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REGULAR ARTICLE

Genetic diversity and growth promoting characteristics of diazotrophic bacteria isolated from 20 genotypes of *Brachiaria* spp.

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Abstract

Background and aims Diazotrophic bacteria, including those of the genus *Azospirillum* and *Nitrospirillum*, colonize *Brachiaria* genotypes and contribute to plant development through nitrogen fixation, production of phytohormones and bioavailability of nutrients. This study aimed to determine the phylogenetic positioning and evaluate the functional abilities of diazotrophic bacteria isolated from *Brachiaria* genotypes.

Methods Diazotrophic bacterial counting and isolation were carried out with rhizosphere soil and root samples from 20 *Brachiaria* genotypes after inoculation in nitrogen-free semi-solid NFb and LGI media. The isolates were analyzed using 16S rRNA and *nif*H sequences, and tested for their functional abilities to produce auxin and siderophores, to solubilize phosphate and zinc, and to degrade cellulose.

Results The diazotrophic population ranged from 10^2 to 10^8 g^{-1} rhizosphere soil or roots. Sequencing of 16S rRNA from 213 isolates confirmed the presence of genera *Azospirillum* and *Nitrospirillum*, and revealed the presence of 14 other diazotrophic genera. The genus *Nitrospirillum* was detected colonizing all niches of most *Brachiaria* species. A PCA analysis showed a positive correlation between the ability to produce siderophores with the ability to produce IAA; and between phosphate and zinc solubilisation.

Conclusions The results showed a high diversity of diazotrophic bacterial species colonizing 20 *Brachiaria* genotypes and revealed the presence of bacteria with variable growth-promoting characteristics, highlighting their potential as good candidates for the development of biofertilizers.

Keywords Isolation · Functional capacity · Sequencing · Phylogenetic positioning

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Introduction

Pasturelands represent one of the largest ecosystems in Brazil (Guarda and Guarda 2014), with the genus *Brachiaria* occupying about 260 million ha, equivalent approximately to 80% of tropical and subtropical regions of the Brazilian pastures (Valle et al. 2010). This forage grass, constituted by around 100 species, is responsible for most of the 221 million cattle that supply

the domestic demand for meat and the exportation to different countries (USDA 2018). Despite its economic importance, it is estimated that between 50 to 70% of pastures in Brazil present different degrees of degradation (Dias-Filho 2011). This phenomenon can mainly be explained by the low natural soil fertility of tropical regions and the lack of nutrient replacements (Macedo 2005) such as nitrogen, which is considered to be the most limiting element to productivity of *Brachiaria* spp. (Boddey et al., 2004). However, the use of nitrogen fertilizers greatly affects the cost of production, and due to the increasing demand for food, sustainable alternatives such as Biological Nitrogen Fixation (BNF) have to be used. BNF is known to be performed by diazotrophic bacteria that can colonize the rhizosphere, the surface and/or the interior of plant tissues (Compant et al. 2010).

In a review published by Baldani and Baldani (2005) related to the BNF process in forage grasses, the authors indicate that BNF is a renewable and continuous source of nitrogen. In this way, BNF in pastures may play an important role in places where the practice of inoculation with specific associative diazotrophic bacteria may substitute, at least partially, the chemical nitrogen source in intensive systems. *Azospirillum spp.* and *Nitrospirillum amazonense (*older *Azospirillum amazonense)*, are among the most studied groups of diazotrophic bacteria that have been found to be associated with several plant families (Reis et al. 2015), presenting a wide distribution, as they are found in the soil, roots, leaves and stems of plants (Baldani et al. 2014). These genera have been isolated from several forage grasses such as *Brachiaria* sp. (Reis et al. 2001; Reis Junior et al. 2004; Brasil et al. 2005; Silva et al. 2013), *Panicum maximum* (Santos et al. 2013), *Axonopus purpusii*, *Hymenachne amplexicaulis* and *Mesosetum chaseae* (Souza et al. 2017), *Miscanthus sinensis* cv. *giganteus* (Eckert et al. 2001) and *Pennisetum purpureum* (Videira et al. 2012). Other diazotrophic bacterial genera have also been isolated from *Brachiaria* grown in Northeast region (Caatinga biome) of Brazil, and these include *Burkholderia* sp., *Bacillus* sp., *Enterobacter* sp., *Klebsiella* sp., *Sphingomonas* sp., *Pantoea* sp. and *Rhizobium* sp. (Oliveira et al. 2018). Similarly, a high diverse non-nitrogen-fixing bacterial community belonging to genera *Pseudomonas, Pantoea, Acinetobacter* and *Enterobacter* was detected in different Brachiaria plants naturally grown in soil in Nairobi, Kenya (Mutai et al. 2017).

Diazotrophic bacteria can play an important role in the rehabilitation and sustainability of pastures, since they incorporate N through the biological fixation process in amounts that vary from 25 to 50 kg ha^{-1}. These amounts have been measured in *Brachiaria* species and *Panicum maximum* genotypes grown in cylinders filled with ^{15}N labelled soil maintained outside the greenhouse (Boddey and Victoria 1986; Miranda et al. 1990). In addition, diazotrophic bacteria possess the capacity to produce and release plant growth regulators, such as auxins (Reis Junior et al. 2004; Videira et al. 2012), solubilize inorganic phosphate (Rodriguez et al. 2004; López-Ortega et al. 2013), and produce siderophores (Tortora et al. 2011). Further studies on the potential of these diazotrophic bacteria in association with forage grasses are therefore needed in order to understand the role of these microorganisms in plant growth-promoting activities. Recently, a commercial inoculant containing two variants (Abv5 and Abv6) of *Azospirillum brasilense* strain Sp7 was applied with success in maize (Hungria et al., 2010) and later its use was extended to *Brachiaria* pastures. However, these increments occur mostly when inoculant is applied together a half dose of nitrogen fertilizer required by the crop (Hungria et al. 2016), suggesting that other mechanisms than the BNF was acting in the plant-bacteria interaction. Despite of that, it should be chased the development of an inoculant containing efficient nitrogen-fixing strains in addition to other biotechnological functionalities. In this sense, we searched for the occurrence of diazotrophic bacteria (associative and endophytic) in twenty *Brachiaria* genotypes, expecting to find out high diversity of bacterial species/strains containing these functionalities and that could be more efficient when applied in future inoculation studies with some of these *Brachiaria* genotypes that occupy around 80% of the Brazilian pastures. Our hypothesis is that *Brachiaria* genotypes could establish a more specific diazotrophic association considering that the plants have been maintained in the same Germoplasm Active Bank for around ten years.

Material and methods

Origin of the genotypes

The study comprised the analysis of 20 *Brachiaria* genotypes collected at the Germplasm Active Bank

(GAB) of the Centro Nacional de Pesquisa de Gado de Corte (CNPGC), part of the Embrapa project number 02.13.08.004.00.00. The genotypes were *B. decumbens* (Basilisk, D24 / 27, D24 / 45, D24 / 2, 79–10, X009, B001), *B. brizantha* (Marandu, Paiaguás, Piatã, Xaraés and B140), *B. humidicola* (Common, Tupi, Ipyporã, H47), *B. ruziziensis* (Common), *B. arrecta* (A2) and *Brachiaria spp.* (Llanero and Mulato II). The GAB has been maintained for up to 10 years at the same place (geographic coordinates 20°27' S and 54°37' W) in a Typical Red Clay Latosol. The GAB is fertilized annualy with phosphorus and potassium to maintain the equivalent to 4–5 mg P dm^3 and 50–60 mg K dm^3), respectively. In addition, nitrogen (70 Kg N ha^{-1}), sulfur (30 kg S ha^{-1}), micronutrients (40 kg FTE-BR10 ha^{-1}) and dolomitic lime (3 ton ha^{-1}) were applied every year to maintain the nutrient plant demand.

Counting and isolation of diazotrophic bacteria

The plant samples containing rhizosphere soil of the 20 genotypes were transported to Embrapa Agrobiologia, and then properly labeled and transplanted to 6 kg pots containing sterile sand and vermiculite (1:1) and kept in a greenhouse. Nutrient fertilization was performed every 15 days with 30 mL of KH_2PO_4 (35 g L^{-1}); 5 mL of $CaCl_2.2H_2O$ (150 g L^{-1}) and 5 mL of $MnSO_4.H_2O$ (5 g L^{-1}), totalizing six fertilizations, and the pots remained in the greenhouse for up three months.

Counting and isolation followed the methodology described by Baldani et al. (2014) using nitrogen-free LGI and NFb semi-solid media. The NFb (*Azospirillum* species) and LGI (*Nitrospirillum amazonense*) semi-solid media were chosen based on the widespread occurrence of these diazotrophic species in high numbers in association with *Brachiaria* species in Brazil (see introduction). Briefly, the bulk soil close to roots was removed and the rhizospheric soil (soil adherent to roots) was taken for analysis. The roots were washed in tap water to remove the remained soil residues and processed with or without surface disinfection. For the root disinfection process, a concentration of 10 g L^{-1} of Chloramine-T ($CH_3C_6H_4SO_2NNaCl.3H_2O$) was used for 5 min. After disinfection time, the roots were placed in sterile distilled water for 1 min and 30 s, and then transferred to phosphate buffer solution (50 mM, pH 7.0) for 1 min and 30 s, and again in distilled water for 5 min. The disinfected roots were used for the isolation of diazotrophic bacteria considered endophytic. For non-disinfected roots, the samples (10 g fresh tissues) were kept in distilled water for the same time as the one used in the disinfection process. The samples (10 g umid soil or fresh roots) were mixed with 90 mL saline solution, agitated to homogenize it for 5 to 10 min on a rotary shaker (~150 rpm) and then diluted up to 10^{-7}, inoculated in both nitrogen free semi-solid media and incubated at 30 °C for 5 days. Flasks (1 to 3) of the highest counting dilution/repetition, showing subsurface veil pellicles characteristic of diazotrophic bacteria, were transferred to new semi-solid media and purified following the indicated methodology (see Baldani et al. 2014). The final number of diazotrophic bacterial isolates varied according to the richness of the target bacteria in the veil pellicle and the success during the steps of purification due to the presence of scavenger bacteria. Purified cultures were then stored in duplicates in appropriate slant media under mineral oil.

Phylogenetic positioning of the isolates

Amplification of the 16S rRNA gene fragment with the primers 27f (5′-AGA GTTTA TCC TG CTG AG-3′) (Furushita et al. 2003) and Amp2 (5′-AAG GAG GT ATC CAT CCG CA-3′) (Wang et al. 1996) was carried out as described by Videira et al. (2012). For amplification of the *nif*H gene the primers PolF (5'-TC GAY CCS AAR GCB GAC TC-3′) and PolR (5'-ATS GCC ATC ATY TCR CCG GA-3′) were used (Poly et al. 2001). PCR products were purified with the Exo-SAP kit, according to the manufacturer's description.. PCR products were sequenced on the ABI PRISM 3500 Genetic Analyzer automatic sequencer (Applied Biosystems) using the commercial Big Dye® Terminator Cycle Sequencing v3.1 kit from Thermo Fisher Scientific (Cat. No.4337456).

The amplicons were sequenced at both ends (forward and reverse), using the same PCR pair of primers. Sequences of the 16S rRNA and *nif*H genes were analyzed and assembled using the program BioEdit version 7.1.9 (Hall 1999) to check the quality of the amplified sequencing products. A visual inspection of the chromatograms was carried out to identify the sequence quality of the fragments and those with lower quality were deleted. The sequences were then aligned and compared with those deposited in the GenBank database using the BLAST program (Altschul et al. 1997). Phylogenetic trees with 16S rRNA (~1400 bp) and

*nif*H (~ 360 bp) sequences were constructed to verify their phylogenetic position in relation to the species type of each identified strain. Multiple sequence alignment was done using the ClustalX program (Tuimala, 2004) and the sequences were processed by the MEGA6 program (Tamura et al. 2013). The consensus tree was constructed by the Neighbor-Joining method, considering a bootstrap of 1000 replicates. Sequencing of the *nif*H amplified fragments was performed for the 32 strains selected according to their variable functional characteristics and the representative taxonomical species.

Functional capacity of the strains

Biological nitrogen fixation- qualitative evaluation

The ability of the isolates to perform the BNF process was evaluated qualitatively in penicillin flasks containing 7 mL of nitrogen free NFb or LGI semisolid media. The inocula cultures were grown in their respective semi-solid medium. The flasks were incubated for 72 h at 30 °C in the dark followed by analysis of the growth pellicle formed near the surface of the bottles. Three replicates were performed for each bacterial isolate, and this procedure was repeated at least three times.

Solubilization of inorganic phosphate and zinc oxide

The solubility of P by the isolates was evaluated in a NBRIP solid medium (Nautiyal 1999), containing insoluble β-tricalcium phosphate [$Ca_3(PO_4)_2$] or zinc oxide (ZnO), as the sole source of phosphorus and zinc, respectively. A total of 10 μL of bacterial cultures was inoculated onto NBRIP agar plates. The appearance of a clear halo around the colonies on the 12th and 15th days after inoculation was used to measure the phosphate and zinc oxide solubilization, respectively. The criteria used to calculate the solubilization index (SI) were those described by Kumar and Narula (1999). Each isolate was tested three times in NBRIP agar medium to confirm the phenotype of phosphate and zinc solubilization. The strain *Gluconacetobacter diazotrophicus* PAL5 (BR11281) was used as positive control.

Production of siderophores

The production of siderophores was evaluated using the chromium azurol S (CAS) method adapted from Schwyn and Neilands (1987). A characteristic colony of each isolate was inoculated in a test tube containing 5 mL of NFB or LGI liquid medium, without Fe^{3+} iron and 1.0 g L^{-1} of $(NH_4)_2SO_4$ for induction of the production of siderophores or chelating compounds of iron. The tubes were incubated at 180 rpm, 30 °C for 48 h. After incubation, three aliquots of 10 μL each were inoculated onto NFb or LGI plate [without Fe^{3+} iron and 1.0 g L^{-1} of $(NH_4)_2SO_4$] containing 1% CAS. Bacteria were incubated for 3 days at 30 °C. The intensity of the production of siderophores was classified as low, medium or high according to the size of the halo. It was also evaluated the type of siderophores produced by the isolates, being the blue/yellow color for catecholate type and blue/pink color for hydroxamate type (Schwyn and Neilands 1987).

Cellulolytic activity

The cellulolytic activity of the bacterial strains was evaluated according to Kasana et al. (2008). Ten μl culture was inoculated in triplicate onto a plate containing CMC agar medium [(0.2% $NaNO_3$, 0.02% K_2HPO_4, 0.05% $MgSO_4$, 0.5% KCl, 0.2% sodium CMC (carboxymethylcellulose), 0.02% peptone and 1.7% agar)]. Plates were incubated for four days at 30 °C. After incubation, the plates were flooded with iodine solution (2.0 g KI, 1.0 g iodine diluted in 300 mL distilled water) for five minutes. The excess solution was drained and the presence of halo around the bacterial colonies indicated a positive result for the cellulolytic activity. The halo was measured with the aid of a digital caliper and the cellulolytic activity index (CI) was calculated as proposed by Hankin and Anagnostakis (1975) and classified according to Dantur et al. (2015).

Production of indole compounds

Bacterial strains were analyzed for the synthesis of indole compounds according to the microplate method proposed by Sarwar and Kremer (1995). Each bacterial isolate was cultured in a tube containing 5 mL of liquid DYGS for 24 h under 30 °C. Subsequently, a 20 μL aliquot of each tube was transferred to another tube containing liquid NFb or LGI culture medium, both without the pH indicator, and L-tryptophan (100 μg mL^{-1}) was added and incubated under shaking at 180 rpm for a period of 72 h, in an environment

without light and with an ambient temperature of 30 °C. Aliquots (1 mL) were then withdrawn from each tube and centrifuged at 12,000 rpm for 5 min. After that, 150 μL of the supernatant from each sample was added to three wells (96 wells) and mixed with 100 μL of the Salkowski reagent. The samples were incubated in the dark for 30 min at room temperature and after this period, readings were taken using a spectrophotometer (Labsystems iEMS Reader MF) at a wavelength of 540 nm and the absorbance data were processed by the program Ascent for iEMS Reader MF. The strain *Azospirillum brasilense* Sp245 was used as a positive control. The readings were standardized by means of a standard curve previously obtained with known concentrations of 3-indole-acetic acid (25 to 600 μg mL^{-1}). All samples were analyzed in triplicate and the result was an average of three readings.

Statistical analysis

The functional information was used to calculate a Principal Component Analysis (PCA) displaying how similar the different isolates are in their functional potential. The characteristics included in the analysis were the production of siderophores, production of IAA, Zn solubilization, and the ability of consuming CMC. The PCA was calculated with the correlation matrix since the variables are expressed in different units. The correlations between the different variables and the coordinate axes was displayed in the form of arrows in the PCA plot. All analyses were done with the package vegan Oksanen et al. (2019) implemented in the R platform (R Core Team 2018).

Results

MPN count and isolation of diazotrophic bacteria in semi-solid NFb and LGI media

The estimated number of diazotrophic bacteria present in the rhizosphere soil and in the roots of the plant genotypes differed according to the sample used for isolation [non-disinfected root (NDR), surface disinfected roots (DR) and rhizosphere soil (RS)], as well as the semi-solid medium (NFb and LGI). In general, the population size of viable diazotrophic bacteria colonizing the 20 *Brachiaria* genotypes ranged from 10^2 to 10^8 bacteria per gram of fresh soil or fresh root tissues. The largest population of diazotrophic bacteria was observed in the *B. decumbens* genotype Basilisk counted in NFb medium and *B. humidicola* genotype H47 counted in the LGI medium (Table 1). In contrast, lower diazotrophic population or no detection (below the minimum detection level) was observed in some genotypes, mainly in the *B. ruziziensis* R134 and *B. humidicola* genotype Tupi counted in both semisolid media. Concerning the niche occupation in the brachiaria forages, the bacterial counting showed a tendence of similar bacterial population in the non-disinfected and disinfected roots, except for disinfected roots that were slightly higher for Basilisk and H47. The rhizospheric soil diazotrophic population was lower as compared to roots and in some cases, non-detectable population was observed for some genotypes counted in both culture media (Table 1).

A total of 213 diazotrophic isolates (110 in NFb medium and 103 in LGI medium) were obtained from the different niches (rhizospheric soil, surface disinfected root and non-disinfected root) of the 20 brachiaria genotypes (Table 1). The highest percentage (22.53%) was obtained from NDR followed by 18.78% for RS and 10.32% for the DR isolated in semi-solid NFb medium, while in the LGI medium the percentage of isolates was 20.65% for NDR, 14.55% for DR and 13.14% for RS. The sum of isolates obtained in both media also varied with the forage grass genotype, with the genotypes *B. decumbens* cv. X009 (25), cv. 24 /27 (20), cv 79–10 (19) and cv. D24/45 (18) showing the highest number of isolates. In contrast, *Brachiaria ruziziensis* cv. R134 (1), *B. decumbens* cv. D24/2 (3) and *B. humidicola* cv. Tupi (4) presented the lowest number of isolates (Table 1). The number of bacterial isolates also varied with *Brachiaria* species, with an average quite similar (14.6 and 13), for *B. decumbens* and *B. arrecta,* respectively followed by *Brachiaria spp*, *B. brizantha* and *B. humidicola* with an average of 9.5, 9.4 and 7.8, respectively while only one representing bacterial isolate was obtained from *B. ruziziensis* (Table 1).

A Venn dendrogram analysis involving the niche of the diazotrophic bacterial genera isolation and each group of *Brachiaria* genotypes (*B. brizantha, B. humidicola, B. decumbens* and *Brachiaria spp* plus *B. arrecta*) showed the occurrence of the bacterial genera randomized among the *Brachiaria* species (Fig. S1). The genera *Nitrospirillum* and *Azospirillum* were common to to all three niches of the *B. brizantha* and *B. decumbens* while

Table 1 Most Probable Number of cultivable diazotrophic bacteria (number of cells per gram of fresh soil or roots) associated with 20 *Brachiaria* genotypes and number of isolates from each plant niche using nitrogen-free NFb and LGI semi-solid culture media

Brachiaria species	Genotype	NFb culture medium						LGI culture medium						Total number of isolates in both semisolid media/ genotype
		Number of bacteria $\times 10^3$ / g fresh roots or soil			Number of isolates			Number of bacteria $\times 10^3$ / g fresh roots or soil			Number of isolates			
		NDR	DR	RS	NDR	DR	RS	NDR	DR	RS	NDR	DR	RS	
B. arrecta	A2	-[a]	45	45	–	2	1	140	0.25	45	5	2	3	13
B. brizantha	B140	150	25	9.5	2	1	4	150	1500	-[a]	3	1	–	11
B. brizanta	Marandu	9500	1500	150	2	1	3	950	-[a]	-[a]	1	–	–	7
B. brizantha	Paiaguás	140	-[a]	-[a]	3	–	–	140	-[a]	0.75	6	–	2	11
B. brizantha	Piatã	140	140	25	2	1	1	140	30	1.5	1	1	2	8
B. brizantha	Xaraés	140	7.5	45	2	2	2	140	140	25	2	1	1	10
B. decumbens	Basilisk	14,000	45,000	250	2	1	3	750	-[a]	-[a]	4	–	–	10
B. decumbens	D24/2	1500	-[a]	-[a]	2	–	–	2	-[a]	-[a]	1	–	–	3
B. decumbens	D24/27	140	140	140	4	1	4	3	0.07	140	2	3	6	20
B. decumbens	D24/45	140	3	45	2	3	6	-[a]	25	250	–	4	3	18
B. decumbens	79–10	140	140	140	7	1	2	140	140	140	3	3	3	19
B. decumbens	X009	140	110	110	8	1	7	4	110	-[a]	4	5	–	25
B. decumbens	B001	25	-[a]	0.25	1	–	2	0.45	0.07	-[a]	2	2	–	7
B.humidicula	Comum	110	-[a]	140	3	–	2	-[a]	200	140	–	2	2	9
B.humidicula	H31	140	140	-[a]	3	2	–	140	2.5	2	2	2	3	12
B.humidicula	H47	95	-[a]	-[a]	1	–	–	45,000	-[a]	-[a]	5	–	–	6
B. humidicula	Tupi	-[a]	95	-[a]	–	3	–	-[a]	-[a]	0.04	–	–	1	4
Brachiaria spp.	Ipyporã	140	140	140	3	1	1	140	140	140	1	4	1	11
Brachiaria spp.	Mulato II	140	140	140	1	1	2	140	3	150	2	1	1	8
B.ruziziensis	R134	-[a]	2.5	-[a]	–	1	–	-[a]	-[a]	-[a]	–	–	–	1
Total number of isolates from each niche/semisolid media					48	22	40				44	31	28	213

-[a] Below the minimum detection level; NDR: non-disinfected root, DR: Surface disinfected root and RS: Rhizospheric soil. Isolation was performed with two *Brachiaria* specimens from each genotype analysed using the three highest dilution flasks that presented positive pellicle in the semi-solid medium

Nitrospirillum genus was also common to the three niches of the other *Brachiaria* species. The *B. decumbens* presented the highest number of genera colonizing the three niches while no single genus was found colonizing only the rhizosphere soil of *B. brizantha* and *B. decumbens.* The genus *Pseudomonas* seems to be more commonly found colonizing the root interior of *Brachiaria* species, except for the *Brachiaria* spp. The genus *Rhizobium* was also found colonizing the root interior of the *Brachairia* species: *B. decumbens, B. humidicola* and *Brachiaria* spp. In contrast, the genus *Herbaspirillum* was detected associated with the rhizosphere soil of *B. brizantha* while the *Lysinobacillus* was associated with *B. humidicola* (Fig. S1).

Molecular characterization and diazotrophic bacterial diversity analysis

Sequences of the 16S rRNA subunit from the bacterial isolates showed a high level of similarity (97% and 99%) to sequences deposited in the NCBI database. It was possible to taxonomically identify 213 isolates at the genus level, indicating a high bacterial diversity constituted by 16 genera, belonging to the Proteobacteria classes (alpha, beta and gamma) and Bacilli, therefore confirming the semi-selectivity of the semisolid NFb and LGI culture media (Fig. 1). The genus *Nitrospirillum* (36.3%), followed by *Azospirillum* (33.0%), *Bacillus* and *Stenotrophomonas* (6.5%),

respectively were highly represented among the isolates of the 20 *Brachiaria* genotypes at harvest time. Other genera with lower number of isolates were also found associated with these *Brachiaria* genotypes including *Rhizobium, Pseudomonas, Paraburkholderia, Gluconacetobacter, Bosea, Kosakonia, Zoogloea, Herbaspirillum, Lysinibacillus, Ochrobactrum, Phytobacter and Sphingobium* (Fig. 1). The sequences of these 213 strains were submitted to the Genbank® database (National Center for Biotechnology Information - NCBI) under accession numbers MK542907 to MK543119.

Phylogenetic analyses indicated the presence of 78 strains closely related to *Nitrospirillum amazonense* species, 8 strains to *Azospirillum brasilense*, 27 strains to *A. lipoferum*, 24 strains to *A. formosense*, 6 strains to *A.oryzae* and 3 strains to *A. melinis* (Fig. 2). In addition to these phylogenetically related species, sequences close to *Paraburkholderia tropica*, *Paraburkholderia silvatlantica*, *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae* and *Stenotrophomonas pavanii* were also identified (Fig. 2). Other bacteria phylogenetically close to the species *Stenotrophomonas maltophilia*, *Phytobacter diazotrophicus* and *Ochrobactrum anthropi* were identified among the diazotrophic isolated strains. Strains taxonomically related to genera *Bacillus, Lysinibacillus, Pseudomonas*, *Rhizobium*, *Sphingobium*, *Bosea*, *Kosakonia* and *Zoogloa* were also identified (Fig. 2 and Table S1).

Although all strains had been isolated using the nitrogen-free semi-solid media and presumily considered diazotrophic bacteria, the 213 strains were subjected to amplification of the *nif*H gene using primers described by Poly et al. (2001). The PCR reaction showed amplified product of the expected size (~ 360 bp) for most of the strains, except for 33 strains that did not show the target amplified gene (data not shown). Analysis of the *nif*H gene fragments of 32 representative strains showed that only 2 (*Pseudomonas nitritireducens* strain NRB021 and *Bacillus aryabhattai* strain NRB108) did not present the band of the expected size. Sequencing of strains previously identified based on the 16S rRNA gene indicated that 8 strains present 100% similarity with *nif*H gene of species *Nitrospirillum amazonense* (Fig. 3a). This high identity was also observed for a strain from *Burkholderia silvatlantica, Stenotrophomonas maltophilia*, *Phytobacter sp.* and *Pseudomonas kuykendallii* (Fig. 3), thus corroborating the results obtained in the analysis of the 16S rRNA gene (Fig. 3b). In addition, sequencing of the *nif*H gene from 6 strains showed that they are closely related to species *A. brasilense* and/or *A. formosense* as identified by the 16S rRNA. The other strains showed similarity with the *nif*H gene of *A. melinis*, *A. lipoferum* and *A. oryzae* and to that observed for the 16S rRNA gene, but also presented distinct clusters (Fig. 3a, b).

Evaluation of the functional capacity of strains

Plant growth-promoting characteristics (solubilization of Pi and Zn, cellulolytic capacity and, siderophore and indole production) were tested among the 213 diazotrophic strains aiming to identify strains with the

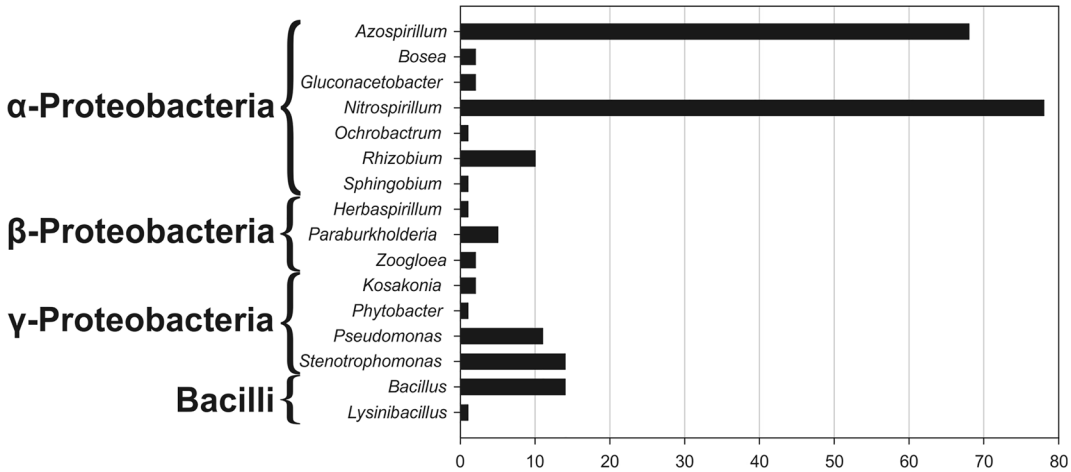

Fig. 1 Distribuition of Proteobacteria classes and genera associated with 20 *Brachiaria* genotypes, maintained in a Germoplasm Active Bank, isolated from disinfected and non-disinfected roots and rhizosphere soil using the nitrogen-free NFb and LGI semisolid media

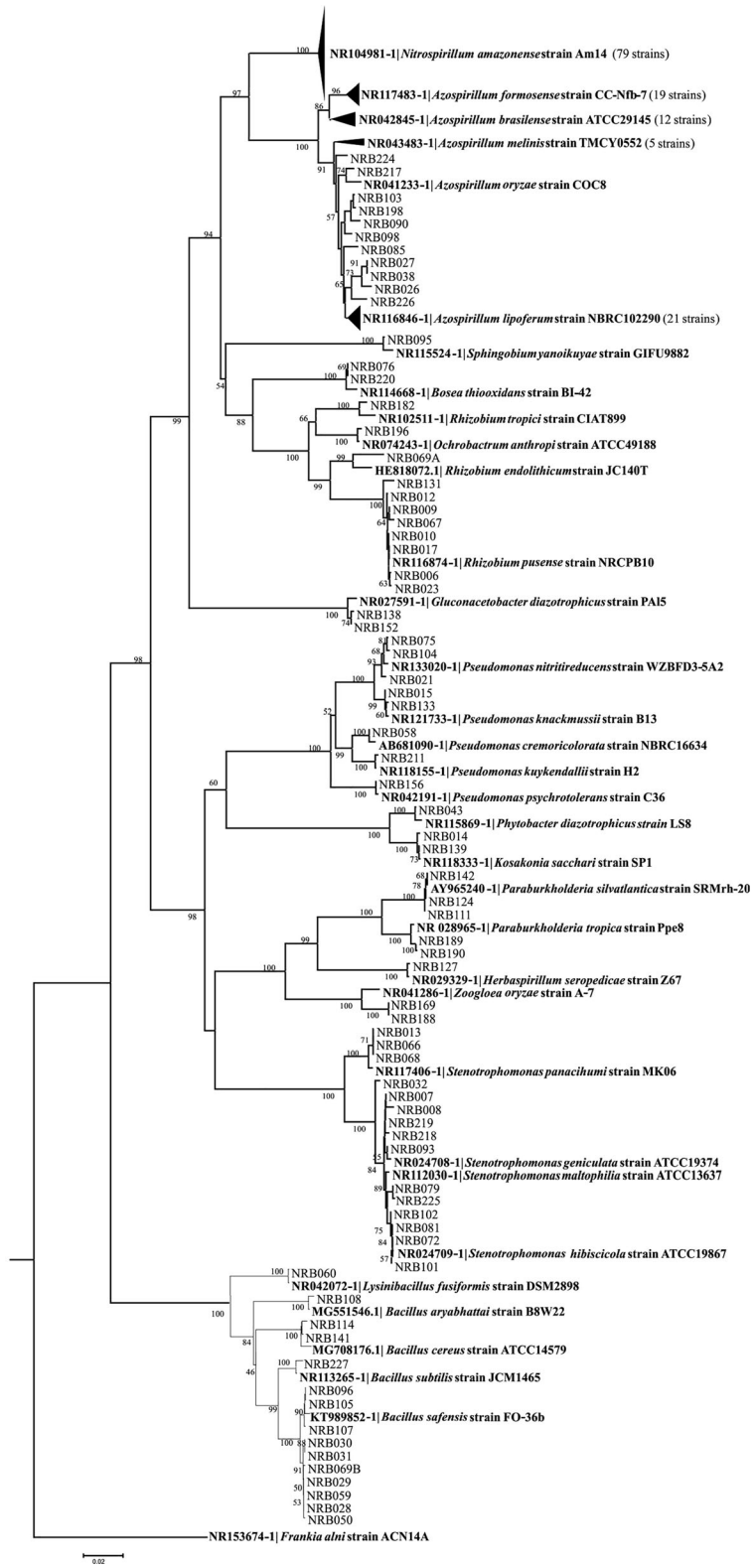

Fig. 2 Phylogenetic tree based on 16S rRNA gene sequences (~ 1450 bp), including the bacterial type strain deposited in the NCBI database. Phylogeny was based on clustering of sequences according to the Neighbor-joining algorithm and Kimura model using the MEGA6.1 program. Numbers located in the branches indicate the percentage of 1000 sub-samples (bootstrap). The scale represents the number of mutations per nucleotide position. The *Frankia alni* 16S rRNA gene sequence was used for tree rooting

potential to be used as biofertilizers in some *Brachiaria* genotypes studied here.

Solubilization of inorganic phosphate and zinc oxide

Out of the 213 strains, 84 (39.44%) presented the capacity to solubilize inorganic phosphate (Fig. 4a). According to Madhaiyan et al. (2004), the phosphate solubilization index (SI) can be classified into levels, high (SI > 3), medium (2 < SI <3) and low (SI <2). Out of the strains that showed the capacity to solubilize inorganic phosphate, 8.33% presented a medium level and the rest (91.67%) showed a low level of Pi solubilization (Fig. 4a). Among the strains classified as medium SI, the strain closely related to *A. formosense* NRB004 presented the highest SI (2.6) followed by *Rhizobium pusense* NRB010, *Pseudomonas nitritereducens* NRB075, *Bosea thiooxidans* NRB076, *A. lipoferum* NRB085, *N. amazonense* NRB180 and *A. formosense* NRB214 that presented a SI greater than 2.0. (Fig. 4b, Table S1). In contrast, the *R. pusense* NRB009 (1.01) and *Bacillus aerius* NRB031 (1.12) showed the smallest SI Few strains phylogenetically related to species *A. brasilense*, *A. melinis*, *A. oryzae*, *Stenotrophomonas maltophilia*, *Phytobacter diazotrophicus* and *P. silvatlantica* also presented low SI (Fig. 4b, Table S1).

In relation to the ability of a strain to solubilize zinc oxide, only 20 strains showed this functional activity, of which 10 strains showed high ability, 9 presented low and 1 showed a medium ability to solubilize ZnO (Fig. 4c). Among the bacterial species, the highest number of strains was observed for the phylogenetically related to *A. formosense* species followed by *A. lipoferum*, *A. brasilense*, *N. amazonense* and *Stenotrophomonas maltophilia* (Fig. 4d). Many of these strains also have the capacity to solubilize inorganic phosphate. The strain phylogenetically related to *A. formosense* NRB082 showed the highest zinc oxide solubility index (5.13), superior to strain PAL5 (4.51) used as positive control. An example of in vitro halo formation characteristic of solubilization of inorganic phosphate (A) and zinc oxide (B) by strains NRB081 and NRB082 grown in NBRIP medium containing insoluble β-tricalcium

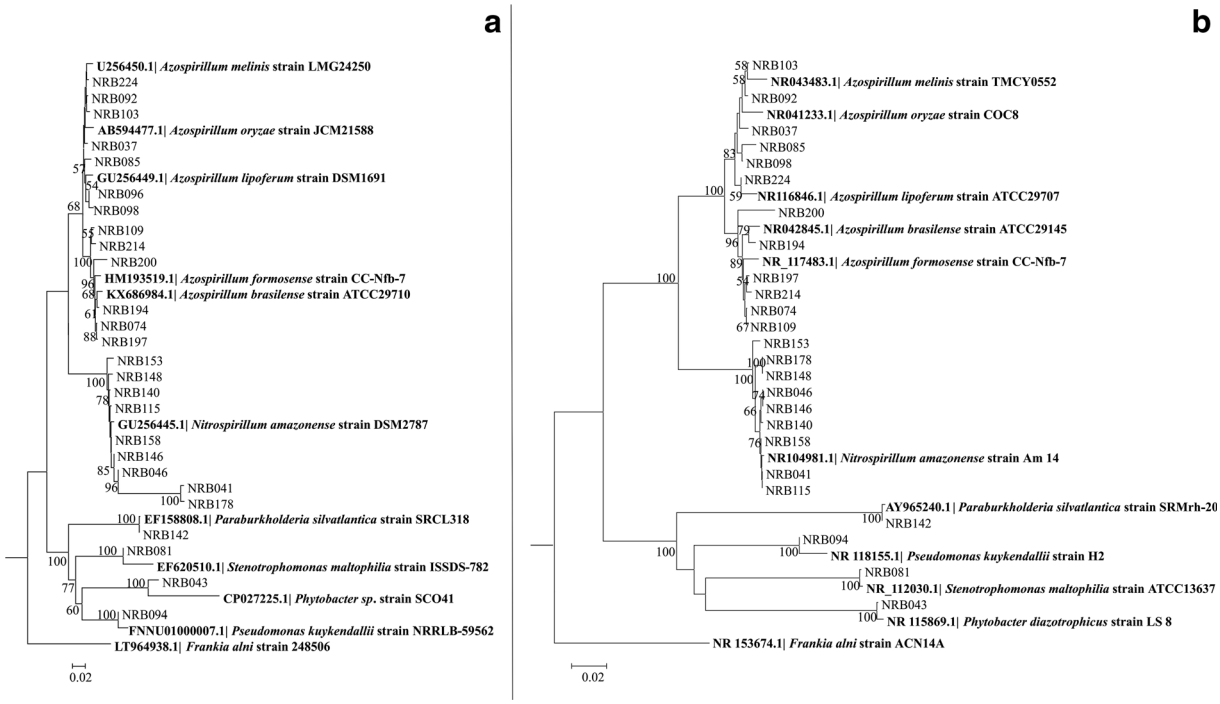

Fig. 3 Comparison of the phylogenetic trees based on sequences of the *nif*H gene (~ 360 bp) (**a**) and the 16S rRNA (**b**) of a group of strains selected based on their physiological and phylogenetically positioning among the 213 characterized strains. Phylogeny was based on clustering of sequences according to the Neighbor-joining algorithm and Kimura model using the MEGA6.1 program. Numbers located in the branches indicate the percentage of 1000 sub-samples (bootstrap). The scale represents the number of mutations per nucleotide position. The *Frankia alni nif*H and 16S rRNA gene sequences were used for tree rooting

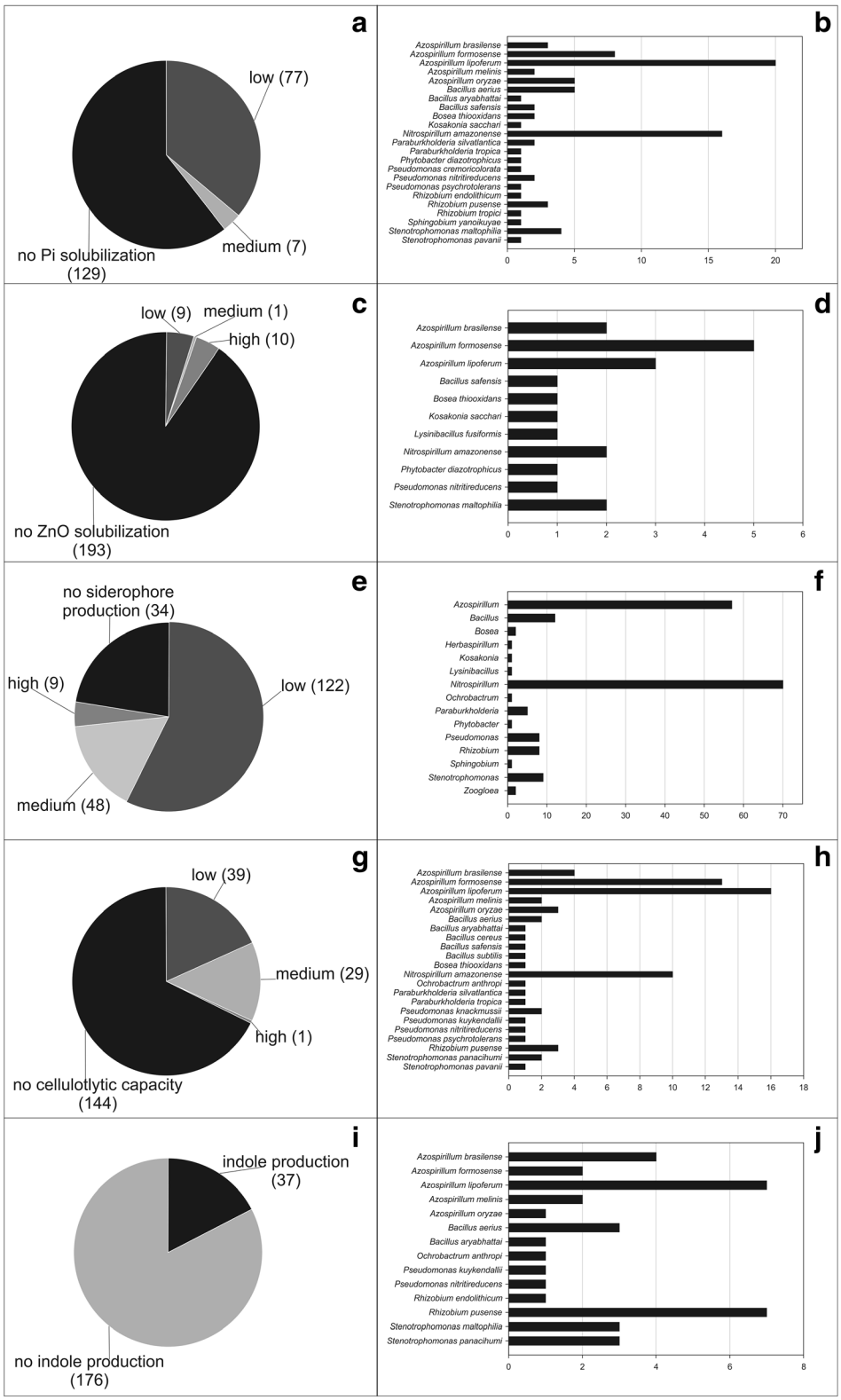
a
low (77)
medium (7)
no Pi solubilization (129)
b
Azospirillum brasilense
Azospirillum formosense
Azospirillum lipoferum
Azospirillum melinis
Azospirillum oryzae
Bacillus aerius
Bacillus aryabhattai
Bacillus safensis
Bosea thiooxidans
Kosakonia sacchari
Nitrospirillum amazonense
Paraburkholderia silvatlantica
Paraburkholderia tropica
Phytobacter diazotrophicus
Pseudomonas cremoricolorata
Pseudomonas nitritireducens
Pseudomonas psychrotolerans
Rhizobium endolithicum
Rhizobium pusense
Rhizobium tropici
Sphingobium yanoikuyae
Stenotrophomonas maltophilia
Stenotrophomonas pavanii
0 5 10 15 20
c
low (9)
medium (1)
high (10)
no ZnO solubilization (193)
d
Azospirillum brasilense
Azospirillum formosense
Azospirillum lipoferum
Bacillus safensis
Bosea thiooxidans
Kosakonia sacchari
Lysinibacillus fusiformis
Nitrospirillum amazonense
Phytobacter diazotrophicus
Pseudomonas nitritireducens
Stenotrophomonas maltophilia
0 1 2 3 4 5 6
e
no siderophore production (34)
high (9)
low (122)
medium (48)
f
Azospirillum
Bacillus
Bosea
Herbaspirillum
Kosakonia
Lysinibacillus
Nitrospirillum
Ochrobactrum
Paraburkholderia
Phytobacter
Pseudomonas
Rhizobium
Sphingobium
Stenotrophomonas
Zoogloea
0 10 20 30 40 50 60 70
g
low (39)
medium (29)
high (1)
no cellulotlytic capacity (144)
h
Azospirillum brasilense
Azospirillum formosense
Azospirillum lipoferum
Azospirillum melinis
Azospirillum oryzae
Bacillus aerius
Bacillus aryabhattai
Bacillus cereus
Bacillus safensis
Bacillus subtilis
Bosea thiooxidans
Nitrospirillum amazonense
Ochrobactrum anthropi
Paraburkholderia silvatlantica
Paraburkholderia tropica
Pseudomonas knackmussii
Pseudomonas kuykendallii
Pseudomonas nitritireducens
Pseudomonas psychrotolerans
Rhizobium pusense
Stenotrophomonas panacihumi
Stenotrophomonas pavanii
0 2 4 6 8 10 12 14 16 18
i
indole production (37)
no indole production (176)
j
Azospirillum brasilense
Azospirillum formosense
Azospirillum lipoferum
Azospirillum melinis
Azospirillum oryzae
Bacillus aerius
Bacillus aryabhattai
Ochrobactrum anthropi
Pseudomonas kuykendallii
Pseudomonas nitritireducens
Rhizobium endolithicum
Rhizobium pusense
Stenotrophomonas maltophilia
Stenotrophomonas panacihumi
0 2 4 6 8

Fig. 4 Diazotrophic bacteria isolated from 20 *Brachiaria* genotypes showing different functional characteristics. Bacteria with phosphate solubilization activity (**a**), ZnO activity (**c**), siderophore production (**e**), cellulolytic activity (**g**), indol acetic acid activity (**i**) and the closely related bacterial species for each function (**b**, **d**, **f**, **h**, **j**), respectively)

phosphate ($Ca_3(PO_4)_2$) or zinc oxide (ZnO) is shown in Fig. S2.

Production of siderophores

Out the 213 strains, 179 strains (84.04%) showed a change in blue/yellowish color (Fig. S3) or blue/pink (Fig. S4) on the CAS plate medium indicating the production of siderophores. One hundred and twenty-two strains (68.16%) presented low intensity, forty-eight (26.82%) showed medium intensity, and nine (5.03%) indicated high production of siderophores (Fig. 4e). Among the bacterial species, the majority of strains was closely related to *N. amazonense* species followed by *A. formosense* and *A. lipoferum* species (Fig. 4f). Many other strains phylogenetically related to other genera such as *Zoogloea*, *Stenotrophomonas*, *Sphingobium*, *Rhizobium*, *Pseudomonas*, *Phytobacter*, *Paraburkholderia*, *Ochrobactrum*, *Lysinibacillus*, *Kosakonia*, *Herbaspirillum*, *Bosea*, *Bacillus* were also detected as siderophore producer (Fig. 4f).

The highest catecholate type siderophore production intensities were observed with phylogenetically related to strains *of P. nitritireducens*, *A. lipoferum*, *R. pusense*, *P. silvatlantica*, *N. amazonense*, *Zoogloea oryzae* and *A. formosense* (Fig. 4f and Table S1). The production of siderophore was so intense in *A. lipoferum* strain NRB034 that it completely altered the color of the agar plate culture medium (Fig. S3). On the other hand, all strains obtained using the semi-solid LGI medium produced hydroxamate -type siderophores since the final color of the plate medium was pink (Schwyn and Neilands 1987) (Fig. S4).

Determination of cellulolytic activity

Out of the 213 tested strains, 69 (32.39%) presented cellulolytic capacity, including representatives phylogenetically related to genera *Azospirillum* (38), *Nitrospirillum* (10), *Bacillus* (6), *Pseudomonas* (5), *Rhizobium* (3), *Stenotrophomonas* (3), *Paraburkholderia* (2), *Ochrobactrum* (1) and *Bosea* (1) (Fig. 4g). Among the 38 strains belonging to *Azospirillum* genus, one strain presented high activity, 15 showed medium activity while 25 had low cellulolytic capacity (Fig. 4h). The NRB011 strain (*A. formosense*) presented the highest cellulolytic activity with a cellulolytic index (CI) of 6.8, followed by the strains NRB033 (*A. lipoferum*) and NRB214 (*A. formosense*), which presented a CI of 4.92 and 4.62, respectively. However, only the strain NRB011 showed a CI higher than the positive control *G. diazotrophicus* PAL5 (5.38). The strains with the lowest rates of cellulose degradation were closely related to the genus *Azospirillum* (NRB027) and *Stenotrophomonas* (NR032 and NRB008). Concerning to the genus *Nitrospirillum,* seven strains showed medium cellulolytic capacity and three with low, with a CI ranging from 1.21 to 3.88. Examples of in vitro cellulolytic activity of strains isolated from *Brachiaria* genotypes are presented in Fig. S5.

Production of indole acetic acid (IAA)

Among the 213 strains tested for their ability to produce this phytohormone, it was observed that only 17.37% were able to produce indoles (Fig. 4i), mainly strains related to the *Rhizobium pusense, A. lipoferum* and *A. brasilense* species. Other species closey related to genera *Stenotrophomonas, Bacillus, Pseudomonas* and *Ochrobactrum* were also detected producing indols (Fig. 4j). The intrinsic ability to produce these substances in the presence of tryptophan varied greatly among strains. Values ranged from 13.96 to 470.53 μg IAA mg^{-1} protein, with an average of 137.87 for *Azospirillum* strains, 187.48 for *Rizobium*, 327.75 for *Bacillus*, 113.45 for *Stenotrophomonas*, 84.75 for *Pseudomonas* and 13.96 μg for *Ochrobactrum* strain (Table S1).

Overall, analysis of of the functional characteristics of the 213 diazotrophic bacterial strains showed that 39% were able to solubilize inorganic phosphate, 9% solubilized zinc oxide, 84% produced siderophores, 32% had cellulolytic activity, 18% were capable of producing indoles and 85% presented the fragment of the *nif*H gene of the expected size (Table S1). There was no clear relationship between the phylogenetic positioning of the isolate, from where it was isolated, and the presence of a given function. However, the PCA allowed us to identify some patterns such as the positive correlation between the ability to produce siderophores with the ability to produce IAA; and between phosphate and Zinc solubilisation Fig. 5). This correlation is indicated by the small angle

between the arrows in the PCA biplot. A group of isolates with NRB023 (*R. pusense*), NRB028, NRB029 and NRB030 (*B. aerius*), NRB037 (*A. oryzae*), NRB033 and NRB097 (*A. lipoferum*), are among those that produced more IAA, and all of them were able to produce siderophores, either in a small or intermediate level. Some were able to solubilise phosphate and some able to degrade CMC. The isolate NBR085, an *A. lipoferum* isolated from the rhizosphere of *B. humidicola*, stands out among the isolates as possessing the ability to carry out all functions measured, with high rates of phosphate and zinc solubilization, CMC degradation, and siderophore production. It was also able to produce IAA in intermediate doses among the isolates and to fix N in semi-solid media (*nif*H present). The isolate NRB074, an *A. brasilense* isolated from *B. brizantha*, was also able to produce considerable amounts of siderophores, fix nitrogen, and solubilize zinc and phosphate. However, it was not able to degrade CMC.

Discussion

Diazotrophic bacterial population and genetic diversity analysis

The results of the present study showed that the diazotrophic population associated with the 20 *Brachiaria* genotypes, maintained in a Germoplasm Active Bank (GAB), ranged from 10^2 to 10^8 bacteria per gram of rhizosphere soil/fresh tissue sample, with the largest population found colonizing the roots (surface or interior). Reis Junior et al. (2004) observed similar results in the roots and rhizosphere soil of three *Brachiaria* genotypes cultivated in soils of Goiás and Minas Gerais states (Cerrado Biome), as well as in the study carried out by Santos et al. (2013) on forage grasses grown in the semi-arid Northeast region (Caating Biome) of Brazil. Large diazotrophic bacterial population (10^2 to 10^6 bacteria g^{-1} fresh tissues) has also been observed in two elephant grass genotypes grown in soil of Rio de Janeiro state (Atlantic forest biome) (Videira et al. 2012) and, in the study with the tropical molasses grass (*Melinis minutiflora)* grown as pioneer plants in poor soils in a tropical region of China (Peng et al. 2006). Therefore, the results indicate that, independently of soil type, there is a direct relationship between the Poaceae and these diazotrophic bacteria (associative and endophytic) as attributed by Baldani and Baldani (2005).

The bacterial colonization of the root system is normally influenced by the release of exudates (Baudoin et al. 2003; Mommer et al. 2016) and variations that occur in the microbial population of the rhizosphere are highly dependent on the plant species and genotype (Barea et al. 2005; Richardson et al. 2009). Therefore, an important aspect to be considered in the selection of

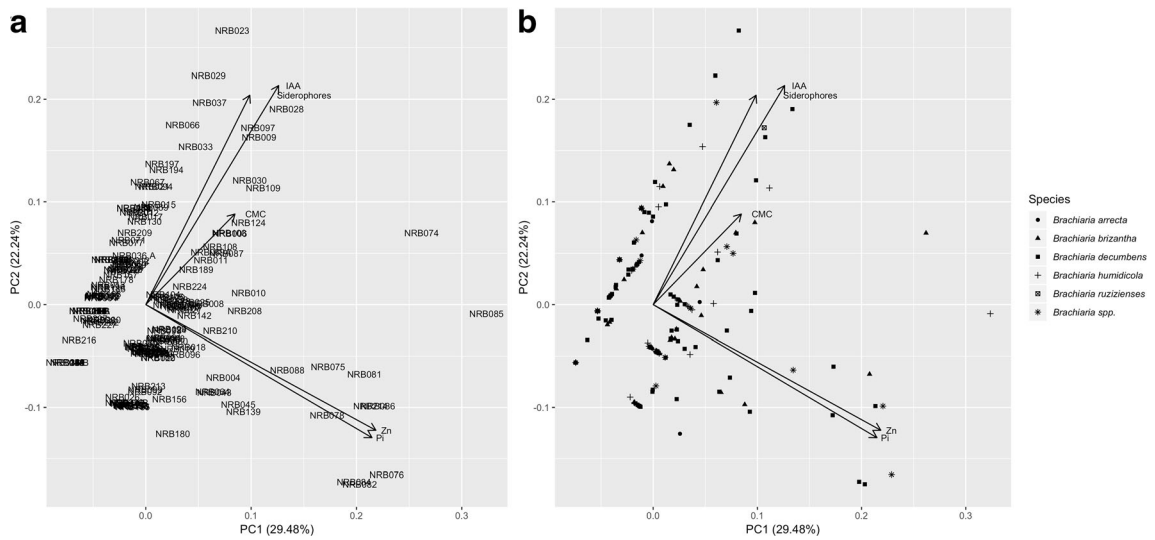

Fig. 5 Principal component analysis (PCA) of five functional activity (Pi and ZnO solubilisation, siderophore and indol acetic acid production, cellulolytic activity) of the 213 diazotrophic strains isolated from 20 different *Brachiaria* genotypes. Each number represents an individual strain (**a**) while the symbols represent the *Brachiaria* species the strains was isolated (**b**). The arrows indicated the functional activity showing the positive correlation between them

promising strains is the more tight relationship between plant and bacteria (Baldani and Baldani 2005). In our study, the GAB has been maintained in the same soil and place for around 10 years and therefore a structural native diazotrophic bacterial population could have been established and possibly modulated by the different *Brachiaria* species/genotypes. The highest diazotrophic population detected in the *B. decumbens* genotype Basilisk counted in NFb medium could be an indicative of this more specific type of association. The genotype Basilisk was the first *Brachiaria* grass introduced in Brazil in the early 70 (Mateus et al. 2013) and therefore could have developed a good interaction with the diazotrophic bacterial community along the years. In contrast, the diazotrophic bacteria population present in the *B. ruziziensis* genotype R134 was below the minimum detection level of the method (MPN) for both nitrogen-free semi-solid media. No clear explanation why the diazotrophic bacteria was so low in that genotype that could not be detected by the method despite the genotype has been maintained in the same GAB. Further studies applying molecular methods should be done to respond the missing information. Concerning the other genotypes, the relationship between the diazotrophic bacterial populations varied with species and the *Brachiaria* genotypes and could be detected in most of the genotypes independently of the semi-solid media.

Analyzes based on partial 16S rRNA and *nif*H gene sequences confirmed the presence of the genera *Azospirillum* and *Nitrospirillum* colonizing roots (surface and interior) and rhizospheric soil of 20 *Brachiaria* genotypes. Interesting, 15 other diazotrophic bacterial genera, belonging to the Proteobacteria classes (alpha, beta and gamma) and Firmicutes, were also identified using the nitrogen-free semi-specific semisolid NFb and LGI media. Among them, we highlight strains phylogenetically related to species *Paraburkholderia silvatlantica, Stenotrophomonas maltophilia, Herbaspirillum seropedicae, Gluconacetobacter diazotrophicus, Stenotrophomonas pavanii, Phytobacter diazotrophicus, Ochrobactrum anthropi, Zoogloea oryzae, Rhizobium pusense*. The species *O. anthropi*, *P. diazotrophicus* and *Z. oryzae* were found, for the first time, to be associated with *Brachiaria* genotypes. These results corroborate the studies carried out by Chalita et al. (2013) who also observed an enormous diversity of diazotrophic bacteria associated with *B. brizantha* and *B. humidicola* grown in Cerrado soils. Many of these diazotrophic bacterial species were also identified associated with *B. decumbens* and *B. humidicola* grown in the Northeast arid region of Brazil (Oliveira et al. 2018). In contrast, the use of rich media allowed the isolation of many bacterial strains belonging to different genera, mainly of *Pseudomonas, Pantoea*, *Acinetobacter* and *Enterobacter* species, from native *Brachiaria* grass grown in Kenya (Mutai et al. 2017). In addition to the interdiversity of the isolates, an intradiversity was also observed between the *Azospirillum* and *Nitrospirillum* strains (Figs. S6, S7, S8, S9 and S10). This fact corroborates the studies carried out by Reis Junior et al. (2006) and Azevedo et al. (2005), that found an intradiversity among *Nitrospirillum amazonense* (older *Azospirillum amazonense*) strains isolated from *Brachiaria* and rice, sorghum and maize, respectively.

The strains NRB074, NRB109, NRB194, NRB197, NRB200 and NRB214 are closely related to the species *A. brasilense* and/or *A. formosense* based on the partial 16S rRNA and *nif*H gene sequences, but each phylogenetic tree presented a different topology (Fig. S8). High similarity of the 16S rRNA and *nif*H genes was observed between the species *A. brasilense* and *A. formosense* (Lin et al. 2012). In contrast, other strains isolated from *Brachiaria* genotypes showed similarity with the *nif*H gene of *A. melinis*, *A. lipoferum* and *A. oryzae*, as well as for the 16S rRNA gene, but formed distinct clusters. This is due to the lack of evolutionary synchronism between the analyzed genes in the different species of *Azospirillum*, despite the high level of similarity of these genes (Liu et al. 2012). Gaby and Buckley (2014) also reported that the genetic divergence of the *nif*H and 16S rRNA genes generally produces different results. This event should be further investigated by analysing the complete clusters formed by the *A. formosense* and *A. brasilense* strains isolated from *Brachiaria* genotypes, considering that only one strain (CC-Nfb-7) was used in the description of *A. formosense* species by Lin et al. (2012). There is no clear explanation why the specific band of the *nif*H gene was not amplified in 33 strains isolated from both nitrogen free semisolid media and that clustered together to species already reported to contain nitrogen-fixing strains such as *Bacillus aerius, B. cereus*, *Bosea thiooxidans*, *Nitrospirillum amazonense*, *Stenotrophomonas maltophilia* and *S. panacihumi*. Except to the *N. amazonense* species, that is a widespread diazotrophic bacterium found associated with

many grasses and other plants (Reis et al. 2015), the other species are constituted mostly by non-nitrogen-fixing strains and therefore additional studies are required including the use of degenerated *nif* primers.

The fact that the genera *Nitrospirillum* and *Azospirillum* presented the highest number of isolates is probably due to the use of the semi-selective LGI and NFb media for these diazotrophic species, respectively. In addition, their wide ecological distribution among various forage grasses (Reis Junior et al. 2004; Brasil et al. 2005; Videira et al. 2012; Santos et al. 2013; Silva et al. 2013; Souza et al. 2017) have also contributed to the frequency of isolation. Interesting was the occurrence of strains closely related to other *Azospirillum* species not yet reported in association with *Brachiaria* cultivated in Brazil, therefore expanding the possibility to explore the potential of these strains in association with *Brachiaria*. Additionally, it is was interesting the occurrence of a group of bacteria phylogenetically related to *Rhizobium*, a saprophytic bacteria that live in symbioses with legumes (Masson-Boivin et al. 2009). Oliveira et al. (2018) have also isolated strains of the genus *Rhizobium* from *B. decumbens* grown in the Northeast region of Brazil and reported a specificity between both partners. However, no further study was done to demonstrate this event. It seems that bacteria belonging to genus *Rhizobium* are quite commonly found colonizing grass plants such as observed for rice (Singh et al. 2006; Peng et al. 2008) and maize (Gutiérrez-Zamora and Martınez-Romero 2001; Ilyas et al. 2008).

Growth promoting characteristics

Our study showed that 84 strains were able to solubilize inorganic phosphate, 20 strains presented the capacity to solubilize zinc oxide while 10 strains showed both functional capacities. These strains were identified as belonging to the genera *Azospirillum*, *Nitrospirillum*, *Paraburkholderia, Pseudomonas*, among others. The ability of these genera to solubilize P and ZnO in vitro has been demonstrated by several authors (Fasim et al. 2002; Rodriguez et al. 2004; Estrada et al. 2013; Goteti et al. 2013; López-Ortega et al. 2013; Gupta et al. 2016; Nandal and Solanki 2017). These functional characteristic has been attributed to the release of several organic acids, with gluconic acid being the major one responsible (Rodriguez et al. 2004; Lin et al. 2006; Saravanan et al. 2007). It is known that phosphorus is one of the main nutrients that limit the development of roots and, consequently, the growth of plants, particularly in tropical soils (Cerrado biome) such as those cultivated with *Brachiaria* that occupy approximately 76 million ha (ABIEC, 2018).

The production of siderophores by associative diazotrophic bacteria can improve plant growth, either by increasing the availability of nutrients through iron uptake or by preventing the growth of soil pathogens due to iron limitation (Miethke and Marahiel 2007; Chaiharn et al. 2009; Sayyed and Chincholkar 2009). Our study showed that besides *Azospirillum* (catecholate type) and *Nitrospirillum* (hidroxamate type), other species have also shown the ability to produce siderophores, mainly the catecholate type. The production of siderophores by strains within the genus *Azospirillum* has also been observed by other authors (Saxena et al. 1986; Pedraza et al. 2004; Manivannan and Tholkappian 2013; Tortora et al. 2011). In the case of *Nitrospirillum*, there are no reports in the literature on siderophore production within this genus; however, Schwab et al. (2018) reported the presence of 2 copies of *ton*B and more than 50 genes encoding TonB dependent receptors, including 11 which are probably involved in iron uptake in *Nitrospirillum amazonense* strain CBAmC. The detection of siderophores among the other diazotrophic species identified in the present study indicated the presence of both types of siderophores with a high production of catecholate type by bacteria closely related to *P. tropica*, *B. safensis*, *B. aerius*, *S. maltophilia*, *O. anthropi*, *P. nitritireducens* strain and oxalate type by *P.silvatlantica*, *H. seropedicae*, *P. diazotrophicus, B. cereus*, *S. panacihumi*, *Z. oryzae* strains. The production of siderophores has also been observed by other authors using some bacteria of similar genera, such as *Burkholderia* (Lewenza et al. 1999), *Pseudomonas* (Saranraj et al. 2013; Kamran et al. 2017), *Rhizobium* (Carrillo-Castañeda et al. 2002) and *Kosakonia* (Kamran et al. 2017).

Differences among the isolates regarding the ability to produce siderophores, should be taken into account in the selection of strains for plant growth-promoting experiments. Strains with high siderophore production activity (e.g. NRB034, NRB021, NRB148, NRB158, NBR230) should be further investigated for their ability to confer resistance to plant diseases. The production of siderophores by associative bacteria can improve plant growth, both by increasing the availability of nutrients through iron uptake and by preventing the growth of soil

pathogens due to iron limitation (Chaiharn et al. 2009; Sayyed and Chincholkar 2009). Thus, of the bacterial strains capable of producing siderophores should be further evaluated for their contribution to the control of phytopathogens and the development of plants in agriculture.

Bacterial strains that present cellulolytic activity can play a very important role in the endophytic colonization of the plant tissues, thus presenting an important ecological role in the interaction with plants (Hurek et al. 1994). Corroborating our study, Mostajeran et al. (2007) and Mehdipour-Moghaddam et al. (2010) also observed the cellulolytic capacity in strains of *Azospirillum*. In addition to this genus, cellulolytic capacity was also detected in strains of the genus *Bacillus* and *Pseudomonas*, and this capacity in both genera has been reported in the literature (Verma et al. 2001; Prakamhang et al. 2009; Reetha et al. 2014). Furthermore, the ability to degrade cellulose was also observed in some *N. amazonense* strains, however there is no report in the literature showing this capacity in the genus *Nitrospirillum,* including the sequenced genome of *N. amazonense* strain CBAmC (Schwab et al. 2018*)* and in a strain isolated from rice (Elbeltagy et al. 2000).

The closely related *A. formosense* strains NRB011 and NRB214 and *A. lipoferum* strain NRB033 should be further investigated due to their high biotechnology potential in the control of *Fusarium*, *Rhizoctonia*, and *Pythium*, causal agents of collection rot in *Brachiaria* (Duarte et al. 2007). In addition, these strains also presented the highest rates of cellulolytic activity and the ability to produce siderophores. It is known that bacteria producing cellulase-like enzymes and other hydrolytic enzymes may benefit their host by inhibiting fungal pathogens by degrading cell wall cells with the action of enzymes such as β-1,4-glycanases, leading to lysis of the cell wall and consequently the death of this pathogen (Sturz et al. 2000; Dobbelaere et al. 2003).

Our study also showed that the indole production was detected in strains belonging to the genera *Azospirillum*, *Rhizobium*, *Bacillus, Stenotrophomonas*, *Pseudomonas* and *Ochrobactrum*. Similar results were observed by Roesch et al. (2007) and Santos et al. (2015) working with different strains of the genus *Azospirillum.*. Pedraza et al. (2004) working with different diazotrophic bacteria observed that all bacterial strains produced IAA, but the *Azospirillum* strains presented higher values, with *A. brasilense* UAP14 producing the highest level (27.36 μg IAA mg protein^{-1}). There is no explanation why the *N. amazonense* strains (total of 78) isolated from *Brachiaria* genotypes did not show indole acetic acid activity, although this characteristic has already been demonstrated in *N. amazonense* strains isolated from *Brachiaria* (Reis Junior et al. 2004) as well as from rice (Rodrigues et al. 2008).

It has been reported that IAA production by bacteria that colonize the rhizosphere, including fre-living and associative, is responsible for the stimulation of the growth and proliferation of the secondary roots, and for the pathogenesis in several plants (Glick 2012). Shigenaga and Argueso (2016), in a review, reported that auxins could act in the signaling of the plant defense process, as is already known for jasmonic acid and salicylic acid. Therefore, the use of diazotrophic bacteria that present the ability to synthesize IAA in the formulation of inoculants may play an important role in the control of diseases (Kazan and Manners 2009; Tian et al. 2017), besides stimulating the germination of the seeds and promoting the growth of secondary roots.

In conclusion, the present study showed a large cultivable population of diazotrophic bacteria colonizing rhizospheric soil and roots (associative and endophytic) of the twenty genotypes of *Brachiaria*, varying according to plant genotype, the sample and the culture medium used for isolation. There was no specific association of a bacterium genus and a *Brachiaria* genotype as for example the genus *Nitrospirillum* was detected associated with all *Brachiaria* species and colonizing the three sampled niches. In addition, it was observed that many of the identified genera were associated with the *B. decumbens* species and colonizing either the root (surface or interior) or the rhizosphere soil. The molecular analysis of the 213 bacterial strains confirmed the presence of diazotrophic bacteria closely related to the species *Nitrospirillum amazonense* and several species belonging to the genus *Azospirillum*. In addition, the presence of *Paraburkolderia*, *Phytobacter*, *Gluconacetobacter*, *Herbaspirillum, Bacillus*, *Pseudomonas*, *Stenotrophomonas* and others in the 20 *Brachiaria* genotypes was detected. Our study revealed, for the first time, the occurrence of the genera *Phytobacter* and *Ochrobactrum* colonizing this forage grass. There was no clear relationship between the phylogenetic positioning of the isolate, from where it was isolated, and the presence of a given function. However, the PCA allowed us to identify some patterns such as the positive correlation between the ability to produce siderophores with the ability to produce IAA; and between phosphate and zinc

solubilisation. In addition, the functional activities of the bacterial strains revealed the presence of one strain (*A. lipoferum* strain NRB085) showing all the functional characteristics and twelve strains with five functional characteristics, thus indicating that these strains are potentially good candidates for field inoculation tests envisaging the development of biofertilizers. Furthermore, the results should assist in the development of ecologically efficient and sustainable agricultural practices.

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