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Associative diazotrophic bacteria from forage grasses in the Brazilian semi-arid region are effective plant growth promoters

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Abstract. The study of plant growth-promoting bacteria (PGPB) can identify outstanding bacteria for crops. For forage grasses adapted to drylands, the selection of PGPB can increase the field performance of pastures. The aim of this study was to isolate, and characterise at molecular, biochemical and symbiotic levels, diazotrophic bacteria obtained from buffel grass (*Cenchrus ciliaris*), sorghum (*Sorghum bicolor*) and Tifton 85 (*Cynodon* spp.) from Brazilian semi-arid region fields. Field-grown plants were collected, and the roots were surface-disinfected, crushed and inoculated in a semi-solid medium. After the formation and confirmation of microaerophilic pellicles, the bacteria were isolated and purified. All bacterial isolates were subjected to *nifH* gene amplification and identified by their partial 16S rRNA gene sequences. The bacteria were evaluated for the production of auxins and siderophores, calcium phosphate solubilisation, and diazotrophic ability as 'in vitro' plant growth-promotion traits. A plant inoculation assay was conducted to assess the plant growth-promotion abilities of the bacterial isolates. Twenty-one bacterial isolates harboured the *nifH* gene (*nifH*$^+$), among which nine were obtained from sorghum, eight from buffel grass, and four from Tifton 85. The bacterial isolates were classified as *Bacillus* (8), *Stenotrophomonas* (7), *Agrobacterium* (4), *Cellulomonas* (1) and *Paenibacillus* (1). All were shown to be auxin producers, with 14 isolates showing diazotrophic capacity 'in vitro'. Fourteen isolates increased plant N content, but the bacterial strains ESA 392 and ESA 398 (*Bacillus*), ESA 397 and ESA 407 (*Stenotrophomonas*), and ESA 401 (*Agrobacterium*) were shown to promote both plant growth and N nutrition. These strains are candidates for further assays to evaluate their agronomic performance under field conditions, aiming inoculant production for forage grasses in drylands.

Additional keywords: biological nitrogen fixation, Caatinga, plant growth promotion traits, strain selection.

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Introduction

The north-eastern region of Brazil is home to 65.6% of the country's sheep and 92.8% of its goats, constituting more than 22 million animals (IBGE 2017). Most of these animals are raised in the semi-arid belt on family-based farms lacking expensive technologies for animal nutrition (Voltolini 2011). In drylands, most sheep and goats are not supplied food and they often graze natural shrub vegetation known as 'Caatinga', a Brazilian stepic savannah that does not have the food quality necessary to achieve high bodyweight (Moreira *et al*. 2006; Moreira and Guimarães Filho 2011). In this context, the development of highly productive pasture lands is important to increase animal production in the region.

Generally, forages require high amounts of fertilisers for suitable growth and development. The grasses in pastures require large amounts of nitrogen (N), resulting in hundreds of kilograms of N fertilisers being applied per hectare, leading to high N losses, and the greenhouse-gas emissions (Signor and Cerri 2013; Mazzetto and Barneze 2016). The development and implementation of new, sustainable technologies for pastures in the Brazilian semi-arid region is needed, and to this end, the selection of new, locally adapted plant growth-promoting bacteria (PGPB) is a promising tool (Hungria *et al*. 2016; da Silva *et al*. 2018).

The PGPB comprise several bacterial *taxa* that are able to influence plant growth positively through several different mechanisms such as biological nitrogen fixation (BNF), phosphorus and potassium mobilisation, biological pest and disease control, and plant hormone regulation (Boddey *et al*. 1997; Glick 2005; de Souza *et al*. 2015). Among these processes, BNF is one of the best understood, and N_2-fixing bacteria can easily be obtained using semi-solid media (Baldani *et al*. 2014).

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Efforts to select for diazotrophic PGPB in Brazil culminated in the official recommendation and use of *Azospirillum brasilense* strains for maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.) and *Brachiaria* spp. (Hungria *et al*. 2010, 2016). Recently, these bacteria were also demonstrated to be efficient plant growth promoters for other pasture grasses, such as sorghum (*Sorghum bicolor* (L.) Moench) (da Silva *et al*. 2018), Marandu grass (*Urochloa brizantha* (Hochst. ex A.Rich.) R.Webster) (Leite *et al*. 2019*a*) and Mombasa grass (*Megathyrsus maximus* (Jacq.) B.K.Simon & S.W.L.Jacobs.) (Leite *et al*. 2019*b*).

Studies involving the selection of diazotrophic PGPB for forages have been conducted in vastly different regions of Brazil, including the mid-west (Reis Junior *et al*. 2004; Brasil *et al*. 2005; Souza *et al*. 2017), south-east (dos Santos *et al*. 2017) and north-east (dos Santos *et al*. 2013; Moreira *et al*. 2013; Antunes *et al*. 2017; da Silva *et al*. 2018), indicating the effectiveness of prospective studies for locally adapted N_2-fixing isolates.

Few reports are available regarding the diversity and efficiency of PGPB from forage grasses in the semi-arid region of Brazil. Diazotrophic isolates from buffel grass (*Cenchrus ciliaris* L.), andropogon (*Andropogon gayanus* Kunth) and Tanzania grass (*Panicum maximum* Jacq. cv. Tanzânia) were previously obtained and phenotypically characterised from Patos, Paraíba State (dos Santos *et al*. 2013). At the same location, Moreira *et al*. (2013) isolated and characterised *Azospirillum*-like bacteria from the same plant species. Both studies characterised and evaluated the cultural diversity and seasonal fluctuation of diazotrophs but did not evaluate their molecular and plant growth-promotion abilities. More recently, a comprehensive study evaluated the molecular diversity and plant growth-promotion potential of field-grown sorghum in Petrolina, Pernambuco State, the results of which pointed towards an *Enterobacter* sp. isolate (ESA 57) as the most promising plant growth promoter (da Silva *et al*. 2018). However, a comprehensive study evaluating the molecular characteristics, plant growth-promotion abilities and symbiotic efficiency of those bacteria were not conducted for multiple field-grown grasses in the Brazilian semi-arid region.

Among the crop pastures grown in the Brazilian drylands, buffel grass, Tifton 85 (*Cynodon* spp.) and sorghum are the most widely distributed. Buffel grass and sorghum are cropped in rainfed systems, whereas Tifton 85 is recommended for use in irrigated pastures (Voltolini 2011). This partition covers the majority of the livestock systems in the Brazilian semi-arid region, and the isolation of bacteria from these different plants could be applied in different crop systems in the region. In this context, we hypothesised that field-grown buffel grass, sorghum and Tifton 85 harbor a diversity of efficient PGPB. Thus, the aim of this study was to isolate and evaluate the molecular diversity, plant growth-promotion abilities and characteristics of PGPB from field-grown buffel grass, Tifton 85 and sorghum in Petrolina municipality, Brazilian semi-arid region. This is more suitable because the isolation of the bacteria were conducted from plants in the same experimental field.

Methods and materials

Isolation of bacteria

Samples of roots of buffel grass (cv. Biloela), Tifton 85 and forage sorghum (cv. BRS Ponta Negra) were collected in the Caatinga Experimental Field, an experimental area of Embrapa Semiárido, Petrolina, Pernambuco State (9°03′45″S, 40°19′37″W), in May 2015. For the sampling, three replicate samples of each species, two plants per replicate, were collected.

The roots were washed with tap water and surface-disinfested by immersion in 1% (v/v) sodium hypochlorite for 10 min, followed by 10 washes in deionised autoclaved water. The disinfected roots were weighed (10 g) and crushed with 90 mL 0.85% (w/v) NaCl solution in a common blender. A serial dilution from 10^{-1} to 10^{-6} was performed, and for each dilution, 100 µL was inoculated into flasks containing 7 mL BMGM semi-solid medium (per L: 1 g glucose, 2 g malic acid, 1 g mannitol, 400 mg K_2HPO_4, 400 mg KH_2PO_4, 200 mg $MgSO_4$, 20 mg $CaCl_2$, 2 mg $NaMoO_4$, and 10 mg $FeSO_4$; 2 g agar, pH 6.0) (Estrada-de Los Santos *et al*. 2001) and incubated at 28°C for 10 days. Each serial dilution was inoculated in triplicate. Afterwards, the less-concentrated dilutions that developed a typical microaerophilic pellicle (MP) were separated, re-inoculated in the same medium, and incubated at the same conditions. The flasks with positive MP were streaked onto plates containing Dyg's medium (per L: 2 g glucose, 2 g malic acid, 1.5 g peptone, 2 g yeast extract, 500 mg K_2HPO_4, 500 mg $MgSO_4$, 1.5 g glutamic acid, and 15 g agar; pH 6.5) (Rodrigues Neto *et al*. 1986).

Both BMGM and Dyg's are nonselective culture media, and the isolation of diazotrophic isolates in BMGM semi-solid, coupled with the use of Dyg's solid media, can retrieve a large diversity of diazotrophic bacteria (Fernandes Júnior *et al*. 2013, 2015). For these reasons, this isolation strategy was applied in the present study. The purified colonies were stored in glycerol at –80°C in the Culture Collection of Microorganisms with Agricultural Interests of Embrapa Semiárido (CMISA).

Molecular analyses

All bacteria grew in liquid Dyg's medium, and DNA was extracted by using the alkaline lysis method as described by Wang *et al*. (1993). All bacteria were subjected to *nifH* amplification, using the primers PolF (TGCGAYCCSAA RGCBGACTC) and PolR (ATSGCCATCATYTCRCCGGA) (Poly *et al*. 2001). Afterwards, nested PCR, where the first PCR product is used as a template for the second reaction, was performed to evaluate the presence of the negative isolates in the first reaction. For nested PCR, the primers NifHfor (ACCCGCCTGATCCTGCACGCAAGG) and NifHrev (ACG ATGTAGATTTCCTGGGCCTTGTT) (Soares *et al*. 2006) were used.

The 16S rRNA gene was amplified by using the universal primers 27F (GAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Weisburg *et al*. 1991), and the PCR products were purified using a Wizard SV Gel and PCR Clean-up System kit (Promega, Madison, WI, USA). Sequencing was conducted at Macrogen (Seoul, South Korea), using an Applied Biosystems 3730*xl* Genetic Analyzer (Thermo Fisher, Waltham, MA, USA) with the primer 27F. The quality of the sequences was verified using Applied Biosystems Sequence Scanner version 2.0 (Thermo Fisher), and the high-quality sequences (QV >20 in 800-bp continuous reads) were compared by using the EzBioCloud database (Yoon *et al*.

2017). The sequences were deposited in the GenBank database of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers MK424592–MK424613.

In vitro *plant growth-promoting traits*

For evaluation of auxin production, the colourimetric procedure described by Sarwar and Kremer (1995) was used, with some modifications. Briefly, the bacteria were grown in liquid Dyg's medium with or without 100 mg L^{-1} of L-tryptophan (L-Trp) for 7 days under constant stirring of 120 rpm at room temperature. The optical density of each culture was adjusted spectrophotometrically at 600 nm (OD_{600}) to 0.3 to standardise the cell concentration.

Subsequently, 1.0-mL aliquots of the OD_{600}-adjusted cultures were centrifuged for 3 min at 6000*g*, after which 150 µL supernatant was added to 96-well ELISA microplates along with 100 µL Salkowski's reagent (1.0 mL 0.5 M $FeCl_3.6H_2O$ and 50 mL 35% (v/v) $HClO_4$). The plates were stored in the dark at room temperature for 30 min, after which the intensity of the reddish coloration was determined spectrophotometrically at 530 nm. The auxin concentration was estimated by using a standard curve with a known concentration of indole acetic acid (Sigma Aldrich, St. Louis, MO, USA).

For siderophore production, the qualitative approach described by Ribeiro and Cardoso (2012) was adapted. Briefly, the isolates were grown in Dyg's medium for 4 days as described above. Afterwards, a 1-mL sample of the culture was centrifuged, and 150 µL supernatant was added to 96-well ELISA microplates along with 150 µL CAS reagent (6.0 mL hexadecyltrimethylammonium bromide (HDTMA), 1.5 mL $FeCl_3.6H_2O$ solution, 4.307 g piperazine, and 6.25 mL 33% (v/v) HCl) (Schwyn and Neilands 1987). The plates were incubated in the dark at room temperature for 30 min, and wells that changed from blue to yellow–orange were considered positive.

The isolates were evaluated for tricalcium phosphate solubilisation in solid medium (Sylvester-Bradley *et al*. 1982). The bacteria were cultured in liquid Dyg's medium for 4 days, centrifuged and resuspended as described above. Subsequently, 10-µL aliquots of the cultures were placed into plates containing GL (glucose–yeast extract) medium (per L: 10 g glucose, 2 g yeast extract with 50 mL K_2HPO_4 and 100 mL $CaCl_2$ (both 10% w/v)), 20 g agar and incubated at room temperature for 6 days. After the incubation period, the diameters of the colonies and the translucent halo surrounding the colonies were measured. The data were used to calculate the solubilisation index via the ratio diameter of halo : diameter of colony (Berraquero *et al*. 1976).

Putative diazotrophic ability was assessed by quantifying the N content in semi-solid medium after MP formation (Kuss *et al*. 2007; Silva *et al*. 2013; Fernandes-Júnior *et al*. 2015). Flasks with 10 mL BMGM semi-solid medium were inoculated with 100-µL samples of the bacterial cultures and incubated at room temperature for 10 days. After the incubation period, the medium was manually homogenised, frozen at –20°C and heated in boiling water for 10 min. Subsequently, the media samples were homogenised again, and 5-mL aliquots were withdrawn to quantify the N content by using the modified Devarda's alloy semi-micro Kjeldahl method (Liao 1981). To calculate the N concentration inputted into the medium by the bacteria, an aliquot of uninoculated BMGM medium was also digested and distilled as a blank.

All 'in vitro' plant growth-promotion traits were assessed with three replications applied in a completely randomised design. For all experiments, the commercial inoculant bacterium *Azospirillum brasilense* (Ab-V5) for *Brachiaria* spp. was also evaluated.

Plant growth-promotion assay

All bacterial isolates were assessed in pot experiments to evaluate their plant growth-promotion abilities. The experiment was performed in 5-L pots filled with a layer sample of red–yellow Ultisol. A soil sample was used for soil chemical analysis (results are shown in the Supplementary Material table S1, available at the journal's website). The model plant sorghum (cv. BRS Ponta Negra) was used.

The seeds were surface-disinfected with 96% (v/v) ethanol for 30 s and 2.5% (v/v) sodium hypochlorite for 5 min; they were then washed 10 times with deionised autoclaved water. For inoculation, the bacterial isolates were grown in liquid Dyg's medium for 4 days as described above. Four seeds were sown per pot, and 1-mL aliquots of the cultures were inoculated over each seed. The experimental treatments included single inoculations of 21 new bacteria and the reference strain Ab-V5. In addition, three uninoculated treatments, one without mineral N and two with the addition of 50 and 100 mg N (NH_4NO_3) per plant per week, starting in the second week, were also tested. For application to each pot, NH_4NO_3 was weighted, dissolved in 100 mL deionised autoclaved water and applied after the daily irrigation.

At 10 days after emergence, the plants were thinned such that a single plant remained per pot. The plants received 500 mL tap water daily and were harvested at 62 days after emergence. The roots were separated from the shoots and carefully washed with running tap water, after which they were separately dried at 65°C in an oven and weighed. The shoots were milled, and the total N concentration was assessed by Devarda's alloy semi-micro Kjeldahl method (Liao 1981). A completely randomised block design was applied with four replications per treatment.

Statistical analyses

All quantitative data were analysed by one-way analysis of variance (ANOVA). Prior to analysis of variance the data were transformed by calculating the square roots of data added to 1 (i.e. $(x + 1)^{0.5}$) to meet the assumptions of a normal distribution of errors and homoscedascity. The Scott–Knott mean range test ($P > 0.05$) was applied to all variables after the ANOVA. Statistical analyses were performed in Sisvar version 5.0 (Ferreira 2011).

Results

Isolation and taxonomic classification of bacterial isolates by partial 16S rRNA gene sequence analysis

Twenty-one bacterial isolates were retrieved after the purification process and confirmation of MP formation, with the sorghum yielding nine, buffel grass eight and Tifton 85 plants yielding four bacterial isolates. Among these bacteria, all isolates

were *nifH*-positive, with 13 yielding amplification products from the first PCR with the PolF/PolR primer pair, whereas eight bacteria showed positive amplification after the nested-PCR reaction using the primer pair NifHfor/NifHrev in the second PCR.

The partial 16S rRNA sequence analysis showed that the bacterial isolates were clustered in the genera *Bacillus* (8), *Stenotrophomonas* (7), *Agrobacterium* (4), *Cellulomonas* (1) and *Paenibacillus* (1), with similarity to the sequences of type strains in the EzBioCloud database ranging from 95% to 100% (Table 1). Buffel grass harboured *Bacillus* (4), *Stenotrophomonas* (3) and *Agrobacterium* (2); sorghum hosted *Stenotrophomonas* (2), *Bacillus* (2), *Agrobacterium* (2) and *Pseudomonas* (1); and Tifton 85 plants were infected by *Bacillus* (2), *Paenibacillus* (1) and *Stenotrophomonas* (1).

In vitro *plant growth promotion traits*

All bacterial isolates were able to synthetise an amount of auxins in the media with or without L-Trp (Fig. 1). In the medium with L-Trp, the average auxin concentration ranged from 30.4 μg mL^{-1} for isolate ESA 407 to 243.0 μg mL^{-1} for ESA 391. In the medium without L-Trp, the bacterial isolates produced from 24.5 μg auxins mL^{-1} (ESA 405) to 115.1 μg mL^{-1} (ESA 410) on average. By contrast, the reference strain Ab-V5 produced, on average, 40.9 and 30.9 μg auxins mL^{-1} in media with and without L-Trp, respectively. Theses averages were lower than observed in 17 new bacterial isolates in medium with or without L-Trp.

Regarding the diazotrophic capacity of all isolates, 14 of 21 bacteria showed the same level of N incorporation in media as the known efficient diazotrophic strain *Azospirillum brasilense* Ab-V5 (Fig. 2), indicating the high diazotrophic potential of the grass-associated bacteria. Positive results for siderophore production were observed only in the Ab-V5 reference strain and for the isolate ESA 408 obtained from Tifton 85. Tricalcium phosphate solubilisation was positive for ESA 402 and Ab-V5 only (both with a solubilisation index of 1.1).

Plant growth promotion assay

Inoculation of newly isolated bacteria, and of the reference strain Ab-V5, improved the growth of roots and shoots as well as N accumulation in sorghum plants (Table 2). Higher averages for root biomass were observed in the plants with full N fertilisation and those inoculated with the ESA 411 (*Stenotrophomonas*) bacterium. However, the plants inoculated with *Bacillus* ESA 392, ESA 398, ESA 402, ESA 401, ESA 400 and ESA 410, with *Stenotrophomonas* ESA 397, ESA 407, ESA 405 and ESA 399, with *Agrobacterium* ESA 396 and ESA 401, with *Paenibacillus* ESA 408, and with *Azospirillum brasilense* Ab-V5 had the same root biomass as observed in the treatments with the 50% N fertilisation rate. These results indicate the potential of these strains to increase sorghum biomass.

The shoots of sorghum plants with 100% or 50% N fertilisation treatments and plants inoculated with *Stenotrophomonas* ESA 397, ESA 407, ESA 399, ESA 405 and ESA 411, with *Bacillus* ESA 392, ESA 398, ESA 402 and ESA 410, with *Agrobacterium* ESA 401, and with *Azospirillum brasilense* Ab-V5 were larger than those in the negative control and in the other inoculation treatments. The N accumulation in the shoots of plants inoculated with *Azospirillum brasilense* Ab-V5, with *Stenotrophomonas* ESA 397, ESA 407, ESA 403 and ESA 399, with *Bacillus* ESA 392, ESA 394 and ESA 398, with *Agrobacterium* ESA 395, ESA 401 and ESA 406, with *Paenibacillus* ESA 408, and with *Cellulomonas* ESA 393 was higher than observed in the negative control treatment and the other seven inoculation treatments.

Table 1. Identification of 21 bacterial isolates obtained from buffel grass (*Cenchrus ciliaris*), sorghum (*Sorghum bicolor*) and Tifton 85 (*Cynodon* spp.) plants in the Brazilian semi-arid region

Bacterial isolate	GenBank accession code	Host	Fragment length (bp)	Closest type strains	Similarity (%)
ESA 391	MK424592	*Cenchrus ciliaris*	1068	*Stenotrophomonas pavanii* (LMG 25348^T)	99.3
ESA 392	MK424593	*Cenchrus ciliaris*	1031	*Bacillus safensis* (FO-36b^T)	98.9
ESA 393	MK424594	*Cenchrus ciliaris*	1009	*Cellulomonas massiliensis* (JC225^T)	97.1
ESA 394	MK424595	*Cenchrus ciliaris*	1028	*Bacillus siamensis* (KCTC 13613^T)	99.5
ESA 395	MK424596	*Cenchrus ciliaris*	884	*Agrobacterium fabrum* (C58^T)	100.0
ESA 396	MK424597	*Cenchrus ciliaris*	950	*Agrobacterium pusense* (NRCPB10^T)	96.4
ESA 397	MK424598	*Cenchrus ciliaris*	1024	*Stenotrophomonas maltophilia* (NCTC10257^T)	99.3
ESA 398	MK424599	*Cenchrus ciliaris*	1135	*Bacillus siamensis* (KCTC 13613^T)	99.3
ESA 399	MK424600	*Sorghum bicolor*	1094	*Stenotrophomonas pavanii* (LMG 25348^T)	98.5
ESA 400	MK424601	*Sorghum bicolor*	1109	*Bacillus velezensis* (NRRL B-41580^T)	99.1
ESA 401	MK424602	*Sorghum bicolor*	1022	*Agrobacterium salinitolerans* (YIC 5082^T)	98.7
ESA 402	MK424603	*Sorghum bicolor*	1082	*Bacillus safensis* (FO-36b^T)	100.0
ESA 403	MK424604	*Sorghum bicolor*	999	*Stenotrophomonas pavanii* (LMG 25348^T)	99.2
ESA 404	MK424605	*Sorghum bicolor*	1091	*Bacillus velezensis* (NRRL B-41580^T)	98.8
ESA 405	MK424606	*Sorghum bicolor*	1134	*Stenotrophomonas pavanii* (LMG 25348^T)	99.3
ESA 406	MK424607	*Sorghum bicolor*	1102	*Agrobacterium salinitolerans* (YIC 5082^T)	98.5
ESA 407	MK424608	*Sorghum bicolor*	993	*Stenotrophomonas bentonitica* (BII-R7^T)	99.1
ESA 408	MK424609	*Cynodon* spp.	1008	*Paenibacillus dongdonensis* (KUDC0114^T)	98.7
ESA 409	MK424610	*Cynodon* spp.	988	*Bacillus aryabhattai* (B8 W22^T)	100.0
ESA 410	MK424611	*Cynodon* spp.	990	*Bacillus velezensis* (NRRL B-41580^T)	99.3
ESA 411	MK424612	*Cynodon* spp.	1000	*Stenotrophomonas pavanii* (LMG 25348^T)	95.0

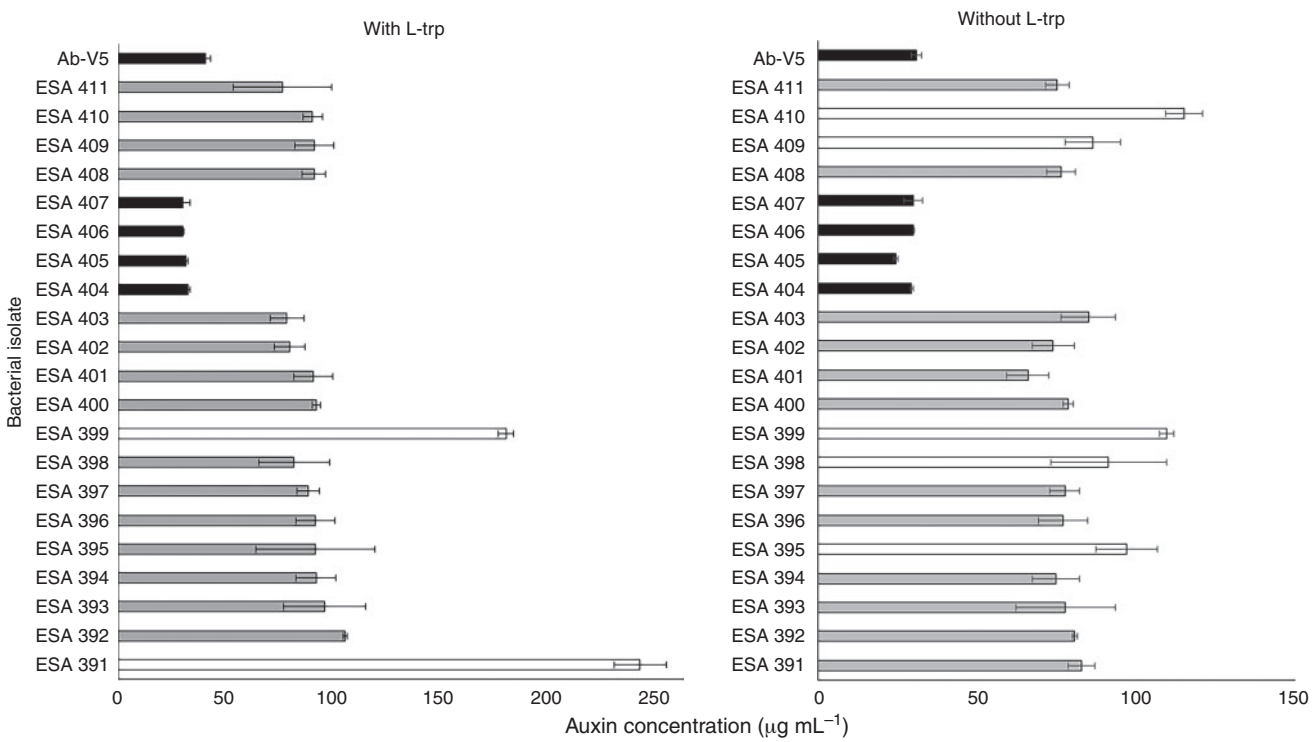

Fig. 1. *In vitro* auxin production by 21 new diazotrophic bacteria from buffel grass, sorghum and Tifton 85 grass and by *Azospirillum brasilense* Ab-V5 in liquid medium with or without L-tryptophan. Data are averages of four replications. Capped lines are mean deviation errors. Bars with the same colour (in the same graphic) do not differ according to the Scott–Knott mean range test.

Discussion

Classification of the isolates at the genus level indicated the presence of five genera, among which *Bacillus* predominated. Members of this genus have the ability to form endospores, which are resistant structures that can withstand severe dehydration for long periods and can germinate when water becomes available (Ambrosini *et al*. 2016). In drylands, previous reports have described the association of *Bacillus* with rainfed native plants such as the Poaceae *Tripogonella spicata* (Nees) P.M.Peterson & Romasch (formerly *Tripogon spicatus* (Nees) Ekman) (Fernandes-Júnior *et al*. 2015) and some cacti (Cactaceae) (Kavamura *et al*. 2013). By contrast, in other crop systems or pristine environments in the Brazilian semi-arid region, isolation efforts did not result in the identification of Firmicutes, whereas the prevalence of other taxonomic clusters such as Enterobacteriaceae (da Silva *et al*. 2018) and Burkholderiaceae (Lima *et al*. 2015) was observed.

In addition to Firmicutes, members of the γ-Proteobacteria genus *Stenotrophomonas* were isolated from the different plants studied. This genus was also obtained from *T. spicata* in the Brazilian Caatinga biome (Fernandes-Júnior *et al*. 2015) and from grasses in dry regions of India (Singh and Jha 2017) and Namibia (Haiyambo *et al*. 2015*a*), as well as from areas with other environmental conditions (Gontijo *et al*. 2018). Four *Agrobacterium* spp. were obtained from buffel grass and sorghum. This genus is endophytically associated with several non-legumes worldwide and exhibits numerous plant-stimulation mechanisms (Bertrand *et al*. 2001; Vendan *et al*. 2010; da Silva *et al*. 2018). However, to the best of our knowledge, this is the first report of the identification of *Agrobacterium* spp. as growth-promoting bacteria isolated from buffel grass. In addition, for the first time, a single isolate belonging to the genus *Cellulomonas* (Actinobacteria, Micrococcales, Cellulomonadaceae) was obtained from buffel grass. *Cellulomonas* was previously isolated from crops grown under different environmental conditions (Egamberdiyeva and Höflich 2002; Zinniel *et al*. 2002), including semi-arid environments (Egamberdieva 2008).

The results of assays to assess plant growth-promoting mechanisms indicated great metabolic variability within the strain culture collection. All isolates produced more auxin than the reference strain in the medium without L-Trp, the primary auxin precursor. These results indicate that all isolates have different metabolic pathways to synthetise auxins, including the primary L-Trp-dependent route, as observed in *Azospirillum*. The production of auxins in culture medium is indicative of the plant growth-promoting abilities of the bacterial isolates (Barazani and Friedman 1999; Brígido and Oliveira 2013; Duca *et al*. 2014).

Only the *Bacillus* sp. isolate ESA 402 was able to solubilise tricalcium phosphate, whereas the *Paenibacillus* isolate ESA 408 was shown to be a siderophore producer. These findings are not in agreement with the results of previous studies conducted in the Brazilian semi-arid region, where the isolation of plant endophytic bacteria resulted in a culture collection with several isolates that were able to act as phosphate solubilisers and/or siderophore producers (Fernandes-Júnior *et al*. 2015; da

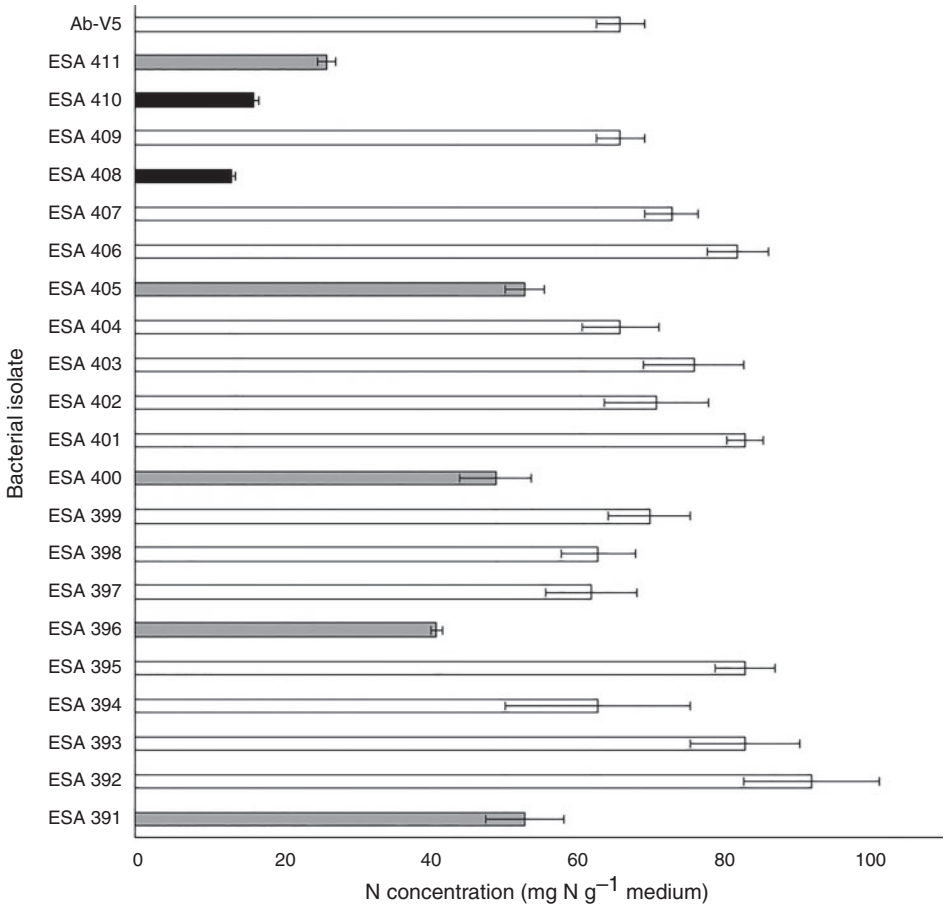

Fig. 2. Nitrogen concentration in BMGM semi-solid medium after microaerophilic growth of 21 new diazotrophic bacteria from buffel grass, sorghum and Tifton 85 grass and of *Azospirillum brasilense* Ab-V5. Data are averages of four replications. Capped lines are mean deviation errors. Bars with the same colour do not differ according to the Scott–Knott mean range test.

Silva *et al*. 2018; de Oliveira *et al*. 2018). Thus, ESA 402 and ESA 408 may be used to generate an inoculant mixture to improve the growth-promotion capacity of a consortium of bacteria.

An equivalent incorporation of N in medium was observed for 14 new bacterial isolates and the known diazotrophic species *Azospirillum brasilense*. In addition, among these 14 isolates, ESA 395, ESA 402 and ESA 409 were ranked in the highest clustering for auxin production (according to the Scott–Knott mean range test), reinforcing their multiple-plant growth-promotion abilities.

In the plant growth-promotion assays, the inoculation of 17 new strains, as well as the reference strain Ab-V5, led to some improvement in sorghum growth and/or N nutrition parameters, reinforcing that these strains are effective PGPB. The plant growth promoted by the bacterial isolates was associated with their putative diazotrophic capacities, because Ab-V5 and seven other N_2-fixing isolates were able to promote N accumulation in plant shoots. Regarding the other plant growth-promotion mechanisms, in auxin production and N_2-fixation assays, ESA 392 (*Bacillus*), ESA 397 (*Stenotrophomonas*), ESA 398 (*Bacillus*), ESA 401 (*Agrobacterium*) and ESA 407 (*Stenotrophomonas*) exhibited the capacity to promote root and shoot growth as well as increase N accumulation in plant shoots, reinforcing the potential of these bacteria to promote sorghum growth.

The bacterial isolates obtained in the present study are similar to PGPB isolates previously obtained from semi-arid environments and they increase the number of plant-associated genera isolated from crops, primarily for buffel grass. The results of the present study, together with those of similar studies, indicate a large diversity and local variability of bacteria associated with soils from the Brazilian semi-arid region, reinforcing the importance of our results regarding grass-associated bacteria in the Brazilian drylands.

The promotion of sorghum growth by bacterial isolates that were selected by assays assessing different plant growth-promotion mechanisms was already described in previous bacterial-isolation studies that used the semi-solid medium strategy (Haiyambo *et al*. 2015*b*; da Silva *et al*. 2018). These data reinforce the effectiveness of our bacterial strains, primarily for the isolates ESA 392, ESA 397, ESA 398, ESA 401 and ESA 407. In Brazil, sorghum, buffel grass and Tifton 85 do not have officially recommended bacterial strains for inoculant

Table 2. Root dry mass (RDM), shoot dry mass (SDM) and total nitrogen in the shoots (TNS) of sorghum plants inoculated with 21 bacteria from three forage grasses and *Azospirillum brasilense* (Ab-V5)
Within columns, averages followed by the same letter are not significantly different ($P > 0.05$) according to the Scott–Knott mean range test ($n = 4$)

Inoculation treatment	RDM	SDM	NAS
	(g plant^{-1})		(mg plant^{-1})
ESA 391	2.27c	9.45b	166a
ESA 392	5.45b	22.92a	213a
ESA 393	3.30b	12.87b	147b
ESA 394	1.02c	7.72b	140b
ESA 395	1.72c	11.17b	158a
ESA 396	0.67c	6.52b	208a
ESA 397	4.40b	17.10a	203a
ESA 398	2.65 b	14.50a	192a
ESA 399	3.57b	16.32a	142b
ESA 400	4.82b	18.42a	168a
ESA 401	5.35b	18.65a	170a
ESA 402	4.42b	16.90a	100b
ESA 403	5.70b	18.77a	226a
ESA 404	2.37c	7.32b	211a
ESA 405	1.40c	10.42b	192a
ESA 406	3.37b	3.77b	144b
ESA 407	3.86b	14.10a	179a
ESA 408	3.05b	12.52b	176a
ESA 409	1.90c	11.30b	131b
ESA 410	5.97 b	20.75a	210a
ESA 411	9.77a	23.08a	113b
Ab-V5	4.15b	15.05a	158a
Negative control	2.92c	16.90a	60b
50% N	5.50b	14.53a	210a
100% N	10.43a	23.76a	223a

production. The results of the present study indicate that our culture collection harbors bacterial isolates (primarily those abovementioned) that should be assessed in further field studies aiming to develop new bacteria isolates for inoculant production according to the Brazilian standards for inoculant recommendations.

Conclusions

Buffel grass, sorghum and Tifton 85 are colonised by different taxa of PGPB. These diazotrophic bacteria, primarily members of the genera *Bacillus*, *Stenotrophomonas* and *Agrobacterium*, are effective at promoting plant growth and N nutrition, indicating that the bacterial isolates ESA 392, ESA 397, ESA 398, ESA 401 and ESA 407 are candidates as bacterial strains for inoculants for grasses in Brazilian drylands.

Conflicts of interest

The authors declare no conflicts of interest.

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