachiaria* spp.: genetic and physiological diversity

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Abstract

J. T. C. Oliveira, G. T. Silva, W. P. S. Diniz, E. F. Fernandes, I. B. Santos, D. R. M. Lima, M.C. Quecine, J. Kuklinsky-Sobral, and F. J. Freire. 2018. Diazotrophic bacteria isolated from Brachiaria spp.: genetic and physiological diversity. Cien. Inv. Agr. 45(3): 277-289. Grass from the genus *Brachiaria* spp. predominates in pastures with low fertile soils. This scenario highlights the importance of the association with microorganisms to foster plant growth, which becomes essential to the successful establishment of this forage in such environments. This study aimed to evaluate the genetic variability and identify the mechanisms of plant growth promotion, *in vitro*, of bacteria associated with *Brachiaria decumbens* Stapf. and *Brachiaria humidicola* (Rendle.) Schweickerdt in Pernambuco, Brazil. We evaluated 20 isolates of diazotrophic bacteria obtained from the endophyte or rhizosphere communities. The genetic characteristics were determined via sequencing the 16S rRNA region, which allowed us to identify ten different bacterial genera: *Bacillus* sp., *Burkholderia* sp., *Enterobacter* sp., *Klebsiella* sp., *Microbacterium* sp., *Pantoea* sp., *Ralstonia* sp., *Rhizobium* sp., *Sinomonas* sp., and *Sphingomonas* sp., with a specificity of the genus *Rhizobium* sp. to *Brachiaria decumbens* Stapf.. The phenotypic and functional characteristics revealed that 100% of the bacterial strains produced indol-3-acetic acid (IAA) with the addition of L-tryptophan, and 60% presented IAA production independent of the L-tryptophan pathway. We also detected that 70% of the isolated bacteria possessed the capacity to solubilize phosphorus. The analysis of the enzymatic output revealed that 30% of the bacterial isolates produced cellulase, 60% produced pectate lyase, 15% produced polygalacturonase, and 30% produced amylase. We also detected the production of N-acyl homoserine lactones in 65% of bacterial strains. In summary, our results showed that plants of *B. decumbens* Stapf. and *B. humidicola* (Rendle.) Schweickerdt interacted with different bacterial genera capable of promoting plant growth.

Keywords: N-Acyl homoserine lactones, plant growth promoting bacteria, plant-microbe interactions.

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Introduction

In Brazil, several species of tropical forage grass are used in the formation of pastures (Aguiar *et al*., 2017). The genus *Urochloa* spp. (=*Brachiaria* spp.) dominates due to its characteristics, such as adaptability and tolerance to different soil types and conditions and flexibility in usage and management, allowing the cultivation of 200 million hectares of pastures in Brazilian territory. However, an increasing percentage of Brazilian pastures already face some level of degradation, mainly due to the lack of nutrient supply (Hungria *et al*., 2016).

Nitrogen (N) plays an essential role in determining crop productivity, primarily related to the cost of implementation and maintenance of crops via inorganic fertilization. Moreover, inorganic fertilization increases soil dynamics and may impact the environment, highlighting the need for seek more viable alternatives to minimize the entry of inorganic fertilizers and prolong the N availability to the plant (Hungria *et al*., 2016).

In this scenario, the application of plant growth-promoting bacteria might contribute to providing a continuous source of N to plants and promote beneficial effects on seed germination, emergence, and plant growth. These bacteria fix nitrogen through many desirable mechanisms, especially the production of phytohormones, such as indole-3-acetic acid (IAA), and the synthesis of lytic enzymes (Araújo *et al*., 2012; Pereira *et al*., 2012; Wemheuer *et al*., 2016).

Plant growth-promoting bacteria may colonize diverse niches in association with plants such as the soil rhizosphere, the surface of plant tissues (epiphytes), and within plant tissue (endophytes). Altogether, this corroborates the high genetic diversity of beneficial bacteria associated with plants and the expression of different plant grown mechanisms of agronomic interest (Compant *et al*., 2010; Mutai *et al*., 2017).

Bacterial diversity changes according to the species of host plants colonized and the niche inhabited (Castanheira *et al*., 2014). Due to several factors that influence this diversity, phylogenetic analyses appear to be tools that enable a better understanding of the role of these microorganisms in the promotion of plant growth, especially in association with forage grass, where the costs of inorganic fertilization can be reduced with the use of these microorganisms (Araújo *et al*., 2012; Cordero *et al*., 2016). Therefore, we aimed to evaluate the genetic variability of diazotrophic bacteria, identify the mechanisms of plant growth promotion, compare isolates of different vegetable species, including *Brachiaria decumbens* Stapf. and *Brachiaria humidicola* (Rendle.) Schweickerdt, and compare colonization niches, rhizosphere communities and root endophytes.

Materials and Methods

We evaluated 20 isolates of diazotrophic bacteria capable of growing in the semisolid medium, NFb, without N supply (Döbereiner *et al*., 1995). The bacteria were previously isolated from plants cultivated in pastures under intermittent grazing of cattle and without inorganic input, localized at the Pau Ferro farm, municipality of Correntes, Pernambuco (09°06'00"S e 36°21'29"W). From a total of 20 selected bacteria, ten were isolated from *Brachiaria decumbens* Stapf., of which five were classified as root endophytes, and the other five were obtained from the rhizosphere (a region of soil placed approximately at a distance of 3–5 mm surrounding the roots). The other half of the bacteria were isolated from *Brachiaria humidicola* (Rendle.) Schweickerdt, six of which were endophytes, and four of which belonged to the rhizosphere.

The identification of bacteria was performed via partial sequencing of the 16S rRNA gene. The DNA extractions were performed using the *PureLink*® Genomic DNA Kits (Thermo Fisher, Massachusetts, USA) and analyzed in agarose

gel (1.00% w/v) with TAE 1x buffer stained with Gel Red (Biotium, California, USA) and 10X *Loading Buffer* (Thermo Fisher, Massachusetts, USA). All DNA presented were photodocumented under ultraviolet light. DNA quantification was performed with NanoDrop 2000® (Thermo Fisher, Massachusetts, USA).

After DNA extraction, the 16S rDNA region was amplified using the primers R1387 (5'-CGGTGTGTACAAGGCCCGGGAACG-3') and P27 (5'-GAGAGTTTGATCCTGGCTCAG-3') (Heuer *et al*., 1997) in a final volume of 50 µl containing 1.50 mM of $MgCl_2$, 0.20 mM of dNTPs, 10X reaction buffer, 0.20 µM of each primer and 0.05 U of Taq DNA Polymerase (Sigma Aldrich, Missouri, USA). The amplification program in the thermocycler constituted an initial denaturation of 10 minutes at 94 °C, followed by 35 cycles: 30 seconds at 94 °C, 1 minute at 62.5 °C, 1 minute at 72 °C, and a final extension phase of 10 minutes at 72 °C.

The integrity of the amplified fragments was evaluated via agarose gel (1.00% p/v) with the presence of a 1 kb DNA Ladder (Fermentas, Massachusetts, USA). After that, the amplicons were purified with polyethylene glycol (PEG 8,000) and sequenced. The obtained sequences from the partial 16S rDNA gene region, amplified by the primer PO27, were used to identify the taxonomical classification via BlastN in the NCBI database (*National Center for Biotechnology Information* – http://ncbi.nlm.nih.gov).

The raw sequences were processed and analyzed using the CLC *Genomics Workbench* (version 7.0.3). The taxonomy assignment for each bacterial isolate was obtained using BLAST analysis within the NCBI database. The nucleotide sequences were aligned, and a single taxon was identified via sequence similarity. To perform the phylogenetic analysis, *Pelistega europaea* Y11890 was included as an outgroup to root the phylogenetic tree. The software MEGA 4 (*Molecular Evolutionary Genetics Analysis*, version 4.0) was used, and the similarity between isolates was determined via the Jaccard coefficient with the method of *Unweighted Pair Group Method with Arithmetic Mean* (UPGMA).

To evaluate both phenotypic and functional mechanisms of plant growth promotion *in vitro*, the isolates of diazotrophic bacteria were cultivated in TSA (*Trypticase Soy Agar*) medium. The analyses were performed with three replicates together with a positive control, the bacterial strain EM303 (*Pseudomonas oryzihabitans*), an endophytic bacterium of soybean, N-fixer, and producer of auxin and lytic enzymes (Kuklinsky-Sobral *et al*., 2004).

To both quantify and identify the biosynthesis of IAA, the bacterial isolates were inoculated in tubes containing liquid TSA medium, with and without the addition of L-tryptophan (5 mM). The incubation occurred under shaking (120 rpm) at 28 °C in the absence of light for 24 hours. After that, each tube was centrifuged at 12.000 *g* for 5 minutes, and the supernatant was added with the Salkowski reagent (2.00% of $FeCl_3$ 0.5 M in 35.00% of perchloric acid) in a proportion of 1.50:0.50 v/v. The samples were again incubated at 28 °C in the absence of light for 30 minutes (Crozier *et al*., 1988; Pereira *et al*., 2012). The quantification of bacterial IAA was performed in a spectrophotometer (530 nm), and the concentration of this phytohormone was estimated using a standard curve with known levels of synthetic IAA: 0.00, 50.00, 100.00, 150.00, 200.00, 250.00, 300.00, and 350.00 µg mL^{-1} diluted in the sterilized culture medium and not inoculated.

To evaluate the potential of this bacteria in solubilizing inorganic phosphate, the bacteria were inoculated in solid medium containing dibasic calcium phosphate as described by Verma *et al*. (2001) with modifications. The bacteria were inoculated in solid medium containing insoluble phosphate (10.00 g L^{-1} of glucose; 5.00 g L^{-1} of NH_4Cl; 1.00 g L^{-1} of NaCl; 1.00 g L^{-1} of $MgSO_4.7H_2O$; 4.00 g L^{-1} of $CaHPO_4$; 15.00 g L^{-1} of agar; pH 7.20). The plates were incubated at 28 °C for 72 hours and then evaluated.

The synthesis of lytic enzymes was evaluated according to the procedures of Mariano and Silveira (2005) for cellulose and amylase production, and the determination of pectin lyase and polygalacturonase followed the procedures of Hankin and Lacy (1984). All the enzymatic tests were incubated at 28 °C for 72 hours. The enzymatic activities were estimated according to the mean of the diameter of the halo of hydrolysis and the colony diameter, both determined with a digital Vernier caliper.

To evaluate the *in vitro* synthesis of signaling quorum sensing molecules, the production of N-Acyl homoserine lactone (AHL) was determined in bioassays between each bacterial strain and the bacteria *Agrobacterium tumefaciens* NTL4 (pZLR4) containing the reporter gene for signaling AHL. *Agrobacterium tumefaciens* NTL4 was inoculated linearly on the surface of a Petri dish containing *Luria Bertani* medium (LB-agar). X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added at 10.00 μg mL^{-1} together with the isolated bacteria inoculated in a perpendicular direction. Each plate was incubated for 48 hours at 28 °C. The presence of *Agrobacterium tumefaciens* blue colonies indicated the production of AHL by the evaluated bacteria (Leite *et al*., 2014; Grönemeyer *et al*., 2012).

The data obtained from the mechanisms of plant growth promotion were analyzed in percentages, and the differences between isolates from different plant species and niches in the variables of AHL production were determined via chi-squared tests (x^2). The quantitative variables of phytohormone production and the synthesis of hydrolytic enzymes were analyzed via orthogonal contrasts using t-tests ($P \leq 0.05$).

Results and Discussion

The 20 valid diazotrophs were grouped into three phyla, five classes, seven orders, eight families (Table 1) and ten genera, with sequences deposited at the National Biotechnology Information Center (NCBI) in Table 2.

The four most common bacterial genera found were *Burkholderia* sp.*, Enterobacter* sp.*, Klebsiella* sp., and *Rhizobium* sp. In *Brachiaria decumbens* Stapf., the bacteria genera *Rhizobium* sp. and *Klebsiella* sp. predominated, whereas, in *Brachiaria humidicola* (Rendle.) Schweickerdt, the genera *Burkholderia* sp. and *Enterobacter* sp. were more prevalent. The root endophytic niche presented as the most common environment for genera *Burkholderia* sp.*, Enterobacter* sp.*,* and *Klebsiella* sp., whereas the rhizosphere niche comprised mainly bacteria from the genus *Rhizobium* sp. (Figure 1).

The phylogenetic tree of the 20 bacterial isolates suggested the formation of four groups (Figure 2). Group 1 comprised the bacteria of the genera *Klebsiella* sp., *Enterobacter* sp., and *Pantoea* sp. Group II clustered the bacteria from the genera *Ralstonia* sp. and *Burkholderia* sp. Group III clustered the bacteria from the genera *Sphingomonas*

Table 1. Taxonomic grouping of 20 diazotrophic bacterial strains isolated from *Brachiaria decumbens* Stapf. and *Brachiaria humidicola* (Rendle.) Schweickerdt in the different niches of root endophytes and rhizospheres.

Phylum	Class	Order	Family
Actinobacteria (2)	*Actinobacteria* (2)	*Actinomycetales* (1)	*Bacillaceae* (1)
Firmicutes (1)	*Alphaproteobacteria* (4)	*Bacillales* (1)	*Burkholderiaceae* (5)
Proteobacteria (17)	*Bacilli* (1)	*Burkholderiales* (6)	*Enterobacteriaceae* (7)
	Betaproteobacteria (6)	*Enterobacteriales* (7)	*Microbacteriaceae* (1)
	Gammaproteobacteria (7)	*Micrococcales* (1)	*Micrococcaceae* (1)
		Rhizobiales (3)	*Ralstoniaceae* (1)
		Sphingomonadales (1)	*Rhizobiaceae* (3)
			Sphingomonadaceae (1)

Table 2. Results of the search for similarities in the National Center for Biotechnology Information (NCBI) GenBank for the partial and complete sequencing of the 16S rDNA gene and the GenBank deposition number of the 20 diazotrophic bacteria isolated from the *Brachiaria decumbens* Stapf. and *Brachiaria humidicola* (Rendle.) Schweickerdt grasses in their different niche of root endophytes and rhizosphere.

Code	Identification	Similarity NCBI (%)	Access Number	Deposition Number
Isolates from *Brachiaria decumbens* Stapf. in the root endophytic niche				
UAGB69	*Enterobacter kobei strain* CIP 105566	97.00%	NR_028993.1	MH537742
UAGB70	*Microbacterium* sp.	96.00%	NR_026160.1	MH537743
UAGB154	*Klebsiella variicola* strain F2R9	98.00%	NR_025635.1	MH537756
UAGB156	*Klebsiella* sp.	96.00%	NR_074729.1	MH537757
UAGB167	*Rhizobium hainanense* strain I66	99.00%	NR_029195.1	MH537758
Isolates from *Brachiaria decumbens* Stapf. in the rhizosphere niche				
UAGB71	*Sinomonas atrocyanea* strain DSM 20127	97.00%	NR_116419.1	MH537744
UAGB80	*Sphingomonas paucimobilis* strain DSM 30198	97.00%	NR_104893.1	MH537745
UAGB139	*Burkholderia cenocepacia* AU 1054 strain AU	98.00%	NR_074686.1	MH537753
UAGB147	*Rhizobium* sp.	94.00%	NR_133049.1	MH537754
UAGB150	*Rhizobium* sp.	93.00%	NR_133049.1	MH537755
Isolates of *Brachiaria humidicola* (Rendle.) Schweickerdt in root endophytic niche				
UAGB1	*Pantoea* sp.	92.00%	NR_102966.1	MH537739
UAGB93	*Burkholderia* sp.	96.00%	NR_102890.1	MH537746
UAGB94	*Burkholderia territorii* strain LMG 28158	97.00%	NR_136496.1	MH537747
UAGB105	*Burkholderia territorii* strain LMG 28158	98.00%	NR_136496.1	MH537748
UAGB106	*Burkholderia lata* strain 383	97.00%	NR_102890.1	MH537749
UAGB110	*Enterobacter* sp.	95.00%	NR_028993.1	MH537750
Isolates of *Brachiaria humidicola* (Rendle.) Schweickerdt in the rhizosphere niche				
UAGB10	*Bacillus anthracis* str. Ames strain Ames	98.00%	NR_074453.1	MH537740
UAGB60	*Klebsiella* sp.	96.00%	NR_074729.1	MH537741
UAGB119	*Enterobacter kobei* strain CIP 105566	97.00%	NR_028993.1	MH537751
UAGB128	*Ralstonia pickettii* 12J strain 12J	98.00%	NR_102967.1	MH537752

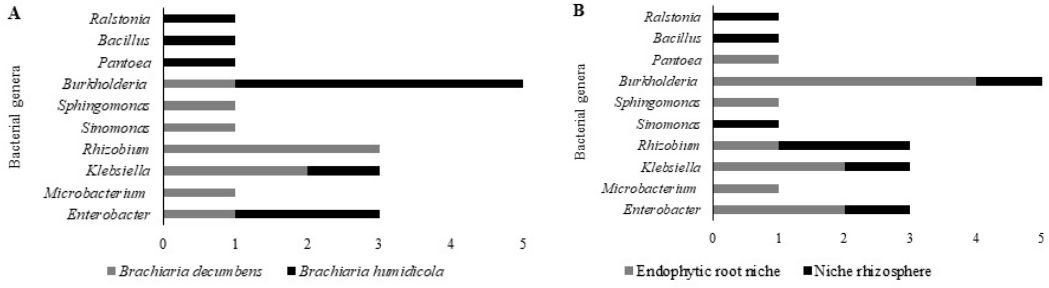

Figure 1. Distribution of the bacterial genera of the 20 diazotrophic bacterial isolates. A=Genera associated with *Brachiaria decumbens* Stapf. and *Brachiaria humidicola* (Rendle.) Schweickerdt; B=Genera associated with the root endophytic niche and the rhizosphere.

sp. and *Rhizobium* sp. Finally, in Group IV, the bacterial genera *Bacillus* sp., *Sinomonas* sp. and *Microbacterium* sp. clustered together. Interestingly, Group III clustered only bacteria isolated from *Brachiaria decumbens* Stapf., whereas, in the other clusters, we observed more heterogeneity of isolates from both species and niches.

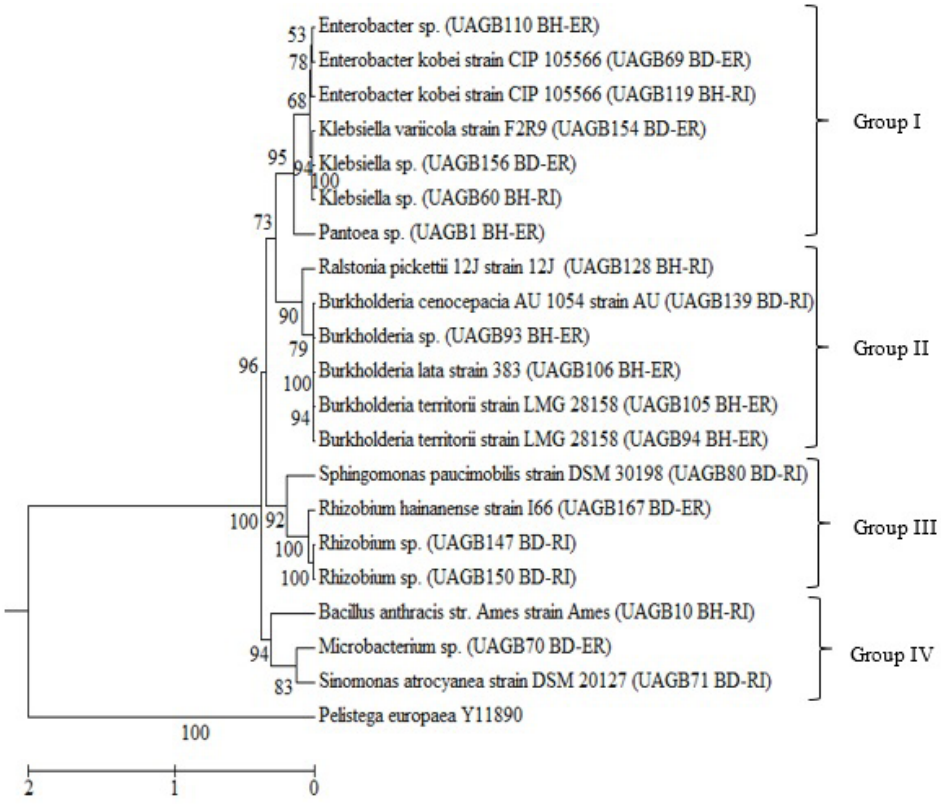

Figure 2. Phylogenetic tree of the partial sequencing of the 16S rDNA built by the method of *Unweighted Pair Group Method with Arithmetic Mean* for the 20 diazotrophic bacteria isolated from the grasses *Brachiaria decumbens* Stapf. (BD) and *Brachiaria humidicola* (Rendle.) Schweickerdt (BH), and from the root endophytic niche (RE) and the rhizosphere (RI). The sequence of *Pelistega europaea* Y11890 was used as an outgroup to root the phylogenetic tree.

Brachiaria decumbens Stapf. presented a specificity to the genes of *Rhizobium* sp., unlike the other bacterial genera with more than one isolate that colonized both plant species (Figure 3A e B). Between the *Brachiaria* plants, the bacteria *Enterobacter kobei* strain CIP 105566 seemed to associate with both species in the different niches of colonization. Evaluating the phylogenetic tree according to plant origin, we did not observe clustering between the root endophytic and rhizosphere niches (Figure 2A e B), which highlights the low specificity of isolation to the plant genotype and niche association.

The bacterial classes G*ammaproteobacteria* and *Betaproteobacteria* comprised 65% of bacterial isolates (Table 1). The bacteria from this class responded to chemotactic factors of root exudates and were thus abundant in rhizospheric soils (Costa *et al*., 2014). However, the profile of exudates differed according to genotype, phenological status, plant nutrition, and seasonality (Murphy *et al*., 2016). The bacterial colonization of the plant (epiphytic and endophytic colonization) persists through continuous rhizosphere colonization. Despite the distinction between niches, there are bacterial populations that flow between them, depending on the environment and the nutritional state of the plant (Luvizotto *et al.,* 2010).

Mutai *et al.* (2017) also reported high heterogeneity when evaluating the bacterial community with mechanisms for plant growth promotion associated with plants from the genus *Brachiaria* spp. in the niches of leaves, roots, and the rhizosphere; namely, *Microbacterium* sp., *Pantoea* sp., *Rhizobium* sp., and *Sphingomonas* sp. Previous studies have also reported other bacterial genera such as *Bacillus* sp., *Burkholderia* sp., *Enterobacter* sp., *Klebsiella* sp., *Ralstonia* sp., and *Sinomonas*

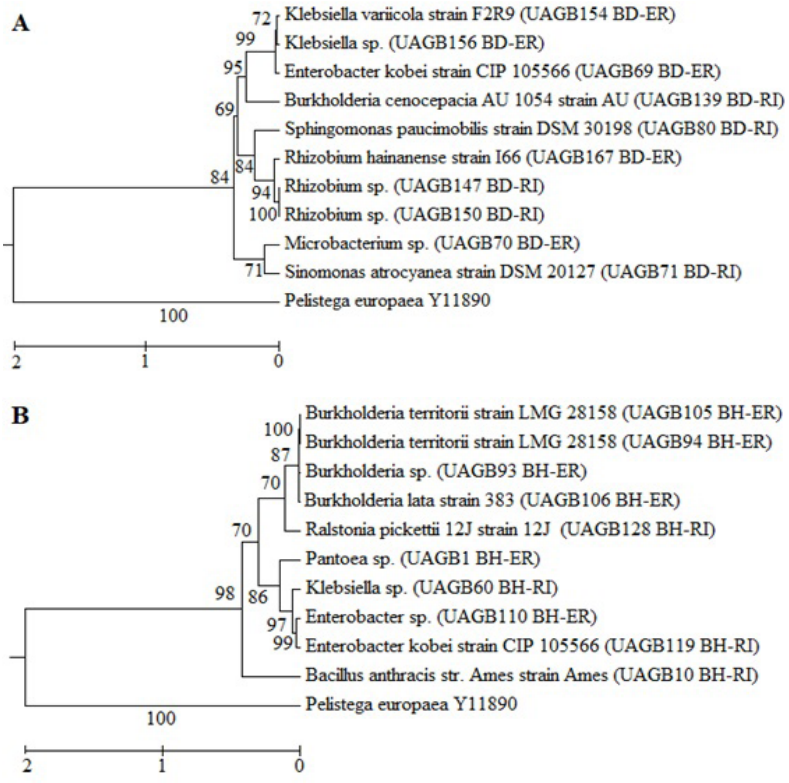

Figure 3. Phylogenetic tree of the partial sequencing of the 16S rDNA for the 20 isolates of diazotrophic bacteria according to the method of *Unweighted Pair Group Method with Arithmetic Mean*. A=Isolates from *Brachiaria decumbens* Stapf. (BD), and B=*Brachiaria humidicola* (Rendle.) Schweickerdt (BH), in the root endophyte niche (RE) and rhizosphere (RI). Sequence from *Pelistega europaea* Y11890 was used as an outgroup to root the phylogenetic tree.

sp. as diazotrophic bacteria of nonleguminous plants (Araújo *et al*., 2012; Pereira *et al*., 2012; Souza *et al*., 2017).

Beyond the capacity of biological nitrogen fixation, all bacterial strains presented other mechanisms for promoting plant growth *in vitro*. All bacterial strains demonstrated the capability of biosynthesizing IAA dependent on L-tryptophan, with concentrations ranging between 162.10 and 2.09 μg mL^{-1}, with the isolates of *Enterobacter kobei* strain CIP 105566 (UAGB69) and *Enterobacter* sp. (UAGB110) corresponding to the maximum and minimum production, respectively. The synthesis of this phytohormone for different metabolic pathways occurred in 60% of isolates, and the concentration of IAA ranged from 26.88 to 0.86 μg mL^{-1} for the bacteria isolated from *Burkholderia lata* strain 383 (UAGB106) and *Enterobacter* sp. (UAGB110), respectively (Table 3).

The synthesis of IAA by bacteria associated with plants occurs via different metabolic pathways, with L-tryptophan acting as a precursor in the majority of them. However, some bacterial genera are capable of synthesizing IAA via metabolic pathways independent of L-tryptophan, but with a smaller production (Richardson *et al*., 2009).

According to Mutai *et al*. (2017), different genera of diazotrophic bacteria isolated from various parts of *Brachiaria* spp. produced IAA *in vitro*, thus corroborating our observed results. Araújo *et al*. (2012) reported peaks of IAA production of 22.4 μg mL^{-1} for the bacteria *Bacillus* sp. isolated from *Brachiaria brizantha*, a value below that observed in 75% of the evaluated strains in our present study. The synthesis of auxin, especially IAA, promotes the growth of secondary roots by microorganisms associated with plants and root elongation. Consequently, this root growth increases the absorption

Table 3. Expression of mechanisms of plant growth promotion *in vitro* for the 20 diazotrophic bacteria associated with *Brachiaria decumbens* Stapf. and *Brachiaria humidicola* (Rendle.) Schweickerdt in the root endophytic and rhizosphere niches.

Code	Bacterial genera	FBN	IAA		SI	EI				AHL
			CLT	SLT		CE	PE	PO	AM	
Isolates from *Brachiaria decumbens* Stapf. in the root endophytic niche										
UAGB69	*Enterobacter kobei strain* CIP 105566	+	162.10	0.00	0.00	0.00	4.74	0.00	0.0	-
UAGB70	*Microbacterium* sp.	+	3.61	0.00	0.00	0.00	7.33	0.00	4.58	-
UAGB154	*Klebsiella variicola* strain F2R9	+	100.08	14.64	2.06	0.00	1.09	0.00	0.00	-
UAGB156	*Klebsiella* sp.	+	67.18	20.80	1.37	0.00	1.18	0.00	0.00	+
UAGB167	*Rhizobium hainanense* strain I66	+	138.64	15.94	0.00	0.00	0.00	0.00	0.00	+
Isolates from *Brachiaria decumbens* Stapf. in the rhizosphere niche										
UAGB71	*Sinomonas atrocyanea* strain DSM 20127	+	113.67	0.00	0.00	0.00	3.76	4.00	2.77	-
UAGB80	*Sphingomonas paucimobilis* strain DSM 30198	+	5.84	0.00	2.04	0.00	1.86	0.00	1.62	-
UAGB139	*Burkholderia cenocepacia* AU 1054 strain AU	+	4.73	2.53	5.48	0.00	2.99	0.00	0.00	+
UAGB147	*Rhizobium* sp.	+	10.56	0.00	4.26	0.00	3.74	4.76	0.00	+
UAGB150	*Rhizobium* sp.	+	5.03	4.00	3.16	1.04	4.39	6.28	2.48	-
Isolates of *Brachiaria humidicola* (Rendle.) Schweickerdt in root endophytic niche										
UAGB1	*Pantoea* sp.	+	35.52	5.47	3.83	0.00	4.26	0.00	1.81	+
UAGB93	*Burkholderia* sp.	+	56.48	0.00	4.12	0.00	0.00	0.00	0.00	+
UAGB94	*Burkholderia territorii* strain LMG 28158	+	63.00	0.00	4.57	0.00	0.00	0.00	0.00	+
UAGB105	*Burkholderia territorii* strain LMG 28158	+	51.79	23.88	3.05	1.69	0.00	0.00	0.00	+
UAGB106	*Burkholderia lata* strain 383	+	49.23	26.33	3.49	1.65	0.00	0.00	0.00	+
UAGB110	*Enterobacter* sp.	+	2.09	0.86	3.73	6.72	0.00	0.00	0.00	+
Isolates of *Brachiaria humidicola* (Rendle.) Schweickerdt in the rhizosphere niche										
UAGB10	*Bacillus anthracis* str. Ames strain Ames	+	13.48	9.21	0.00	1.20	4.86	0.00	1.09	+
UAGB60	*Klebsiella* sp.	+	4.98	0.00	0.00	0.00	2.30	0.00	2.20	+
UAGB119	*Enterobacter kobei* strain CIP 105566	+	3.73	15.60	2.79	1.24	0.00	0.00	0.00	+
UAGB128	*Ralstonia pickettii* 12J strain 12J	+	10.98	8.88	3.05	0.00	0.00	0.00	0.00	-

FBN=Growth capacity in nitrogen-free medium "nitrogen-fixing capacity"; IAA=Production of indole-3-acetic acid (µg mL^{-1}); CTL=Production of IAA in medium added with L-tryptophan; SLT=Production of IAA in medium without L-tryptophan; SI=Solubilization index of inorganic phosphate according to the mean of the hydrolysis halo ad the colony diameter; IE=Enzymatic index relating the mean of the hydrolysis halo and the colony diameter; CE=Cellulase enzyme; PE=Pectate lyase enzyme; PO=Polygalacturonase enzyme; AM=Amylase enzyme; AHL=Production of the signaling quorum sensing molecule N-Acyl homoserine lactone.

of water and nutrient uptake and ultimately leads to plant growth promotion (Machado *et al*., 2013).

Our analysis also showed that 70% of bacterial strains possessed the capacity for inorganic phosphorus (P) solubilization, and the most prominent indexes occurred in strains of *Burkholderia* sp. (UAGB93, UAGB94 and UAGB139) (Table 3). Some microorganisms found in soil and associated with plants play an essential role in P

cycling, hydrolyzing inorganic forms to organic forms and thus facilitating plant assimilation via enzymatic activity, especially acid phosphatase (Verma *et al*., 2001).

While N is the element responsible for the maintenance of productivity and persistence of the pasture, P in the plant tissue plays an essential role in plant establishment and respiration. Moreover, P also influences storage, transport, and energy utilization in the photosynthetic process, protein synthesis, and enzymatic metabolism (Taiz and Zeiger, 2013). At forage grass, in general, phosphate fertilization benefits both shoot and root growth, but there species and/or cultivars of *Brachiaria* are capable of growing in soil with low P availability (Cecato *et al*., 2000). Commonly cultivated in low fertile soils with low P availability, the association between *Brachiaria* and P-solubilizing bacteria might increase the amount of P available for plant uptake and fulfill plant phosphorus requirements.

Araújo *et al*. (2012) reported that *Bacillus* strains associated with *Brachiaria brizantha* were capable of synthesizing phosphatases. In our study, the *Bacillus anthracis* strain Ames (UAGB10) isolated from *Brachiaria humidicola* (Rendle.) Schweickerdt did not solubilize phosphate. These results demonstrated that each isolate possesses characteristics influenced by the place of cultivation, thus highlighting the importance of obtaining local strains to better manage crops according to the local conditions. Luvizotto *et al*. (2010) evaluated strains of *Burkholderia* spp. isolated from sugarcane according to their capacity to solubilize phosphate and observed a range of 1.11 to 6.00, similar to our results.

Our analysis of enzymatic production showed that, from the total bacterial isolates, 30% produced cellulase, 60% produced pectate lyase, 15% produced polygalacturonase, and 35% produced amylase. The highest enzymatic indexes were observed in the strains *Enterobacter* sp. (UAGB10) (6.72), *Microbacterium* sp. (UAGB70) (7.33), *Rhizobium* sp. (AUGB150) (6.28) and *Microbacterium* sp. (UAGB70) (4.58), respectively (Table 3). The lytic enzymes produced by microorganisms revealed their capacity to degrade organic compounds, which is of increased interest for industrial application and textile, food, and paper production. Moreover, these enzymatic capabilities also attract attention for use in agriculture because they allow bacterial penetration and colonization in the plant (Compant *et al*., 2010).

According to the orthogonal contrast analysis, the isolates from *Brachiaria decumbens* Stapf. presented the following mechanisms of plant growth promotion: IAA production in the presence of L-tryptophan and synthesis of the pectate lyase, polygalacturonase, and amylase enzymes. According to the niches, the root endophytes synthesized the highest concentrations of IAA with or without the presence of L-tryptophan and had the highest indexes of phosphate solubilization and cellulase production (Table 4).

Among the niche isolation from *Brachiaria decumbens* Stapf., the root endophytes presented the highest production of IAA with or without the presence of L-tryptophan. On the other hand, the isolates extracted from the rhizosphere possessed the highest indexes of phosphorus solubilization and enzymatic activity from all tested enzymes. Similarly, the isolates from *Brachiaria humidicola* (Rendle.) Schweickerdt, originating from the root endophytic community, presented the highest concentrations of IAA with or without the presence of L-tryptophan, as well as the highest indexes of phosphorus solubilization and the highest production of cellulase (Table 4).

The niches of root endophytes and the rhizosphere are distinct from a physical, chemical, and biological context. In the rhizosphere, root exudates modify the nutritional quality of this niche in comparison to soil, however, with stronger competition between several genera and species of microorganisms present in the soil. In contrast, the endophytic niche is more stable and uniform, with weaker competition between microbes and with more nutrients available.

Table 4. Comparison between the means via orthogonal contrast of the expression of mechanisms of plant growth promotion *in vitro* for 20 diazotrophic bacteria isolated from *Brachiaria decumbens* Stapf. and *Brachiaria humidicola* (Rendle.) Schweickerdt within the niches of root endophytes and rhizosphere.

Average	IAA		SI	EI			
	CLT	SLT		CE	PE	PO	AM
B. decumbens Stapf.	61.14	5.89	1.84	0.10	1.74	2.88	1.14
B. humidicola (Rendle.) Schweickerdt	29.13	9.02	2.86	1.25	0.00	1.14	0.51
Endophytic root niche	66.34	9.90	2.38	0.91	0.21	1.48	0.58
Niche rhizosphere	19.22	4.47	2.31	0.39	1.68	2.65	1.13
B. decumbens - Endophytic root	94.32	10.48	0.69	0.00	0.45	2.42	0.92
B. decumbens - Rhizosphere	27.97	1.30	2.99	0.21	3.02	3.34	1.37
B. humidicola - Endophytic root	41.60	9.97	3.60	1.64	0.00	1.01	0.34
B. humidicola - Rhizosphere	7.59	7.39	1.59	0.57	0.00	1.37	0.80
General	45.14	7.46	2.35	0.68	0.87	2.01	0.83
Brachiaria decumbens Stapf. *vs*. *Brachiaria humidicola* (Rendle.) Schweickerdt							
Test t	15.63**	-3.81**	-4.64**	-25.31**	23.19**	11.47**	8.34**
Endophytic root niche *vs*. Niche rhizosphere							
Test t	22.90**	6.58**	0.33^{ns}	11.62**	-19.56**	-7.65**	-7.16**
Brachiaria decumbens Stapf. - Endophytic root niche *vs*. Rhizosphere niche							
Test t	17.81**	9.63**	-8.85**	-2.60**	-17.14**	-4.421**	-3.40**
Brachiaria humidicola (Rendle.) Schweickerdt - Endophytic root niche *vs*. Niche rhizosphere							
Test t	20.03**	0.73^{ns}	6.42**	11.61**	0.00^{ns}	-4.81*	-7.17*

IAA=Production of indole-3-acetic acid (µg mL^{-1}); CTL=Production of IAA in medium added with L-tryptophan; SLT=Production of IAA in medium without L-tryptophan; SI=Solubilization index of inorganic phosphate according to the mean of the hydrolysis halo ad the colony diameter; EI=Enzymatic index relating the mean of the hydrolysis halo and the colony diameter; CE=Cellulase enzyme; PE=Pectate lyase enzyme; PO=Polygalacturonase enzyme; AM=Amylase enzyme; ^{ns}=nonsignificant; * and ** = significant at 5 and 1% probability by t-test, respectively.

Therefore, bacteria from distinct niches presented different mechanisms of plant growth promotion, likely an outcome of their adaptive capacity, directly reflecting their potential for influencing plant growth in a natural environment (Compant *et al*., 2010; Murphy *et al*., 2016).

We detected the production of AHL in 65% of our bacterial strains (Table 3). The endophytic niche of *Brachiaria humidicola* (Rendle.) Schweickerdt contained isolates with the highest production. The production of AHL autoinducers is linked to the capacity of intercell communication, which is related to mobility regulation, horizontal gene transfer, stress tolerance, enzyme production, and biofilm formation (Pinton *et al*., 2010). The biofilm protects the bacterial population when inoculated in soil or on seeds, thus favoring the maintenance of population densities in such way that the beneficial or deleterious interactions between plant and bacteria initiate (Grönemeyer *et al*., 2012).

Our study reports bacterial isolates with potential application in the management of *Brachiaria* pastures given their capacity to fix nitrogen, produce IAA, solubilize phosphate, synthesize lytic enzymes and N-Acyl homoserine lactone. The next step should be validation this potential by evaluating the performance of these bacteria in forage grass cultivated in soils with low natural fertility in field experiments.

Brachiaria decumbens Stapf. and *Brachiaria humidicola* (Rendle.) Schweickerdt presented interactions with bacteria of the genera *Bacillus* sp., *Burkholderia* sp., *Enterobacter* sp., *Klebsiella* sp., *Microbacterium* sp., *Pantoea* sp., *Ralstonia* sp., *Rhizobium* sp., *Sinomonas* sp., and *Sphingomonas* sp., with specificity for the genus *Rhizobium* sp. to *Brachiaria decumbens* Stapf. plants. Root endophytes and rhizosphere bacteria from *Brachiaria decumbens* Stapf. and *Brachiaria humidicola* (Rendle.) Schweickerdt presented the potential and characteristics for

plant growth promotion via *in vitro* tests for the production of IAA, solubilization of phosphate, synthesis of lytic enzymes, and production of N-Acyl homoserine lactone. The bacteria were diverse according to the different plant species in which they were obtained and their niches.

Resumen

J. T. C. Oliveira, G. T. Silva, W. P. S. Diniz, E. F. Figueredo, I. B. Santos, D. R. M. Lima, M.C. Quecine, J. Kuklinsky-Sobral, y F. J. Freire. 2018. Bacteria diazotrófica aislada de *Brachiaria* spp.: diversidad genética, fenotípica y funcional. Cien. Inv. Agr. 45(3): 277-289. Pastos del género *Brachiaria* spp. predomina en pasturas con suelos poco fértiles. Este escenario resalta la importancia de la asociación con microorganismos para fomentar el cultivo de plantas. Este estudio tuvo como objetivo evaluar la variabilidad genética e identificar los mecanismos de promoción del crecimiento de las plantas, *in vitro*, de bacterias asociadas con *Brachiaria decumbens* Stapf. y *Brachiaria humidicola* (Rendle.) Schweickerdt en Pernambuco, Brasil. Evaluamos 20 aislamientos de bacterias diazotróficas obtenidas de la comunidad de endofitas o rizosfera. Las características genéticas se determinaron mediante la secuenciación de la región 16S rRNA, lo que permitió identificar diez géneros bacterianos diferentes: *Bacillus* sp., *Burkholderia* sp., *Enterobacter* sp., *Klebsiella* sp., *Microbacterium* sp., *Pantoea* sp., *Ralstonia* sp., *Rhizobium* sp., *Sinomonas* sp., y *Sphingomonas* sp., con una especificidad del género *Rhizobium* sp. a *Brachiaria decumbens* Stapf.. Las características fenotípicas y funcionales revelaron que el 100% de las cepas bacterianas producían ácido indol-3-acético (AIA) con la adición de L-triptófano y el 60% presentaban producción de AIA independientemente de la vía de L-triptófano. También detectamos que el 70% de las bacterias aisladas poseían la capacidad de solubilizar el fósforo. El análisis de la producción enzimática reveló que el 30% de los aislados producían celulasa, 60% pectato liasa, 15% poligalacturonasa y el 30% producía amilasa. También detectamos la producción de N-acyl homoserine lactones en el 65% de las cepas bacterianas. En resumen, las plantas de *B. decumbens* Stapf. y *B. humidicola* (Rendle.) Schweickerdt interactuó con diferentes géneros de bacterias capaces de promover el crecimiento de la planta.

Palabras claves: N-acil homoserina lactonas, interacciones planta-microbio, bacterias promotoras del crecimiento de plantas.

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