



Bacteria from tropical semiarid temporary ponds promote maize growth under hydric stress



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ABSTRACT

World climate change has triggered soil water stress and imposed limitations on agricultural production. Plant growth-promoting bacteria (PGPBs) have been an efficient strategy to improve the biological supply and growth of plants under distinct abiotic stress conditions. We hypothesized that the soils from a temporary pond may harbor PGPBs with potential strains which increase maize tolerance to water deficit. We studied rhizosphere and bulk soil of *Mimosa bimucronata* in a temporary pond from semiarid Northeast Brazil to access strains with characteristics to promote plant growth and mitigate abiotic stress for maize crop. We isolated 355 bacterial isolates, from which 96 were selected based on the morphophysiological characterization to assess IAA production (42 % produced over 50 µg mL⁻¹ of IAA), calcium phosphate solubilization (with one isolate achieving medium IS), biofilm and exopolysaccharides production (66 % and 98 % of isolates, respectively). Based on these mechanisms, the 30 most promising bacterial isolates were selected to assess biological nitrogen fixation (74 % of the isolates showed nitrogenase activity greater than 20 C₂H₄.h⁻¹.mg⁻¹), ACC deaminase activity (80 % of isolates) and growth in medium with reduced water activity (8 % of isolates grew in medium with water activity (A_w) of 0.844). We sequenced the 16S rRNA gene from the seven most promising isolates in *in vitro* and *in vivo* assays, which were identified as *Staphylococcus edaphicus*, *Bacillus wiedmannii*, *Micrococcus yunnanensis*, *Streptomyces alboflavus*, *Streptomyces alboflavus*, *Bacillus wiedmannii* and *Bacillus cereus*. *In vivo*, eleven isolates and three bacterial consortia did not differ from the control with nutrient solution, for total leaf area and root dry mass of maize. *S. alboflavus* (BS43) had the best *in vivo* results, not differing from the control with nutrient solution. We highlight the unpublished potential of *Staphylococcus edaphicus* and *Streptomyces alboflavus* in promoting the growth of plants under water stress. In addition, it is the first report of bacteria isolated from a temporary pond in the Brazilian semiarid which promoting plant growth attributes and development.

1. Introduction

Environmental studies predict an increase in the frequency and intensity of drought in the next years due to the global climate changes (Lesk et al., 2016). Drought is one of the main abiotic stresses that has limited agricultural productivity on a large scale (Gornall et al., 2010;

Lesk et al., 2016). In the Brazilian semiarid, the uneven rainfall distribution and changes in the water regime (Montenegro and Ragab, 2012; Rao et al., 2016) reduce yield of crops such as maize (*Zea mays*), which is largely grown in the region as a subsistence crop. In addition, this crop is one of the main cereals in the worldwide market (Ranum et al., 2014). Therefore, the use of drought-tolerant microorganisms has

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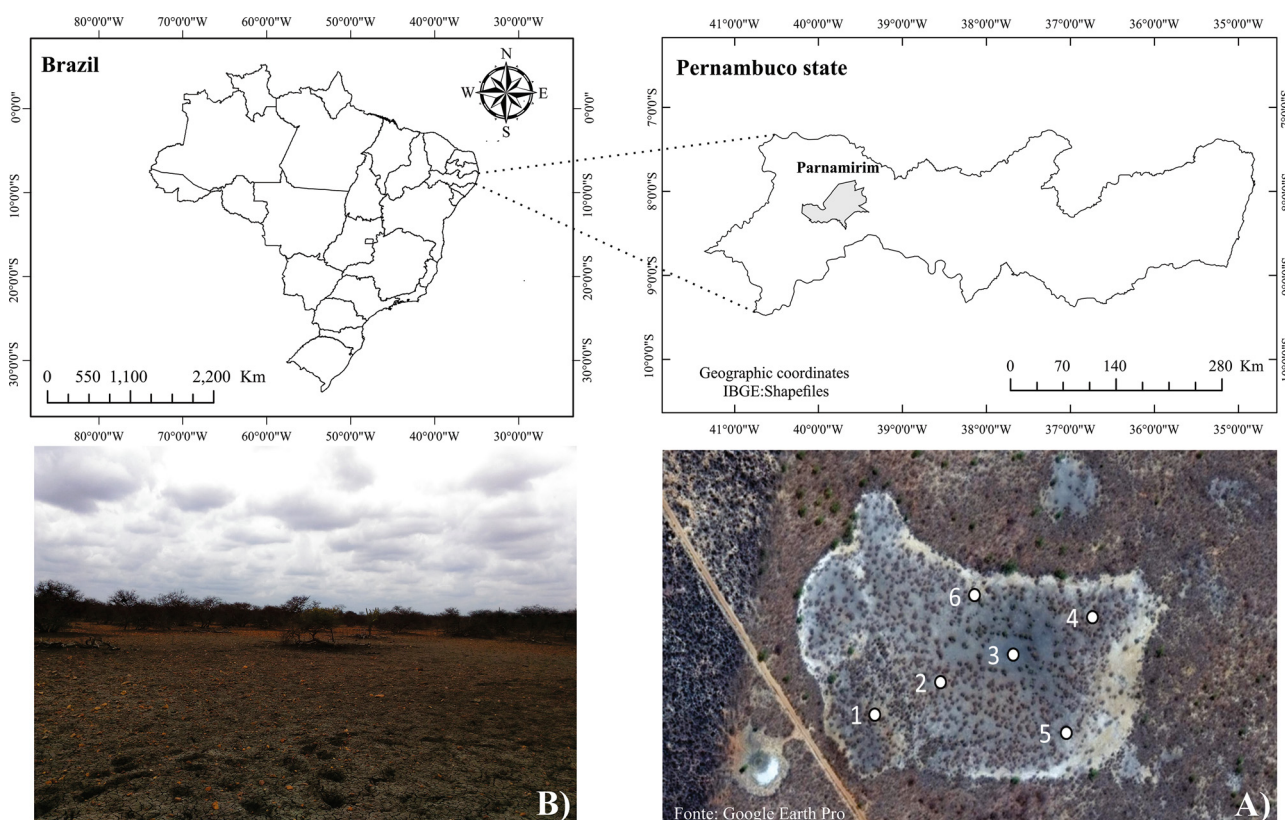


Fig. 1. Geographic location of the municipality of Parnamirim, Pernambuco, Brazil. (A) Distribution of the sampling points (B) in temporary pond.

been a promising strategy to improve the crops development in areas with low water availability (Shahzad et al., 2017). Thus, prospecting bacteria under water stress conditions is a viable option, due to the selection of microorganisms that favors plant adaptation (Bouskill et al., 2016; Cattivelli et al., 2008).

The Brazilian tropical semi-arid region is characterized by high temperatures, with large rainfall distribution temporal irregularities (average of 250–800 mm) and prolonged drought seasons, and comprise a deciduous forest named Caatinga. This is dominated by xerophytic vegetation, composed of a variety of botanical families, mainly represented by Cactaceae, Fabaceae and Euphorbiaceae (Albuquerque et al., 2012). Caatinga's soil and plant microbiome is also an excellent source of biodiversity and biotechnological potential (Kavamura et al., 2013a, 2013b, 2017; Taketani et al., 2017).

Temporary ponds are frequently found in the Northeastern Brazilian semi-arid, which are generally small natural wetlands, formed by flooding and drainage phases. They are considered ecosystems of vital importance to maintain biodiversity, even when they are totally dry during the dry season (Schwartz and Jenkins, 2000; Simões et al., 2008). A species widely distributed and adapted to temporary ponds is maricá (*Mimosa bimucronata* (DC)), a Fabaceae characterized by the ability to make symbiotic associations with arbuscular mycorrhizal fungi and nitrogen-fixing bacteria (Araújo et al., 2017; Stoffel et al., 2016).

Maricá in temporary ponds can harbor bacteria with biotechnological potential, such as the ability to tolerate water deficit, as well as promoting plant growth (Araújo et al., 2017). These plant growth-promoting bacteria (PGPBs), present several mechanisms, such as the production of indole-3-acetic acid (IAA), phosphate solubilization and biological nitrogen fixation (Cohen et al., 2015; Pathak et al., 2017; Shahi et al., 2011). Besides, these mechanisms can also induce drought tolerance by the systemic induced tolerance process, which involves several physiological and biochemical changes in the plant (Yang et al., 2009) like the activity of the enzyme ACC deaminase (Glick, 2014), the

formation of biofilm (Timmusk et al., 2014), the production of exopolysaccharides (Rolli et al., 2015) and the osmotic adjustment (Sarma and Saikia, 2014).

Studies on prospecting for PGPBs and the potential of these bacteria to promote plant growth and alleviate the symptoms of water deficit have been increasingly carried out. For example, a bioprospecting screening of bermudagrass (*Cynodon* spp.) obtained two strains of PGPBs identified as *Bacillus* sp. (12D6) and *Enterobacter* sp. (16i) which delayed the onset of plant drought symptoms when inoculated into the rhizospheres of wheat (*Triticum aestivum*) and maize (*Zea mays*) seedlings (Jochum et al., 2019). *Azotobacter* sp. strains obtained from a screening of rhizospheric soil samples from tropical semi-arid increase shoot dry weight, plant height, chlorophyll content, nitrogen, phosphorous and iron concentration in maize cultivated at 40 percent of field capacity (Shirinbayan et al., 2019). Inoculation of PGPBs in maize during the early growth stages under drought conditions significantly improved plant height, stem diameter, leaf chlorophyll, and root morphology (Lin et al., 2020).

We hypothesize that soils from a temporary pond from tropical semi-arid may harbor PGPBs with the potential to increase the tolerance of maize to water deficit. Our goal was to isolate and evaluate the biotechnological potential of *M. bimucronata* rhizosphere and bulk soil bacteria from a temporary pond in the tropical semi-arid region of Brazil and to evaluate their maize promoting potential under water deficit.

2. Materials and methods

2.1. Site description and sampling strategy

The study area was a temporary pond without anthropogenic disturbance located in Parnamirim, Pernambuco state, Brazil (8°17'05.2" S; 39°54'12.6" W). The local climate is tropical semi-arid (Köppen-Geiger climatic classification: BSh) and the site is inserted in the hinterland depression, with average annual precipitation of 431.8 mm and

hyperxerophilous Caatinga vegetation (Cruz, 2005). The soil samples were collected at six points in both rhizosphere (RS) and bulk soil (BS) of *M. bimucronata* (Fig. 1). Each point was sampled in triplicate at 0–0.2 m depth forming one composite sample per point, totaling 12 samples. The soil was classified as Vertisol (1, 2, 3, 5 and 6) and Gleysols (4) (Fig. 1) according to the criteria established by the Brazilian Soil Classification System (Santos et al., 2018) and WRB (Food and Agriculture Organization (FAO), 2014).

2.2. Bacterial isolates

The bacterial isolation was performed placing 10 g of soil into 90 mL of sterile saline solution (dilution 10^{-1}). From this dilution, a ten-fold serial dilution was performed (10^{-2} to 10^{-5}) and each dilution was then inoculated in King B culture medium (King et al., 1954) at 30 °C for 72 h. The isolates were selected based on their morphological characteristics and streaked until pure colonies were obtained. The same procedure was carried out for the isolation of endospore-forming bacteria. However, for the latter procedure, serial dilutions were carried out in a water bath at 80 °C for 20 min (Bettiol, 1995). All colonies were stored in a freezer at -20 °C, using King B medium, added with 25 % glycerin, in triplicate.

The isolates were grouped based on their phenotypical characteristics (growth time, size, shape, transparency and colony color, presence, amount and type of mucus and colony elevation), at 70 % similarity using the UPGMA method (Unweighted Pair Group Method with Arithmetic Mean), based on the Jaccard coefficient (Hammer et al., 2001), and random representatives of each formed group were selected.

The selected isolates were subjected to Gram determination (Buck, 1982) and characterized in terms of growth promotion mechanisms *in vitro* and *in vivo*. Finally, based on the best results presented from these characterizations, the isolates were selected for molecular analysis.

2.3. Characterization of growth promotion mechanisms *in vitro*

The quantitative assessment of IAA production followed Brick et al. (1991) as modified by Kuss et al. (2007). Briefly, the isolates were grown in triplicate in Tryptone Soya Broth (TSB) medium supplemented with L-tryptophan (5 mM) in the dark for 24 h at 30 °C, and then centrifuged (12,000 rpm, 5 min) to obtain the supernatant. The production of IAA was determined with Salkowski's reagent, using 1:1 ratio of supernatant followed by incubation in the dark for 30 min and evaluated in a spectrophotometer at 520 nm. The values were converted to equivalent concentrations of IAA ($\mu\text{g per mL}$) using a calibration curve.

Bibasic calcium phosphate solubilization was evaluated according to Verma et al. (2001), and aluminum phosphate solubilization, according to Hara and Oliveira (2004). The isolates were inoculated in triplicate, in solid culture medium and incubated at 28 °C for 72 h. The formation of the translucent halo around the colony indicated the phosphate solubilization. The halo was measured and the solubilization index (SI) was calculated according to the following equation: $SI = \text{Diameter}_{\text{halo}} / \text{Diameter}_{\text{colony}}$.

Biological nitrogen fixation (BNF) was determined by the acetylene reduction assay, according to Boddey et al. (1990). The isolates were grown in 100 % TSB (Tryptic Soy Broth) liquid medium in triplicate for 24 h at 28 °C, centrifuged (12,000 rpm) and washed in 0.85 % saline solution three times. Finally, they were resuspended in 1 mL of sterile H_2O and 100 μL of the suspension was inoculated in 5 mL of JNFB medium (Döbereiner et al., 1995) and incubated for 48 h at 30 °C. The tubes were sealed and 1 mL of acetylene was injected, resulting in a 20 % incubation atmosphere, for 1 h at 30 °C. A volume of 1 mL was injected in a Thermo Scientific gas chromatograph, equipped with a flame ionization detector (250 °C) and a N Poropak column (120 °C; Supelco, Bellefonte, Pennsylvania, USA). Negative controls were similarly done

in not incubated JNFB tubes.

2.4. Characterization of drought tolerance mechanisms *in vitro*

The evaluation of biofilm formation followed by O'Toole and Kolter (1998). 100 μL of the bacterial culture were inoculated in 900 μL of TSB culture medium (10 %), and incubated for 96 h at 40 °C. Subsequently, three washes were performed with sterile distilled water (1 mL) and, after drying in air, 1 mL of 0.1 % crystal violet (CV) was added for 15 min and a triple wash was performed again to remove excess dye. Quantification was performed by adding 1 mL of ethanol (95 %) to solubilize the CV-dyed tube and the absorbance (560 nm) was determined in a spectrophotometer.

Exopolysaccharides (EPS) were qualitatively determined according to Paulo et al. (2012). The isolates were grown in TSB medium (10 %) modified by the addition of 10 % sucrose and pH 7.5 (Kavamura et al., 2013a), and inoculated (5 μL) onto 5-mm diameter paper discs disposed in a culture medium for 72 h at 28 °C. EPS production was characterized visually by measuring the EPS halo produced and confirmed using the platinum loop impregnated with the colony in 2 mL of ethyl alcohol. The positive test occurred through the precipitation of the colony and the negative, when the ethyl alcohol presented a cloudy character.

1-aminocyclopropane-1-carboxylate (ACC) deaminase was determined by growing the isolates in a culture medium containing ACC as the only source of nitrogen (Penrose and Glick, 2003). The isolates were cultured in TSB 10 % liquid medium in triplicate for 2 days, centrifuged (12,000 rpm) and washed in 0.85 % saline solution three times and, finally, resuspended in 1 mL of autoclaved H_2O 20 μL of this suspension was transferred to the M9 mineral culture medium with and without the addition of 3 mM ACC as the only source of nitrogen. The isolates were incubated at 28 °C for 10 days and those that showed more accentuated growth in the M9 + ACC medium, compared to the ACC free medium were considered as producers of ACC deaminase.

The ability of the isolates to grow in a medium with reduced water activity was determined by adding sorbitol to the King B culture medium as an osmotic stress simulator. The isolates were grown at 30 °C for 96 h, in five sorbitol concentrations (0 g L^{-1} , 85 g L^{-1} , 285 g L^{-1} , 520 g L^{-1} , and 660 g L^{-1}), producing activity of water (A_w) values corresponding to 0.998; 0.986; 0.957; 0.897 and 0.844 of the non-modified culture medium (Hallsworth et al., 1998).

2.5. Growth promotion of *Zea mays* L. under water stress

The experiment was carried out in greenhouse in completely randomized design, with three replications and 37 treatments: thirty isolates, five bacterial consortia (BC), two controls without inoculation, one being conducted with a complete nutrient solution from Hoagland and Arnon (1950) (CH) and another control with 80 % nitrogen restriction (NRC) of this nutrient solution. The bacterial consortia were formed from the isolates that showed the highest performance on *in vitro* evaluations (Table 1).

Z. mays L. cultivar AG 1051 seeds were sterilized superficially and inoculated using 1 mL of the inoculum per seed, and reinoculated 14 days after planting. Both inoculations were done with the isolates grown in liquid medium King B for 24 h (10^8 UFC. mL^{-1} (DO550 = 0.1)). The seeds were germinated in sterile substrate of sand and vermiculite (2:1) in 2-liter pots. After 20 days of planting, water stress was established for 30 days with soil moisture, on a weight basis, corresponding at 30 % (0.078 g.g^{-1}) of field capacity. The pots were weighed daily to evaluate the weight variation of each pot, and the water lost by evapotranspiration was replenished by irrigation to maintain the pre-established soil moisture (Dourado et al., 2019). The evaluation was performed 50 days after planting. The total leaf area (TLA), shoot dry mass (SDM) and root dry mass (RDM) both obtained after drying in a forced air oven at 60 °C for 72 h were determined. The TLA was obtained by the expression $TLA = 0.75 \times L \times W$, where L and W represent

Table 1

In vitro features of the consortia of plant growth-promoting bacteria isolated from rhizosphere and bulk soil of *Mimosa bimucronata* from a temporary pond in the Semiarid of Brazil, selected for *Zea mays* L. growth promotion under water stress.

Consortia	Isolates	Mechanisms
BC1	ERS16-2; ERS18; BS43-1; RS59-6; EBS4	ARA, ACC, IAA, P-Ca, EPS, A _w
BC2	ERS16-2; ERS18; BS43-1; EBS4	ARA, ACC, IAA, P-Ca, A _w
BC3	ERS16-2; ERS18; EBS4	ARA, ACC, IAA, A _w
BC4	ERS16-2; EBS4	ARA, ACC, A _w
BC5	ERS16-2; RS59-6; RS85; EBS4	ARA, ACC, EPS, BIO, A _w

ARA: acetylene reduction assay (nmol C₂H₄ h⁻¹); ACC: 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase; IAA: Indole-3 acetic acid productions (μg mL⁻¹); IS P-Ca: Calcium phosphate solubilization index; BIO: biofilm production (DO_{560m}); EPS: exopolysaccharides production (mm); A_w: water activity.

total leaf length and width, respectively (Sangoi et al., 2007).

2.6. Identification of isolates by 16S rRNA gene sequencing

Bacterial genomic DNA was extracted by the bead beating method. Briefly, a small amount of the bacterial colony was suspended in 500 μL TE buffer (10 mM Tris HCl, mM EDTA, pH 8.0). Cell lysis was performed on 10 μL 10 % sodium dodecyl sulfate solution (SDS) and 0.1 g (2 beads) of glass. The mixture was stirred for 15 min with a vortex and then 500 μL of a phenol:chloroform:isoamyl alcohol (25:24:1 ratio) mixture was added and centrifuged at 12,000 rpm for 5 min. The supernatant was transferred to a new tube with 40 μL 5 M sodium acetate pH 5.0 and 400 μL ice cold isopropanol, and centrifuged at 12,000 rpm

at 5 min. The pellet was washed with 500 μL 70 % ethanol (–20 °C) and centrifuged at 12,000 rpm for 2 min. Ethanol was removed with a pipette. The pellet was dried at room temperature and eluted in 50 μL of sterile water and stored at –20 °C. The amount and quality of DNA from each sample was checked by 1.0 % agarose gel electrophoresis in 1X TAE buffer (400 mM Tris; 20 mM glacial acetic acid; 1 mM EDTA).

Universal bacterial primers 27 F (5'-AGAGTTTGACCTGGCTCAG-3') and 1492R (5'-GGTACCTGTTACGACTT-3') (Lane, 1991) were used for partial amplification of the 16S rRNA gene. The amplification reaction with a final volume of 50 μL used 2 μL DNA, 1.5 μL MgCl₂ (50 mM), 5 μL 10 X PCR Buffer, 1 μL dNTP's (10 mM), 2 μL of each primer (10 μM), 0.6 μL of Taq DNA Polymerase (5 U/μL) and ultrapure water to complete the volume. The amplification conditions were: initial denaturation of 94 °C for 5 min, 35 cycles of denaturation (94 °C for 40 s) annealing (55 °C for 40 s) and extension (72 °C for 1.5 min) and a final extension of 72 °C, for 7 min. Amplified products were evaluated on 1 % agarose gel and visualized in a transilluminator.

PCR products were sent to Macrogen Laboratory (South Korea) for purification and sequencing and the sequences obtained were compared to the NCBI database. In order to perform the Clustal W alignment, similar sequences were obtained from GenBank (NCBI). Aligned sequences were used for phylogenetic analysis by the Neighbor-Joining method, using Kimura-2 parameters by the MEGA 6 program, applying a bootstrap with a minimum of 1,000 replications, as described by Martins et al. (2015).

3. Data analysis

The parameters evaluated *in vitro* were submitted to Skott-knott test ($p < 0.05$). The data for the *in vivo* experiment were subjected to the homogeneity test and subsequently subjected to analysis of variance

Table 2

In vitro characterization of plant growth promotion mechanisms of bacteria isolated from rhizosphere and bulk soil of *Mimosa bimucronata* from a temporary pond of Brazilian semiarid region

Isolates	Gr	IAA	P-Ca	BIO	EPS	ARA	ACC	A _w
		μg. mL ⁻¹		DO _{560m}	mm	nmol C ₂ H ₄ h ⁻¹ mg ⁻¹		
EBS4	+	1.61 ± 0.72 h	1.95 ± 0.23 c	0.10 ± 0.02 c	9.8 ± 0.29 d	19.38 ± 13.59 b	-	0.844
ERS13-3	+	8.72 ± 1.79 h	1.98 ± 0.07 b	0.18 ± 0.06 c	25.0 ± 2.65 b	68.95 ± 60.88 a	+	0.957
ERS13-9	+	11.23 ± 3.56 h	x	0.87 ± 0.19 a	23.0 ± 1.04 b	x	-	0.897
ERS16-2	+	9.55 ± 2.94 h	2.01 ± 0.10 b	0.05 ± 0.01 c	14.3 ± 0.58 c	96.62 ± 4.64 a	+	0.957
ERS18	+	117.81 ± 4.30 a	x	0.09 ± 0.06 c	8.7 ± 0.58 d	x	-	0.957
BS24	-	1.97 ± 0.87 h	1.74 ± 0.06 c	0.07 ± 0.03 c	20.7 ± 4.04 b	13.81 ± 16.36 b	+	0.957
BS24-1	+	115.91 ± 16.12 a	x	0.27 ± 0.06 b	12.7 ± 4.73 c	32.98 ± 21.15 b	+	0.957
BS28-7	+	114.67 ± 6.45 a	x	0.08 ± 0.00 c	8.0 ± 0.50 d	x	+	0.957
BS28-10	+	115.66 ± 2.09 a	x	0.12 ± 0.04 c	9.0 ± 1.00 d	60.29 ± 15.23 a	+	0.844
BS38	-	16.02 ± 1.00 h	x	0.33 ± 0.41 b	10.2 ± 0.29 d	30.44 ± 3.57 b	+	0.957
BS43	+	10.79 ± 0.25 h	1.77 ± 0.22 c	0.06 ± 0.01 c	8.3 ± 0.58 d	x	+	0.957
BS43-1	+	9.79 ± 0.57 h	2.23 ± 0.20 a	0.08 ± 0.06 c	10.7 ± 3.06	51.75 ± 3.10 a	+	0.957
BS46-1	-	52.74 ± 3.81 e	1.83 ± 0.18 c	0.18 ± 0.08 c	12.0 ± 0.29 c	60.69 ± 8.92 a	+	0.844
RS48	+	83.60 ± 1.24 d	1.10 ± 0.02 f	0.09 ± 0.04 c	13.7 ± 2.89 c	80.90 ± 25.31 a	-	0.957
RS52	+	35.79 ± 5.66 g	1.14 ± 0.00 f	0.20 ± 0.23 c	8.7 ± 0.58 d	x	+	0.957
RS54	-	49.82 ± 10.18 e	1.51 ± 0.15 d	0.16 ± 0.12 c	12.3 ± 1.15 c	61.45 ± 18.83 a	-	0.844
RS59	+	108.35 ± 5.50 b	x	0.17 ± 0.07 c	9.0 ± 0.12 d	x	+	0.897
RS59-3	+	116.18 ± 2.69 a	1.30 ± 0.13 e	0.34 ± 0.21 b	8.0 ± 1.00 d	80.54 ± 17.72 a	+	0.844
RS59-6	+	42.41 ± 6.42 f	x	0.30 ± 0.07 b	32.3 ± 4.51 a	x	+	0.957
RS64	-	108.53 ± 11.36 b	1.27 ± 0.11 e	0.16 ± 0.06 c	14.0 ± 5.29 c	29.00 ± 22.85 b	-	0.844
RS66-1	+	5.66 ± 1.36 h	1.12 ± 0.05 f	0.82 ± 0.03 a	17.0 ± 2.65 c	x	+	0.957
RS66-2	-	32.66 ± 3.78 g	x	0.09 ± 0.09 c	23.7 ± 9.07 b	8.28 ± 9.92 b	+	0.957
RS66-3	-	30.84 ± 2.02 g	1.16 ± 0.07 f	0.85 ± 0.08 a	12.3 ± 1.15 c	98.49 ± 40.48 a	+	0.957
RS67-1	+	42.08 ± 7.06 f	1.28 ± 0.11 e	0.15 ± 0.08 c	11.7 ± 0.58 d	41.98 ± 25.04 b	+	0.844
RS70	+	32.91 ± 6.47 g	1.17 ± 0.14 f	0.09 ± 0.03 c	10.0 ± 4.36 d	x	+	0.844
RS76-1	+	7.89 ± 1.03 h	1.81 ± 0.11 c	0.10 ± 0.06 c	x	x	+	0.957
RS79-1	+	108.17 ± 7.39 b	x	0.09 ± 0.05 c	11.3 ± 1.53 d	x	+	0.957
RS82	-	25.39 ± 2.99 g	1.42 ± 0.08 d	0.11 ± 0.02 c	17.3 ± 8.74 c	x	+	0.957
RS84	+	26.10 ± 1.67 g	1.21 ± 0.06 f	0.12 ± 0.00 c	10.7 ± 0.58 d	81.46 ± 0.85 a	+	0.897
RS85	+	97.27 ± 0.87 c	1.07 ± 0.05 f	0.96 ± 0.04 a	9.3 ± 0.58 d	x	+	0.957

GR: Gram assay: positive (+); Negative (-); Absente (x); IAA: Indole-3 acetic acid productions; IS P-Ca: Calcium phosphate solubilization index; BIO: biofilm production; EPS: exopolysaccharides production; ARA: acetylene reduction assay; ACC: 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase; A_w: water activity. Means followed by the same letter do not differ significantly at the 5% level by the Scott-Knott test.

(ANOVA), followed by the classification of means by Dunnett's test ($p < 0.05$). The parameters evaluated *in vitro* and *in vivo* were submitted to Spearman's correlation. Statistical analyzes were performed using the statistical software R Core Team (2019) version 3.6. The results were expressed as mean $\pm$ standard deviation.

4. Results

4.1. Bacterial isolation and screening

We obtained 355 bacterial isolates of which 131 isolates from rhizosphere soil (RS); 97 isolates from bulk soil (BS); 104 endospore-forming isolates of rhizosphere soil (ERS) and 23 endospore-forming isolates of bulk soil (EBS). The phenotypical grouping formed 42 groups of RS, 27 groups of BS, 12 groups of ERS and 6 groups of EBS at 70 % similarity, and 32 RS, 32 BS, 25 ERS and 7 EBS were selected, totaling 39 isolates from BS and 57 isolates from RS.

4.2. Characterization of growth promotion mechanisms *in vitro*

All 96 isolates evaluated synthesized IAA using tryptophan as a precursor ranging between 1.03 and 118 $\mu\text{g mL}^{-1}$, for isolates EBS5 and ERS18, respectively. About 44 % isolates (42) produced between 1–11 $\mu\text{g mL}^{-1}$ of IAA while 18 % of the isolates (17) produced IAA $> 50 \mu\text{g mL}^{-1}$ (Table 2).

No isolates were able to solubilize aluminum phosphate, while 42 % (40) were able to solubilize bibasic calcium phosphate, with IS ranging from 1.06 to 2.23, for the isolates ERS13 and BS43-1, respectively (Table 2).

66 % (63) of the isolates produced biofilm and 98 % (92) produced EPS, with the highest production were obtained by RS85 and RS59-6, respectively (Table 2). For biofilm, none of the isolates presented $\text{DO}_{560\text{nm}} > 1.0$, whereas 29 % (28) of the evaluated isolates presented $\text{DO}_{560\text{nm}}$ between $\text{DO}_{560\text{nm}}$ 0.2–1.0, while for EPS 38 % (36) produced a halo $< 10 \text{ mm}$, 36 % (35) produced a halo between 10–14 mm and 22 % (21) presented a halo formation $> 14 \text{ mm}$ (Table 2).

Based on the most promising isolates for IAA and EPS production, bibasic calcium phosphate solubilization, formation of biofilm and the production of EPS, 30 isolates were selected to determine BNF and the ability to tolerate water stress *in vitro*, based on the activity of the enzyme ACC deaminase, growth in a medium with reduced water activity and hence, determined the potential *in vivo* under water stress. Based on the best results presented in the *in vitro* and *in vivo* characterization, seven isolates were selected for molecular characterization.

During BNF evaluation, 19 isolates formed a film in semi-solid JNFB medium and nitrogenase activity varied between 8 and 98 $\text{nmol of C}_2\text{H}_4 \text{ h}^{-1}$ for isolates RS66-2 and ERS16-2, respectively (Table 2), and 10 % (2) had $\text{nmol concentrations of C}_2\text{H}_4 \text{ h}^{-1}$ lower than the control treatment, therefore no nitrogenase activity, while 74 % (14) of the isolates showed production $> 20 \text{ nmol of C}_2\text{H}_4 \text{ h}^{-1}$.

Positive activity of the ACC deaminase enzyme was found in 80 % (24) of the 30 isolates evaluated (Table 2), while all were able to grow in water activities up to 0.957. However, only 37 % (11) of the isolates grew in water activity of 0.897, and 27 % (8) in water activity of 0.844. The EBS4 isolate showed greater tolerance to osmotic stress (A_w 0.897 and 0.844) (Table 2).

4.3. Growth promotion of *Zea mays* L. under water stress

An overview of the experiment at 37 days after planting is presented in the Supplementary Fig. S1. Only isolate BS43 did not differ ($p > 0.05$) for all TLA, SDM and RDM evaluated when compared to CH control (Table 3 and Supplementary Fig. S2), while ten isolates (ERS13-9, BS24, BS28-10, BS38, RS59-6, RS64, RS66-2, RS67-1, RS79-1 and RS82) and three consortia (BC2, BC3 and BC4) showed no significant difference ($p > 0.05$), again when compared with the CH

Table 3

Inoculation effect of plant growth-promoting bacteria isolated from rhizosphere and bulk soil of *Mimosa bimucronata* from a temporary pond in the Semiarid of Brazil on the agronomic parameters evaluated in *Zea mays* L. under water stress.

Isolates	TLA (cm^2)	SDM (g)	RDM (g)
CH	885.70 $\pm$ 256.19	1.78 $\pm$ 0.33	1.33 $\pm$ 0.26
NRC	317.49* $\pm$ 73.06	0.46* $\pm$ 0.14	0.41* $\pm$ 0.02
EBS4	337.15* $\pm$ 74.61	0.50* $\pm$ 0.16	0.62 $\pm$ 0.19
ERS13-3	235.03* $\pm$ 96.34	0.45* $\pm$ 0.23	0.39* $\pm$ 0.32
ERS13-9	492.13 $\pm$ 281.31	0.79* $\pm$ 0.37	0.60 $\pm$ 0.41
ERS16-2	492.73 $\pm$ 148.38	0.79* $\pm$ 0.17	0.54* $\pm$ 0.30
ERS18	286.39* $\pm$ 87.32	0.49* $\pm$ 0.03	0.73 $\pm$ 0.41
BS24	492.08 $\pm$ 202.09	0.97* $\pm$ 0.10	0.77 $\pm$ 0.10
BS24-1	389.96* $\pm$ 80.90	0.51* $\pm$ 0.17	0.54* $\pm$ 0.19
BS28-7	430.85* $\pm$ 103.21	0.64* $\pm$ 0.22	0.88 $\pm$ 0.44
BS28-10	578.67 $\pm$ 88.95	0.88* $\pm$ 0.05	0.85 $\pm$ 0.11
BS38	557.98 $\pm$ 188.97	0.66* $\pm$ 0.23	0.70 $\pm$ 0.09
BS43	628.59 $\pm$ 346.39	1.36 $\pm$ 0.15	1.95 $\pm$ 0.29
BS43-1	413.55* $\pm$ 310.78	0.53* $\pm$ 0.10	0.43* $\pm$ 0.04
BS46-1	450.00* $\pm$ 162.23	0.84* $\pm$ 0.26	0.93 $\pm$ 0.05
RS48	362.19* $\pm$ 64.81	0.71* $\pm$ 0.15	0.78 $\pm$ 0.29
RS52	409.92* $\pm$ 61.47	0.82* $\pm$ 0.10	0.70 $\pm$ 0.37
RS54	270.05* $\pm$ 143.43	0.56* $\pm$ 0.34	0.66 $\pm$ 0.42
RS59	373.85* $\pm$ 175.59	0.72* $\pm$ 0.21	0.91 $\pm$ 0.46
RS59-3	287.87* $\pm$ 198.22	0.76* $\pm$ 0.52	0.79 $\pm$ 0.47
RS59-6	541.08 $\pm$ 129.71	0.77* $\pm$ 0.17	0.66 $\pm$ 0.11
RS64	479.66 $\pm$ 251.53	0.75* $\pm$ 0.43	0.72 $\pm$ 0.33
RS66-1	409.03* $\pm$ 83.02	0.57* $\pm$ 0.11	0.66 $\pm$ 0.21
RS66-2	490.80 $\pm$ 7.59	0.70* $\pm$ 0.18	0.74 $\pm$ 0.15
RS66-3	440.56* $\pm$ 212.84	0.97* $\pm$ 0.22	0.80 $\pm$ 0.50
RS67-1	581.00 $\pm$ 139.01	0.90* $\pm$ 0.20	0.93 $\pm$ 0.51
RS70	427.64* $\pm$ 94.66	0.64* $\pm$ 0.18	0.48* $\pm$ 0.24
RS76-1	260.20* $\pm$ 60.38	0.69* $\pm$ 0.38	0.69 $\pm$ 0.25
RS79-1	549.45 $\pm$ 192.72	0.67* $\pm$ 0.28	0.70 $\pm$ 0.16
RS82	542.79 $\pm$ 179.81	1.06* $\pm$ 0.23	0.69 $\pm$ 0.11
RS84	457.75* $\pm$ 139.49	1.02* $\pm$ 0.09	0.56 $\pm$ 0.49
RS85	418.40* $\pm$ 100.76	0.62* $\pm$ 0.28	0.66 $\pm$ 0.09
BC1	420.38* $\pm$ 62.29	0.88* $\pm$ 0.22	0.77 $\pm$ 0.21
BC2	553.25 $\pm$ 2.17	0.82* $\pm$ 0.34	0.99 $\pm$ 0.50
BC3	541.69 $\pm$ 257.01	0.66* $\pm$ 0.31	0.62 $\pm$ 0.39
BC4	584.09 $\pm$ 121.62	0.77* $\pm$ 0.10	0.75 $\pm$ 0.05
BC5	479.81 $\pm$ 30.50	0.60* $\pm$ 0.28	0.44* $\pm$ 0.25

CH: no-inoculation control with nutritive solution; NRC: no-inoculation control with nutrient solution restricted of nitrogen; TLA: total leaf area; SDM: shoot dry mass; RDM: root dry mass. BC: bacterial consortia. Significant values when compared to CH are found with * and when compared to NRC are found in bold according to Dunnett's test ($p < 0.05$).

control, for TLA and SDR (Supplementary Fig. S2). Using NRC as comparison parameter, it was possible to observe that only the CH differed ($p < 0.05$) for all evaluated parameters (TLA, SDM and RDM). However, BS43 stands out for having a difference ($p < 0.05$) in the SDM and RDM parameters (Table 3).

Among the isolates evaluated in promoting of *Z. mays* growth under water stress, the isolate BS43 stood out, being the same capable of producing IAA, solubilizing calcium phosphate, producing EPS, forming biofilm, synthesizing ACC and growing in medium with reduced water activity (0.957) (Table 2). These mechanisms may have contributed to the further development of the root system when compared to CH. Based on a combined comparison of SDM and RDM, isolate BS43 (3.31 g) obtained a total biomass higher than CH (3.11 g).

Table 4 shows the correlation analysis among IAA, P.Ca, EPS, BIO, ARA, ACC and W_a and TLA, SDM, RDM. IAA production had negative and significant correlations with calcium phosphate solubilization ($r = -0.56$, $p < 0.00007$), EPS production ($r = -0.30$, $p < 0.004$) and with water activity ($r = -0.22$, $p < 0.034$), while calcium phosphate solubilization showed a positive correlation with bacteria nitrogen fixation ($r = 0.36$, $p < 0.0005$) and negative correlation with biofilm-forming isolates ($r = -0.21$, $p < 0.043$).

The nitrogen fixing PGPBs showed a negative correlation with water activity ($r = -0.28$, $p < 0.007$). The ACC enzyme showed a positive

Table 4Spearman's correlation coefficients between all parameters *in vitro* (IAA, P.Ca, EPS, BIO, ARA, ACC and Wa) and *in vivo* (TLA, SDM, RDM).

	IAA	P.Ca	EPS	BIO	ARA	ACC	Wa	SDM	RDM	TLA
IAA	1	7.2E-05	0.004	ns	ns	ns	0.034	ns	ns	ns
P.Ca	-0.56	1	ns	0.043	0.0005	ns	ns	ns	ns	ns
EPS	-0.30	-	1	ns	ns	ns	ns	ns	ns	ns
BIO	-	-0.21	-	1	ns	ns	ns	ns	ns	ns
ARA	-	0.36	-	-	1	ns	0.007	ns	ns	ns
ACC	-	-	-	-	-	1	0.003	0.05	ns	0.027
A _w	-0.22	-	-	-	-0.28	0.31	1	ns	ns	ns
SDM	-	-	-	-	-	0.20	-	1	3E-09	2.7E-04
RDM	-	-	-	-	-	-	-	0.67	1	0.0027
TLA	-	-	-	-	-	0.23	-	0.54	0.31	1

ns: not significant; IAA: Indole-3 acetic acid; P-Ca: calcium phosphate solubilization index; EPS: exopolysaccharides production; BIO: biofilm formation; ARA: acetylene reduction assay; ACC: 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase; A_w: water activity; TLA: total leaf area; SDM: shoot dry mass; RDM: root dry mass.

correlation with water activity ($r = 0.31$, $p < 0.003$), with SDM ($r = 0.20$, $p < 0.05$) and with TLA ($r = 0.23$, $p < 0.027$). Among the *in vivo* parameters, a positive correlation was observed between TLA and SDM ($r = 0.54$, $p < 0.00027$) and with RDM ($r = 0.31$, $p < 0.0027$), in addition to a high positive correlation between SDM and RDM ($r = 0.67$, $p < 3E-09$).

5. Molecular characterization of isolates

Isolates EBS4, ERS16-2, ERS18, BS43-1, RS59-6 and RS85 were selected based on the *in vitro* characterization and BS43 was selected based on the *in vivo* results for 16S rRNA sequencing. Of these, three were *Bacillus* (ERS16-2 and RS59-6 – *B. wiedmannii*, and RS85 – *B. cereus*), two *Streptomyces* (BS43 and BS43-1 – *S. albobiflavus*), and one each *Staphylococcus* (EBS4 – *S. edaphicus*), and *Micrococcus* (ERS18 – *M. yunnanensis*) (Table 5).

The highest percentage of similarity was obtained for the isolate ERS16-2 (98.84 %) with the bacterium *Bacillus wiedmannii* (ACC: NR.152692.1). The other PGPBs showed a similarity percentage below 98.7 %, with emphasis on the isolate BS43-1, which presented the lowest similarity index (95.04 %) with the strain *Streptomyces albobiflavus*. The partial sequences of strains EBS4, ERS16-2, ERS18, BS43, BS43-1, RS59-6 and RS85 were submitted to GenBank under access number MT256060, MT256061, MT256062, MT256063, MT256064, MT256065 and MT256066, respectively.

Phylogenetic analysis of the seven strains revealed significant polymorphism among these sequences (Fig. 2). The phylogenetic tree generated revealed that strains ERS16-2 and RS59-6 are in the same clade and more closely related to the sequences of *Bacillus cereus* (NR.074540, NR.1157141 and NR.1155261), *Bacillus proteolyticus* (NR.157735), *Bacillus wiedmannii* (NR.152692), and *Bacillus tropicus* (NR.1577361) than the RS85 strain. Strains BS43 and BS43-1 are in the same clade and closely related to the sequence of *Streptomyces albobiflavus* (NR.044151 and NR.1125221). The strain EBS4 is more closely

Table 5Identification of strains obtained from the rhizosphere and bulk soil of *Mimosa bimacronata*, based on seven bacterial strains selected from the sequencing of the 16S rRNA

Isolates	BLAST similarity	Query Cover	Per. Ident	Access
EBS4	<i>Staphylococcus edaphicus</i>	97 %	96.92 %	NR.156818.1
ERS16-2	<i>Bacillus wiedmannii</i>	94 %	98.84 %	NR.152692.1
ERS18	<i>Micrococcus yunnanensis</i>	96 %	98.47 %	NR.116578.1
BS43	<i>Streptomyces albobiflavus</i>	90 %	97.27 %	NR.044151.1
BS43-1	<i>Streptomyces albobiflavus</i>	99 %	95.04 %	NR.044151.1
RS59-6	<i>Bacillus wiedmannii</i>	94 %	97.27 %	NR.152692.1
RS85	<i>Bacillus cereus</i>	96 %	96.81 %	NR.074540.1

EBS: endospore-forming isolates of bulk soil; ERS: endospore-forming isolates of rhizosphere soil; BS: isolates from bulk soil; RS: isolates from rhizosphere soil

related to the sequences of *Staphylococcus edaphicus* (NR.156818) and *Staphylococcus saprophyticus* (NR.1156071), while strain ERS18 to the *Micrococcus yunnanensis* (NR.116578) and *Micrococcus aloeverae* (NR.1340881).

6. Discussion

We reported the use of beneficial bacteria to improve the health and growth of plants under water stress. This approach is recognized as one of the main strategies to help plant tolerance to drought and sustainably feed the growing global population (Tiwari et al., 2018). It is difficult, though, to obtain PGPBs tolerant to water stress due to a multitude of factors such as the host plant, mechanisms for promoting bacterial growth, adaptability and functioning in the environment (Kumar et al., 2014; Zachow et al., 2013).

A possible strategy for obtaining bacteria tolerant to water stress is the use of naturally dry environments for screening (Gowtham et al., 2020; Jochum et al., 2019; Ullah et al., 2019), due to the selection of adaptation mechanisms that guarantee their survival (Cattivelli et al., 2008). For this reason, we opted to extend our research for PGPBs resistant to water stress, to unexplored dry environments such as the temporary pond of the Brazilian tropical semiarid region. The advantages of using PGPBs obtained in naturally dry environments to increase plant biomass under water stress have already been reported for several crops (Kasim et al., 2013; Mishra et al., 2017; Paul and Lade, 2014; Vejan et al., 2016).

Phylogenetic analysis of the 16S rRNA gene sequence revealed that they belonged to four genera: *Bacillus*, *Streptomyces*, *Staphylococcus* and *Micrococcus*, with Firmicutes being the most represented phylum (EBS4, ERS16-2, RS59-6 and RS85) followed by the phylum Actinobacteria (ERS18, BS43 and BS43-1) (Table 5). The predominance of the phylum Firmicutes in the Brazilian semiarid region was also reported by Kavamura et al. (2013b). The presence of these phyla in dry environments is a widely observed phenomenon (Chodak et al., 2015; Fuchslueger et al., 2016; Hartmann et al., 2017), due to the ability to use recalcitrant carbon sources, solubilize nutrients (Hartmann et al., 2017; Mohammadipanah and Wink, 2016), form spores, and thicker peptidoglycan layer, which can increase water stress resistance (Schimel et al., 2007). Kavamura et al. (2013a) reported the predominance of bacteria belonging to the phylum Firmicutes showing mechanisms to promote growth and tolerance to water stress in the Brazilian semiarid region.

The isolates showed different performances *in vitro* (Table 2). We observed a high variability in the expression of mechanisms by isolates of different genera and isolates of the same genus, as observed mainly among those belonging to the genus *Bacillus*. According to Gaiero et al. (2013) the significant expression of one mechanism does not always coincide with the significant expression of other mechanisms between species and bacterial strains.

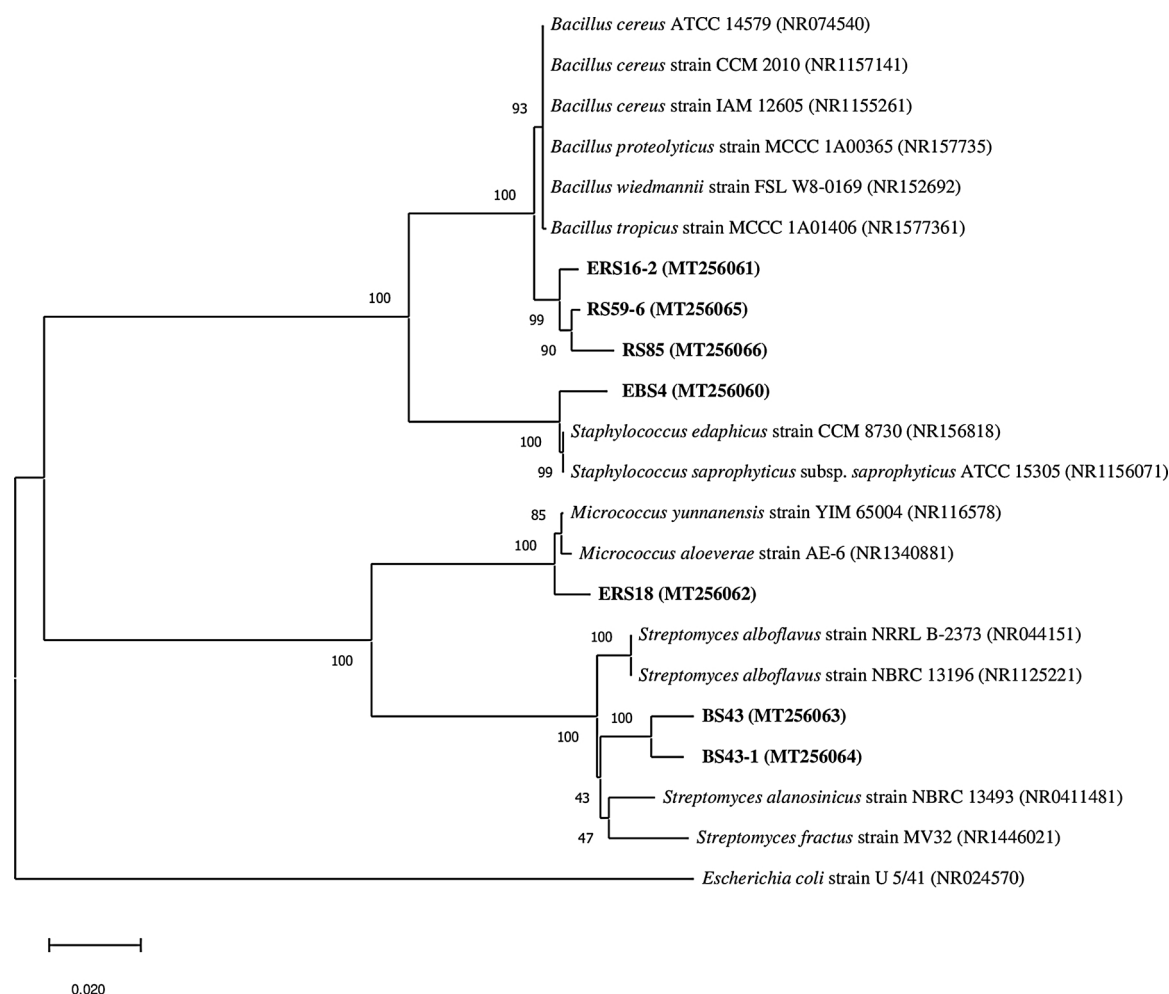


Fig. 2. Phylogenetic relationship based on the partial sequence of the 16S rRNA gene of seven bacterial strains promoting growth *Zea mays*, with the strains obtained from the alignment of the sequences with the NCBI database. The scale bar at the bottom indicates the number of differences in the composition of the base among the sequences. The accession numbers for the 16S rRNA sequences are in parentheses.

Regarding growth promotion mechanisms, the most promising *in vitro* isolates (ERS18, BS43-1 and ERS16-2) represented three genera: *Micrococcus* (IAA), *Streptomyces* (P-Ca) and *Bacillus* (BNF), respectively. The production of IAA by *Micrococcus yunnanensis* has also been reported by Sukweenadhi et al. (2015), the potential for solubilization of calcium phosphate by *Streptomyces* spp by Wahyudi et al. (2019) and the potential of BNF by *Bacillus* spp by Govindasamy et al. (2010). The importance of these mechanisms in promoting plant growth is well known (Kim et al., 2012; Niu et al., 2018; Wang et al., 2014). Some studies report the direct contribution of IAA production and indirect contribution of phosphate solubilization and BNF, tolerance to water stress by improving plant health (Forni et al., 2017; Kumar et al., 2016; Nakbanpote et al., 2014; Sukweenadhi et al., 2015; Wang et al., 2014).

About the mechanisms that can induce drought tolerance, the most promising isolates *in vitro* (EBS4, RS59-6, RS85) represented two genera: *Staphylococcus* (Wa) and *Bacillus* (EPS, BIO, ACC), respectively. The 16S rRNA sequence of the EBS4 isolate showed 96.92 % similarity to *Staphylococcus edaphicus*, recently described as a new species, which may harbor genes for resistance to environmental stresses (Pantůček et al., 2018). Our work is the first report on the potential for promoting plant growth by *Staphylococcus edaphicus*, where we highlight the high resistance to water stress. The production of EPS is responsible for forming and spreading biofilms (Kumar et al., 2016), which may explain the high biofilm formation by our isolates (RS59-6 and RS85). These mechanisms act by forming a hydrated microenvironment around the root, which retains water for a longer time (Rolli et al.,

2015; Sandhya et al., 2009), increasing resistance to stress water (Naseem and Bano, 2014; Vardharajula et al., 2011).

Although PGPBs use several mechanisms to promote plant growth under water stress, possession of the enzyme ACC deaminase is essential (Glick, 2014). In our study, 80 % of the strains evaluated showed activity of the enzyme ACC deaminase, with the sequenced isolates representatives of the genera *Bacillus* and *Streptomyces*. According to Niu et al. (2018), stressful habitats for host plants may favor the selection of isolates with ACC deaminase activity. However, this scenario contributes to high concentrations of ethylene, resulting in growth inhibition or even death of the plant. The enzyme ACC deaminase is responsible for cleaving the plant's ethylene precursor, ACC, into α -ketobutyrate and ammonia, resulting in longer roots and sprouts (Glick, 2014).

However, mechanisms that favor tolerance to water stress *in vitro* are not expressed *in vivo*, necessarily (Chauhan and Nautiyal, 2010; Podile et al., 2013). Thus, the assessment of PGPBs in promoting plant growth in controlled environments is primordial (Rana et al., 2011). The contribution of PGPBs to the growth of *Z. mays* under water stress has been verified by several studies (Kaushal and Wani, 2016; Niu et al., 2018). Among our treatments, only BS43 (*Streptomyces alboflavus*) significantly increased SDM and RDM when compared to NRC (Table 3). Our study showed that the increase in SDM may be correlated with the activity of the enzyme ACC deaminase (Table 4), as seen by Naveed et al. (2014) and Etesami et al. (2015).

Only the biocontrol potential of *Streptomyces alboflavus* has been

described as mechanisms for promoting plant growth (Wang et al. 2013a, b). Here, we found that *S. albobiflavus* was able to produce IAA, solubilizing calcium phosphate, producing EPS, forming biofilm, synthesizing ACC deaminase and growing in medium with reduced water activity (0.957) (Table 2), but these mechanisms are clearly not correlated to better development of *Z. mays* under water stress. Other isolates also contributed to the development of *Z. mays* under water stress (Table 3), such as RS59-6 (*Bacillus wiedmanni*), which showed no difference ($p < 0.05$) with CH, for the agronomic parameters TLA and RDM. The potential of *Bacillus* spp. obtained from the Brazilian semiarid in promoting the growth of *Z. mays* under water stress was also verified by Kavamura et al. (2013a).

Overall, this study revealed that PGPBs obtained from rhizosphere and bulk soil of *Mimosa bimucronata* from a temporary pond in tropical semiarid contribute to the adaptation and drought tolerance of *Z. mays*. This demonstrates that dry natural environments are promising for prospecting for drought-tolerant bacteria with the potential to promote plant growth.

7. Conclusions

Our study is the first report that demonstrates that soils of a temporary pond in the tropical semiarid region of Brazil harbor several bacteria that exhibit high tolerance to water stress and have characteristics that promote plant growth under water stress. In addition, this study indicates that *Streptomyces albobiflavus* and *Staphylococcus edaphicus* have mechanisms to promote plant growth and tolerance to water stress.

We demonstrated that *Streptomyces albobiflavus* has multiple mechanisms for promoting plant growth, which establish a greater resistance of *Zea mays* to water stress.

The potential of the most promising PGPBs obtained in our experiment is being evaluated in non-sterile soil and will be evaluated in field conditions. The aim of this study is the indication of inoculants and the development of microbial consortia that improve plant growth in areas affected by water deficiency.

CRediT authorship contribution statement

Victor Lucas Vieira Prudêncio de Araújo: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Mario Andrade Lira Junior:** Conceptualization, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Funding acquisition. **Valdomiro Severino de Souza Júnior:** Conceptualization, Resources, Writing - original draft. **José Coelho de Araújo Filho:** Conceptualization, Resources, Writing - original draft. **Felipe José Cury Fracetto:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft. **Fernando Dini Andreote:** Methodology, Resources, Writing - original draft. **Arthur Prudêncio de Araujo Pereira:** Methodology, Formal analysis, Writing - original draft. **José Petrônio Mendes Júnior:** Methodology, Formal analysis, Writing - original draft. **Felipe Martins do Rêgo Barros:** Methodology, Formal analysis, Writing - original draft. **Giselle Gomes Monteiro Fracetto:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Visualization, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.micres.2020.126564>.

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