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Screening of Brazilian cacti rhizobacteria for plant growth promotion under drought

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ABSTRACT

Drought is one of the major problems worldwide. The search for new and efficient microorganisms, from unexplored environments, to be used in association with plants to alleviate the negative effects imposed by water stress, is an interesting alternative. Thus, cacti-associated bacteria from the Brazilian semi-arid region were isolated based on their ability to grow in medium with reduced water availability. Strains were tested for the production of exopolysaccharides (EPS), as well as *in vitro* plant growth promotion traits. A great proportion of the isolates belong to the genus *Bacillus*. From a total of forty-eight bacteria, 65% were able to grow in medium with reduced water availability (0.919A_w), exopolysaccharide production was observed for 65% of the strains. The production of indole acetic acid (IAA) exceeding 51 μg mL^{-1} was observed for 4% and the high solubilization of Ca–P was verified for 6% of the isolates. No strain was able to produce hydrogen cyanide (HCN), 71% produced ammonia and 79% showed a halo of carboxymethyl cellulose (CMC) degradation. *Zea mays* L. growth promotion under water stress (30% of field capacity) was achieved by two strains of *Bacillus* spp. This is the first report to describe cacti-associated bacteria from Brazilian semi-arid with plant growth-promoting abilities.

Introduction

Water is one of the most limiting factors for plant development, as well as for all life forms. Drought is a natural and complex phenomenon that affects several parts of the world, causing social, economic and environmental negative impacts (Macedo et al. 2010). Agricultural losses due to drought are quite substantial. One alternative for growing plants under dry conditions is the use of xerotolerant microorganisms associated to crops. These microorganisms can be found in environments where they are constantly submitted to water stress, as the scenario found in the Brazilian northeast. It has a semi-arid climate, which is considered to have an aridity index of 0.2–0.5 and rainfall ranging from 200 to 800 mm (*United Nations Environmental Programme*, UNEP 2007). There are two well defined seasons: rainy and dry, with the predominance of a dry and hot weather. It comprehends a unique biome called Caatinga, a poorly explored environment so far. This biome is characterized by typical vegetation with the presence of a relatively low tree layer (up to 5 m high), with thin spiny trees and shrubs, deciduous in the dry season (Queiroz 2006). The vegetation is also characterized by the presence of plants belonging to the Cactaceae family, whose members remain green even during the summer. These cacti had been modified throughout evolution, with some unusual evolutionary features such as changes in vegetative structures (loss or reduction of leaves); the cortex and the medulla are transformed into a tissue suitable for water storage; side branches are transformed into clusters of spines (Anderson 2001). One cactus widely distributed in the Caatinga is *Cereus jamacaru*, known as "mandacaru" (Fig. 1A). It has an arboreal appearance, with a cylindrical trunk with four to six ribs and innumerous spiny branches. Two other cacti, *Melocactus* sp. (Fig. 1B), known as "cabeça-de-frade" and *Pilosocereus gounellei* (Fig. 1C), known as "xique-xique" also have a wide distribution. The first possesses a cone-shaped shoot and can reach up to 22 cm high. It has ten edges with areolas of spines arranged in groups of five to seven, the flowers are red and the fruit is pink (Barbosa 1998). The second one is usually found in rocky and stony soils, more specifically in "Caatinga de lajedo" (Fig. 1D) (Taylor and Zappi 2004). It is a species with a columnar shape, forming a set of branches like a chandelier. Its height can vary from short stature, up to small tress with 3–4 m high (Gorelick 2009).

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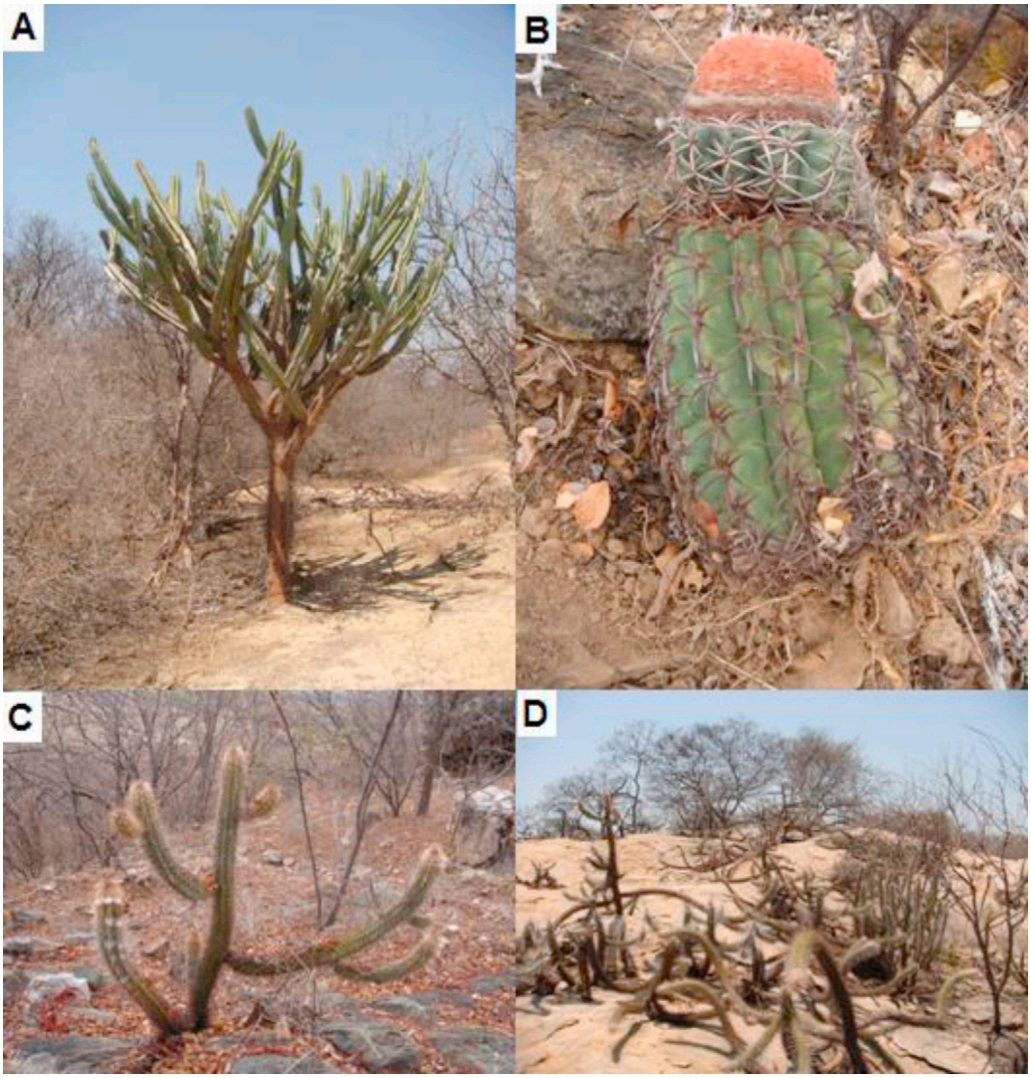

Fig. 1. Cacti found in the Caatinga biome of the Brazilian semi-arid region. A – *Cereus jamacaru*; B – *Melocactus* sp.; C – *Pilosocereus gounellei*; D – *P. gounellei* commonly found in rocky and stony soils ("Caatinga de lajedo").

Due to the high biodiversity potential, this biome possibly harbors novel microorganisms with interesting and desirable characteristics like the ability to tolerate some environmental stressful conditions, *e.g.* drought, as well as to promote plant growth. Microorganisms can survive under drought conditions through several mechanisms such as the production of exopolysaccharides (EPS) (Nocker et al. 2012), biofilm formation (Chang et al. 2007) and osmolytes production in order to avoid cell water loss (McNeil et al. 1999). Besides, microorganisms can also offer plant protection against desiccation through the maintenance of a moist environment and conducive to root development, supply of nutrients, hormones, also acting as plant growth-promoters.

The mechanisms by which plant growth-promoting rhizobacteria (PGPR) act, can be whether direct or indirect (Saraf et al. 2011). Phytohormone production like indole acetic acid (IAA), gibberellin, cytokinin and ethylene; phosphate solubilization; nitrogen fixation and siderophore production are all considered direct mechanisms. The main auxin produced by plants is IAA that can also be produced by soil inhabiting bacteria. It stimulates root proliferation that increases nutrient uptake, thus promoting plant growth (Lambrecht et al. 2000). Phosphorus is a highly distributed nutrient in soils; however, it can bind easily to other elements, like Fe, Al and Ca, depending on soil pH, being inaccessible for plant absorption (Lindsay et al. 1989). Phosphate solubilization can be performed by some fungi and bacteria through the production of organic acids that dissolve phosphate by anion exchange or binding to Fe or Al, turning phosphorus available (Omar 1998). Among the indirect mechanisms, it is possible to consider antibiosis by volatile production, such as hydrogen cyanide (HCN) and ammonia; competition; parasitism, with the production of enzymes such as chitinases, glucanases and cellulases (Kai et al. 2009; Minaxi et al. 2012; Sindhu and Dadarwal 2001).

Therefore, due to their multiple traits, the search for new PGPR becomes interesting, once they can be used as inoculants for biofertilization, phytostimulation and biocontrol purposes (Bloemberg and Lugtenberg 2001) in agriculture, forestry and environmental recovery (Lucy et al. 2004).

In this way, the aim of this work was to identify and characterize novel rhizobacteria from three Brazilian cacti, *C. jamacaru*, *P. gounellei* and *Melocactus* sp. able to grow at reduced water content, exhibiting plant growth-promotion traits for future application in agricultural improvements under drought conditions.

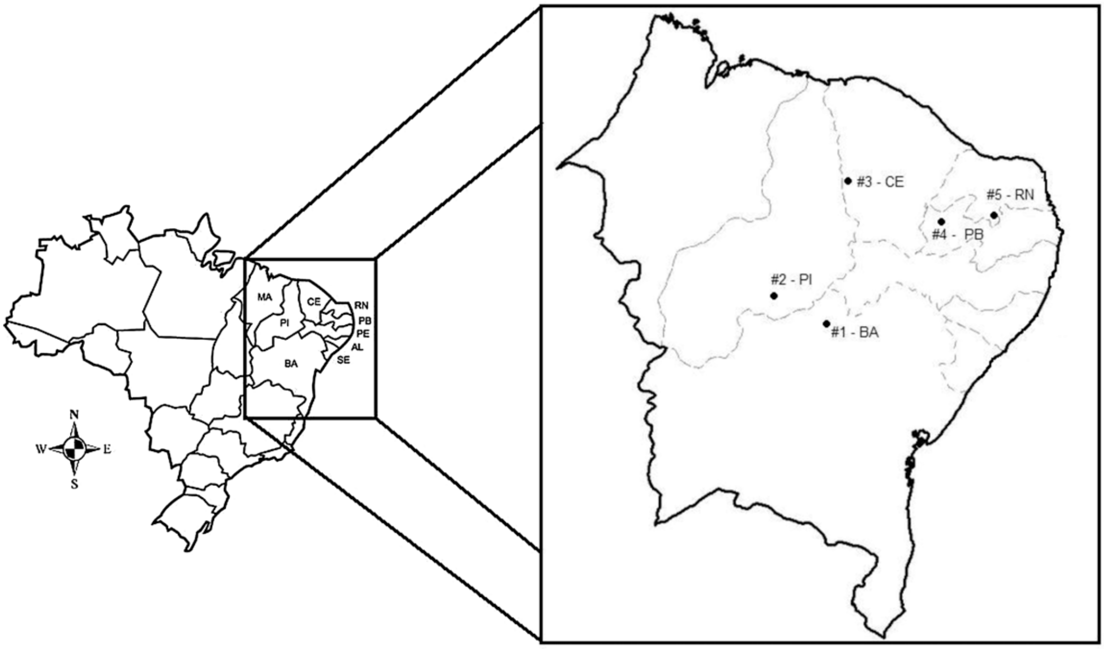

Fig. 2. Map of Brazil, showing in the detail the distribution of the sampling sites comprising five states (Bahia (BA), Piauí (PI), Ceará (CE), Paraíba (PB) and Rio Grande do Norte (RN)) of the Caatinga biome from the semi-arid region of Brazilian northeast.

Materials and methods

Bacterial isolation and identification

Sampling of soil and rhizosphere of *C. jamacaru*, *P. gounellei* and *Melocactus* sp., members of the Cactaceae family, was conducted in five different sites delimited along Caatinga biome from Brazilian semi-arid (5–10°S and 37–43°W), during the dry season. Each site corresponds to one state (Fig. 2).

The bacterial isolation was performed placing 1 g of rhizosphere soil into 10 mL of saline buffer (NaCl 0.8%; KCl 0.02%; Na_2HPO_4 0.14%; KH_2PO_4 0.024%) under aseptic conditions. The suspension obtained was serial diluted and then inoculated in Tryptone Soya Agar (TSA) medium (10%) at 28 °C. Mesophilic bacteria having different morphological characteristics were selected and stored at −80 °C for further analysis. Bacteria were selected based on their ability to grow in TSA medium (10%) with additional sorbitol in five different concentrations ($0\,g\,L^{-1}$; $85\,g\,L^{-1}$, $285\,g\,L^{-1}$, $520\,g\,L^{-1}$ and $660\,g\,L^{-1}$) to simulate a water stress at 40 °C. The higher the values of sorbitol added the less the activity of water (A_w) of the medium. Sorbitol was added based on Hallsworth et al. (1998). The added sorbitol produced values of A_w corresponding to 0.998, 0.986, 0.957, 0.897 and 0.844, respectively.

Bacteria were identified by Fatty Acid Methyl Ester (FAME) analysis and/or 16S rRNA sequencing. FAME analysis was conducted by MIDI-MIS method as proposed by Sasser (1990). The similarity index (SI) equal or higher than 0.5 and separated by a minimum value of 0.1 between the first and the second hit in the database (Kunitsky et al. 2006), were used to classify strains at species level; whereas lower similarity indexes were used for affiliation at higher taxonomic levels, such as genera or families. Genomic DNA was obtained from pure cultures, according to Sunnucks and Hales (1996). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal primers 1492R (5′-TAC GGY TAC CTT GTT ACG ACT-3′) and 27F (5′-GAG AGT TTG ATC CTG GCT CAG-3). Samples were purified using Exo-ap. Then, sequencing reaction was performed with three primers 1492R, 27F and qPCR (5′-CCT ACG GGA GGC AGC AG-3′) for each sample. Sequencing analysis was achieved using ABI 3500 Genetic Analyzer (Applied Biosystems). The quality of sequences was checked using FinchTV 1.4.0 program (Geospiza Inc.) and the three sequences of each sample were manually merged into one sequence using BioEdit 7.1.3.0 (Hall 1999). Sequences were analyzed using Ez Taxon, a web-based tool used for the identification of prokaryotes (Chun et al. 2007). The sequences were aligned using ClustalW (Higgins et al. 1994) in Mega 5.01 (Tamura et al. 2011) and the aligned sequences were used for the construction of dendrograms, using Neighbor-Joining method (Saitou and Nei 1987) according to the model of Jukes–Cantor (Jukes and Cantor 1969) with a bootstrap of 1000 replicates.

In vitro assay for drought tolerance

Bacterial growth under reduced water availability

Strains obtained from medium with 0.957A_w (the only that displayed bacterial growth), were grown in TSA medium (10%) with $405\,g\,L^{-1}$ of sorbitol, producing a lower A_w value, corresponding to 0.919A_w at 40 °C.

Exopolysaccharide production

The qualitative determination of exopolysaccharide production was performed according to Paulo et al. (2012). Each strain was inoculated onto 5-mm diameter paper discs disposed in a medium (2% yeast extract; 1.5% K_2HPO_4; 0.02% $MgSO_4$; 0.0015% $MnSO_4$; 0.0015% $FeSO_4$; 0.003% $CaCl_2$; 0.0015% NaCl; 1.5% agar) modified by the addition of 10% of saccharose, pH value of 7.5. The production was characterized by the size of the halo produced and its slime appearance. The production of EPS was confirmed by mixing a portion of the mucoid substance in 2 mL of absolute ethanol, where the formation of a precipitate indicated the presence of EPS (Paulo et al. 2012).

Screening of bacterial isolates for in vitro plant growth promotion traits

IAA production. The production of IAA was determined by the colorimetric methodology described by Gordon and Weber (1951) with some modifications. Aliquots of 100 μL of each bacterium at a concentration previously adjusted to 10^8 cells mL^{-1} (OD$_{550\,nm}$ = 0.1) were inoculated into 10 mL of Tryptone Soya Broth (TSB) medium (10%) amended with 5 mM L-tryptophan, in the dark. After a 48-h period of incubation at 28 °C, cells were harvested by centrifugation (10,000 rpm for 5 min) and 750 μL of the supernatant was treated with 750 μL of Salkowski reagent (50 mL of perchloric acid (35%) and 1 mL of $FeCl_3$ solution (0.5 M)) for 30 min, under light protection. Spectrophotometer was reset using negative control: 750 μL of medium without inoculum and 750 μL of Salkowski reagent. Optical density was taken at 530 nm with the use of spectrophotometer (UV-1601 PC, Shimadzu). Development of pink to red color indicates IAA production. Concentration of IAA produced by cultures was compared to a standard curve of commercial IAA (Hi-media).

Quantitative determination of phosphate solubilization

The detection of available phosphate was performed with a colorimetric method according to Nautiyal (1999) with some modifications. Test-tubes with 10 mL of NBRIP (National Botanical Research Institute's Phosphate) medium (1% glucose; 0.5% $Ca_3(PO_4)_2$; 0.5% $MgCl_2 \cdot 6H_2O$; 0.02% KCl; 0.025% $MgSO_4 \cdot 7H_2O$; 0.01% $(NH_4)_2SO_4$) were inoculated with 100 μL of 10^8 cells mL^{-1} (OD$_{550\,nm}$ = 0.1) of each isolate, in triplicate. After incubation at 180 rpm, 28 °C for 15 days, 1000 μL of each sample were transferred to microtubes of 1.5 mL capacity and centrifuged at 10,000 rpm for 5 min. Aliquots of 145 μL of supernatant of each sample were added to 570 μL of distilled water and 285 μL of ammonium molybdate–vanadate reagent (5% ammonium molybdate and 0.25% ammonium vanadate, 1:1 (v/v)) (Malavolta et al. 1989; Silva 1999). Spectrophotometer was reset using negative control with 145 μL of NBRIP medium without inoculum, 570 μL of distilled water and 285 μL of the ammonium molybdate–vanadate reagent. Optical density was taken at 420 nm (UV-1601 PC, Shimadzu) after 10 min of reagent addition. Standard curve was obtained using a stock solution of KH_2PO_4 (0.0875%) (0.1 mg P mL^{-1}). Results are given in absorbance, and P concentration in μg mL^{-1}.

NH_3 production

Ammonia production was detected according to Cappuccino and Sherman (1992). Freshly grown cultures were inoculated in test-tubes with 10 mL of peptone water and incubated for 48 h at 28 °C. After incubation, 1 mL of each culture was transferred to microtubes of 1.5 mL capacity, and 50 μL of Nessler's reagent (10% HgI_2; 7% KI; 50% aqueous solution of NaOH (32%)) were added in each microtube. The development of faint yellow color indicates a small amount of ammonia and deep yellow to brownish color indicates maximum production of ammonia (Dey et al. 2004).

HCN production

HCN detection was performed according to Bakker and Schippers (1987), where all isolates were streaked on TSA (10%) with additionally glycine (4.4 g L^{-1}). After 24 h of growth at 28 °C, Petri dishes were inverted and on each cover, an autoclaved filter paper soaked with picric acid (0.5%) and Na_2CO_3 (2%) solution was put onto it. Petri dishes were sealed and incubated at 28 °C for 48 h. The HCN production is indicated by changes in coloration from orange to red. A strain of *Pseudomonas* sp. was used as a positive control.

Cellulase production

The ability to degrade cellulose was performed in carboxymethyl cellulose (CMC) medium (0.2% $NaNO_3$; 0.1% K_2HPO_4; 0.05% $MgSO_4$; 0.05% KCl; 0.2% carboxymethyl cellulose salt; 0.02% peptone; 1.7% agar) where 5 μL of each isolate was inoculated in plates with the medium, in quadruplicate. After the incubation at 28 °C for 48 h, plates were stained with iodine (0.666% KI; 0.333% iodine) for 5 min (Kasana et al. 2008). Colony and halo diameters were measured and cellulolytic index (CI) was calculated based on the ratio of halo diameter and colony diameter (Teather and Wood 1982).

Plant growth promotion experiment

Zea mays seeds (DKB 455) were surface sterilized and inoculated with each strain by mixing for 2 h in one-day-old inoculum (10^8 UFC mL^{-1} (DO$_{550}$ = 0.1)). Control treatment was achieved by mixing the seeds with sterilized saline solution (0.85%). For pot experiment, soil was collected, air-dried, sieved (2-mm/10-mesh) and analyzed for physical–chemical characteristics. Soil was autoclaved for 1 h. The soil was clay, pH 4.1; 26 g dm^{-3} of organic matter (Table 1). The correction was performed with nutrient inputs of NPK at 0.11 × 0.16 × 0.043 g L^{-1}, respectively, and the pH was adjusted with 2.9 g L^{-1} of lime before filling the pots. Twenty inoculated seeds were sown in soil-filled pots (3 kg of soil per pot). Five seedlings were kept in each pot after germination. The pots were arranged randomly with five repeats at ambient light and temperature, in a greenhouse. After the germination, to simulate a water stress, plants were left to grow with 30% of water field capacity. Evaluation was performed after thirty days of sowing. The leaf area, stalk length and shoot dry biomass were determined.

Statistical analysis

Greenhouse experiments were conducted in a completely randomized design. Data were subjected to One-Way ANOVA followed by a classification of means with Dunnett's test in order to compare the treatments with the control (uninoculated plants). All statistical analysis was performed with Assistat 7.6 beta (Silva and Azevedo 2002).

Results

Isolation, selection and bacterial characterization

Bacterial growth was only visualized up to the concentration of 285 g L^{-1} of sorbitol (0.957A_w). Thus, forty-eight bacterial strains, isolated from this medium, were identified (Table 2). A great majority of them (73%) belongs to the Bacillaceae family, which comprehends endospore-forming bacteria. In the present work, strains characterized by FAME profile and/or 16S rRNA gene sequencing showed similarity to ten *Bacillus* species: *B. aryabhattai*, *B. atrophaeus*, *B. cereus*, *B. megaterium*, *B. methylotrophicus B. pumilus*, *B. safensis*, *B. solisalsi*, *B. subtilis* and *B. tequilensis*, and two other members from the same family: *Brevibacillus reuszeri* and *Virgibacillus pantothenticus* Strain LMA3 was characterized by 16S rRNA gene sequencing with 99.6% of similarity to *B. aryabhattai*, with five divergent nucleotides (nt) in a total of 1385.

Two other families, Paenibacillaceae and Enterobacteriaceae that correspond each one to 8.3%, are the second largest group, with *Paenibacillus cineris* and *P. lentimorbus*; *Enterobacter helveticus*, *Pantoea cypripedii* and *P. stewartii.* There is also a smaller frequency (4.2%) of Micrococcaceae family, represented by *Arthrobacter nicotianae* and *A. defluvii* and 2.1% (each) of members of the Gordoniaceae, Promicromonosporaceae and Nocardiaceae families, represented by *Gordonia amarae*, *Cellulosimicrobium cellulans* and *Nocardia* sp., respectively.

In vitro assay for drought tolerance

Bacterial growth under reduced water availability

Among the isolated strains, 65% were able to grow in medium with reduced water availability (0.919A_w). These isolates include:

Table 1
Characteristics of the soil used for the plant growth promotion experiment.

pH	O.M. ($g\,dm^{-3}$)	Ca ($mmol\,dm^{-3}$)	Mg ($mmol\,dm^{-3}$)	H + Al ($mmol\,dm^{-3}$)	S.B. ($mmol\,dm^{-3}$)	C.E.C. ($mmol\,dm^{-3}$)	K ($mmol\,dm^{-3}$)	V (%)	P ($mg\,dm^{-3}$)	B ($mg\,dm^{-3}$)	Cu ($mg\,dm^{-3}$)	Fe ($mg\,dm^{-3}$)	Mn ($mg\,dm^{-3}$)	Zn ($mg\,dm^{-3}$)
4.1	26	4	1	80	5.4	85	0.5	6	2	0.21	1.4	54	2.4	0.2

Bacillus spp., *Brevibacillus* sp. and *Virgibacillus* sp. Concerning *Bacillus* spp. only strains LMA5, LMA31 and LMA38 (similar to *B. cereus*) did not grow in this medium. Absence of growth was also observed for members of the Enterobacteriaceae, Paenibacillaceae and other families.

Exopolysaccharide production

Concerning this ability, 65% of the strains were able to produce EPS. This was observed for *Bacillus* spp., *Brevibacillus* sp., *Virgibacillus* sp., *Paenibacillus* sp., *Cellulosimicrobium* sp., *Pantoea* sp., *Enterobacter* sp. and one strain belonging to the Enterobacteriaceae family. Seven *Bacillus* strains showing similarity to *B. subtilis*, *B. tequilensis* and *B. cereus* were not able to produce EPS at 28 °C, however, when submitted to 40 °C, it was possible to observe a slight production (data not shown).

Screening of bacterial isolates for in vitro plant growth promotion traits

IAA production and phosphate solubilization ability

Around 30% of the strains showed IAA production above 1.0 $\mu g\,mL^{-1}$. Only two strains LMA28 (*Pantoea* sp.) and LMA68 (*Arthrobacter* sp.) produced a high amount of IAA: 113.57 and 135.22 $\mu g\,mL^{-1}$, respectively. All *Bacillus* spp. displayed a production lower than 50 $\mu g\,mL^{-1}$.

Phosphate solubilization above 1000 $\mu g\,mL^{-1}$ was observed for 6% of the strains, corresponding to the strains LMA28 (*Pantoea* sp), LMA29 (*Enterobacter* sp.) and LMA49, all belonging to the Enterobacteriaceae family. *Bacillus* strains seem to be median Ca–P solubilizers, falling within the range of 29 to 500 $\mu g\,mL^{-1}$. Strains LMA53 (*Paenibacillus* sp.) and LMA71 (*Cellulosimicrobium* sp.) were able to solubilize around 24.95 and 33.75 $\mu g\,mL^{-1}$, respectively.

Indirect mechanisms: NH_3, HCN and cellulase production

HCN production was not detected for any of the strains. The production of ammonia was checked for 71% of the isolates. Three *Paenibacillus* spp., LMA11 (*Virgibacillus* sp.), LMA67 (*Gordonia* sp.), LMA71 (*Cellulosimicrobium* sp.) and 21% of *Bacillus* spp. did not show ammonia production. The degradation of cellulose was not verified for 21% of the strains, corresponding to LMA4, LMA30 and LMA73 (*Bacillus* spp.), LMA32 and LMA53 (similar to *P. cineris*), three strains of the Enterobacteriaceae family (strains LMA28, LMA29 and LMA49), LMA67 (*Gordonia* sp.) and LMA74 (*Nocardia* sp.). It was observed a higher frequency of the isolates with cellulolytic index (CI) ≥ 4. They are equivalent to LMA1, LMA18, LMA22 (*Bacillus* spp.) and LMA55 (*Paenibacillus* sp.).

Plant growth promotion experiment

For plant growth promotion experiments, seven strains have been selected based on their *in vitro* abilities (Table 3). The phylogenetic relationship among them is shown in Fig. 3. Strains belonging to the Firmicutes were grouped into two clades. Three strains were inserted into clade I, which is supported by a bootstrap value of 100. It belongs to the *B. subtilis* group. Strains LMA22 and LMA52 showed similarity of 99.9% (1 divergent nucleotide (nt) of 1048) and 99.85% (2 nt of 1296) to the type strain *Bacillus tequilensis*, respectively. Strain LMA33 showed similarity with *B. subtilis* subsp. *subtilis*, *B. methylotrophicus* and *B. amyloliquefaciens* subsp. *plantarum* with 99.49%, 99.92% and 99.76%, and 6 nt of 1167; 1 nt of 1246 and 3 nt of 1246, respectively. Three strains were grouped into clade II, with a bootstrap of 100, belonging to the *Bacillus megaterium* group, with similarity to the type strains varying from 98.35% to 100%, corresponding to 20 nt of 1029. Finally, one strain was grouped into clade III (*P. cypripedii*), phylum Proteobacteria, with similarity to the type strain of 97.19% and 35 nt of 1246. Probably this strain corresponds to an undescribed species, due to the low similarity.

Table 2
Identification of the strains obtained from soil and rhizosphere of *Cereus jamacaru*, *Melocactus* sp. and *Pilosocereus gounellei* through FAME analysis and 16S rRNA sequencing.

Strain	FAME		16S rRNA		
	Closest species	Similarity index	Closest species	Similarity	nt div/total[a]
LMA1	*Bacillus megaterium*	0.739	–	–	–
LMA2	*Bacillus megaterium*	0.616	–	–	–
LMA3	–	–	*Bacillus aryabhattai*	99.6%	5/1385
LMA4	–	–	*Bacillus safensis*	99.9%	1/1392
LMA5	*Bacillus* sp.	0.458	*Bacillus cereus*	99.9%	1/1250
LMA10	*Bacillus megaterium*	0.827	–	–	–
LMA11	*Brevibacillus reuszeri*	0.758	–	–	–
LMA11′	*Virgibacillus pantothenticus*	0.642	–	–	–
LMA12	*Bacillus megaterium*	0.873	–	–	–
LMA15	–	–	*Bacillus aryabhattai*	99.9%	1/1286
LMA16	*Bacillus megaterium*	0.669	–	–	–
LMA18	*Bacillus atrophaeus*	0.692	–	–	–
LMA20	–	–	*Bacillus solisalsi*	97.8%	15/671
LMA21	–	–	*Bacillus aryabhattai*	99.1%	10/1168
LMA22	–	–	*Bacillus tequilensis*	99.9%	1/1048
LMA27	*Brevibacillus reuszeri*	0.624	–	–	–
LMA28	–	0.854	*Pantoea cypripedii*	97.2%	35/1246
LMA29	–	0.798	*Enterobacter helveticus*	99.4%	6/944
LMA30	*Bacillus pumilus*	0.507	–	–	–
LMA31	*Bacillus cereus*	0.586	–	–	–
LMA32	–	–	*Paenibacillus cineris*	99.6%	5/1386
LMA33	–	–	*B. methylotrophicus*	99.9%	1/1246
LMA34	*Bacillus megaterium*	0.569	–	–	–
LMA38	*Bacillus cereus*	0.599	–	–	–
LMA40	*Bacillus megaterium*	0.566	–	–	–
LMA41	*Bacillus megaterium*	0.524	–	–	–
LMA42	*Bacillus* sp.	0.645	*Bacillus aryabhattai*	99.3%	9/1373
LMA43	*Bacillus megaterium*	0.651	–	–	–
LMA44	–	–	*Pantoea stewartii*	95.4%	59/1294
LMA46	*Arthrobacter nicotianae*	0.815	–	–	–
LMA47	*Bacillus megaterium*	0.844	–	–	–
LMA48	*Bacillus* sp.	0.407	*Bacillus aryabhattai*	100%	0/1279
LMA49	–	0.590	–	–	–
LMA52	–	–	*Bacillus tequilensis*	99.9%	2/1296
LMA53	–	–	*Paenibacillus cineris*	99.9%	1/1281
LMA55	*Paenibacillus lentimorbus*	0.543	–	–	–
LMA56	–	–	*B. methylotrophicus*	99.5%	7/1360
LMA57	*Bacillus subtilis*	0.531	–	–	–
LMA58	*Bacillus subtilis*	0.643	–	–	–
LMA61	*Paenibacillus lentimorbuss*	0.586	–	–	–
LMA63	*Bacillus* sp.	0.503	*Bacillus aryabhattai*	99.8%	3/1322
LMA64	–	–	*Bacillus aryabhattai*	99.9%	1/1405
LMA67	*Gordonia amarae*	0.876	–	–	–
LMA68	–	–	*Arthrobacter defluvii*	98.8%	14/1146
LMA69	*Bacillus megaterium*	0.824	–	–	–
LMA71	*Cellulosimicrobium cellulans*	0.853	–	–	–
LMA73	*Bacillus pumilus*	0.635	–	–	–
LMA74	*Nocardia* sp.	0.766	–	–	–

[a] nt div/total – number of divergent nucleotides by the number of total nucleotides.

Table 3
In vitro features of the strains selected for *Zea mays* L. growth promotion under water stress.

Strain	Identification	0.919A_w	IAA	Ca–P solubilization	EPS	NH_3	Cellulase
LMA3	*Bacillus* sp.	+	+	++	+	+	++
LMA15	*Bacillus* sp.	+	+++	+++	–	+	+
LMA22	*Bacillus* sp.	+	+	++	–	++	++++
LMA28	*Pantoea* sp.	–	++++	++++	+	++	–
LMA33	*Bacillus* sp.	+	+	+	+++	–	++
LMA42	*Bacillus* sp.	+	+++	+++	+	++	+++
LMA52	*Bacillus* sp.	+	+	++	–	+	++

0.919A_w: ability of bacteria to grow in medium with reduced water availability; IAA: <1 μg mL^{-1} (+), 1–10 μg mL^{-1} (++), 11–50 μg mL^{-1} (+++), >51 μg mL^{-1} (++++); Ca–P solubilization: <50 μg mL^{-1} (+), 50–100 μg mL^{-1} (++), 101–500 μg mL^{-1} (+++), >501 μg mL^{-1} (++++); EPS in saccharose medium pH 7.5 at 28 °C: – (no production), halo $\leq$ 10 mmØ (+), halo of 10–14 mmØ (++), halo $\geq$ 14 mmØ (+++); cellulase based on cellulolytic index: CI = 0 (–), CI < 2 (+), 2 $\leq$ CI < 3 (++), 3 $\leq$ CI < 4 (+++), CI $\geq$ 4 (++++); NH_3 production based on the intensity of brownish color: low (+), medium (++), high (+++).

As suggested by Stackebrandt and Ebers (2006), similarity values lower than 98.7% can define a new bacterial species. However, a polyphasic approach is necessary to describe this strain.

Three strains of *Bacillus* spp. (LMA3, LMA42 and LMA52) and one strain of *Pantoea* sp. (LMA28) significantly ($p < 0.01$) increased the leaf area of *Z. mays* L. when compared to the control (Table 4). The strain LMA3 provided the highest increase in the leaf area of *Z. mays* L. (81%), followed by LMA28 (77.7%), LMA42 (75%) and LMA52 (67.5%). One strain of *Bacillus* spp. (LMA3) showed a significant increase ($p < 0.01$) of stalk length in 17%. Two strains: LMA3 and

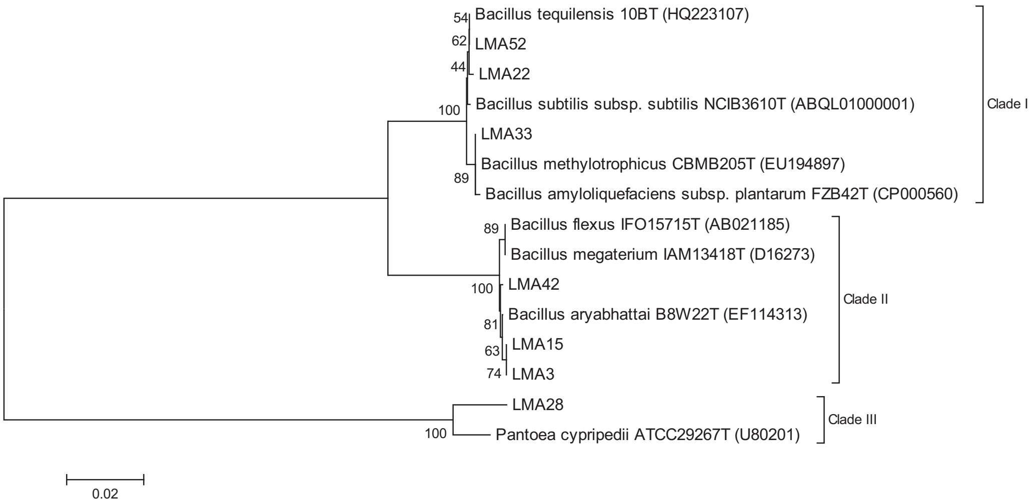

Fig. 3. Phylogenetic relationship based on partial 16S rRNA gene sequence of seven bacterial strains selected for plant growth promotion, with their best matches in the EZ-Taxon database. Alignment was constructed using Mega 5.01, followed by clustering using Neighbor-Joining distance and Jukes-Cantor model. A bootstrap analysis was performed with 1000 replicates, and values indicate the percentage of clustering matches. The scale bar at the bottom indicates the number of differences in base composition among sequences.

LMA15, were able to increase shoot dry biomass of *Z. mays* L., in a significant way ($p < 0.01$) in 66.3% and 56.7%, respectively when compared to the control. Thus, only one strain LMA3 (*Bacillus* sp.) increased the three analyzed plant parameters. Besides, the inoculation of this strain seemed to have protected the plant against the negative effects of desiccation (Fig. 4).

In the attempt to find a correlation among IAA and phosphate solubilization with plant parameters, IAA production positively correlated to stalk length ($r = 0.48$; $0.01 \leq p < 0.05$) as well as phosphate solubilization ($r = 0.45$; $0.01 \leq p < 0.05$). For other parameters, no such correlation has been observed.

Discussion

It is known that all microorganisms need water in order to maintain their physiological activities and up to date no organism has developed the ability to survive without water (Potts 1994). The value of A_w that inhibits any cellular activity depends on each organism (Connon et al. 2007). There are few studies about the concept of xerophilism, because its definition is a complicated task. For bacteria, minimum values of A_w, where growth can be observed, are defined: $0.900A_w$ (for a large percentage of bacteria) and some haloarchaea can grow up to $0.750A_w$ (Grant 2004). Thus, in the present study, bacteria grown in medium with reduced water availability can be considered as xerotolerant, once they tolerate and do not need exclusively this kind of medium to grow. Thus, an initial selection of microorganisms based on their ability to grow in medium with reduced water availability, is an interesting approach.

Table 4
Growth promotion of *Zea mays* L. under water stress (30% of field capacity), by seven selected bacterial strains. Mean values obtained for twenty-five replicates. Statistically significant values according to Dunnett's test at 5% of probability are found in bold.

Treatment	Leaf area (cm^2)	Stalk length (cm)	Shoot dry biomass (g)
Control	62.53	7.52	0.2610
LMA3	**113.19**	**8.80**	**0.4340**
LMA15	97.67	8.45	**0.4090**
LMA22	100.43	7.47	0.3610
LMA28	**111.10**	7.99	0.3830
LMA33	80.75	6.92	0.3090
LMA42	**109.45**	8.45	0.3450
LMA52	**104.73**	7.66	0.3730

The genus *Bacillus* is deeply studied and the high frequency of *Bacillus* spp. detected in this study can be attributed to the ability of endospore formation, that enable bacteria to thrive unfavorable environmental conditions such as heat, radiation, dehydration and starvation (Nicholson et al. 2000). Some *Bacillus* spp. have also been isolated from desert regions like the Sinai desert (Othman et al. 2003), semi-arid regions and associated to cacti (Puente et al. 2009). Cacti-associated microorganisms can be considered as thermotolerant, halotolerant and xerotolerant (Puente et al. 2004) due to the conditions the plants are exposed to. Some *Bacillus* species characterized in this work are still poorly studied, like *Bacillus aryabhattai*, also detected in semi-arid regions in Mexico (Aguirre-Garrido et al. 2012). There are few works concerning microorganisms from arid and semi-arid areas. Hanna et al. (2012) have isolated microorganisms associated to several plants from a desert in Egypt, detecting members similar to those obtained in this study: *B. megaterium* and *B. pumilus*, as well as *B. polymyxa*, *B. macerans*, *B. licheniformis*, members of the Enterobacteriaceae family like *Enterobacter agglomerans*, *E. sakazakii*, *E. cloacae*, *Serratia adorifera*, *S. liquefaciens*, *Klebsiella oxytoca* and *Pantoea* sp.

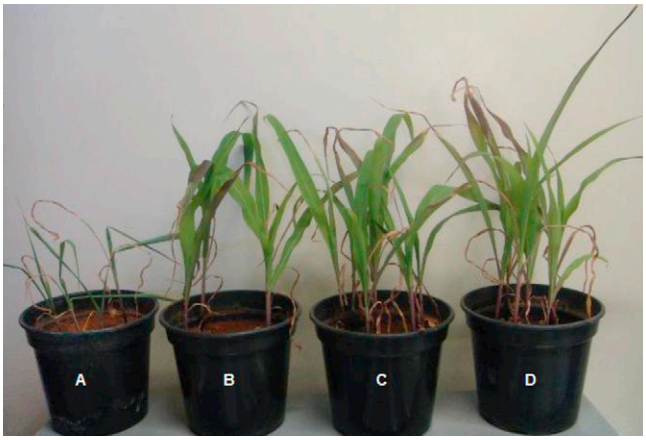

Fig. 4. Results of plant growth promotion under water stress (30% of water field capacity). A – Uninoculated plant; B and D – plants inoculated with two different strains of *Bacillus* spp., LMA52 and LMA3, respectively. C – plant inoculated with one strain of *Pantoea* sp. (LMA28). Inoculated plants seemed to have been protected against the negative effects of desiccation.

Exopolysaccharides are produced by a wide variety of microorganisms and they accumulate in cells' surfaces. Their use has been associated to an adaptation mechanism to stressful environmental conditions such as saline soils, temperature variation and water stress (Coronado et al. 1996). Chang et al. (2007) suggested that a strain of *Pseudomonas putida* produce an EPS, called alginate, which influences the development of biofilm and the physical–chemical properties of EPS itself, in response to water-limiting conditions. These responses facilitate the maintenance of a hydrated microenvironment, protecting the microorganisms against dehydration. When inoculated in plants, EPS-producing microorganisms can help plant survive under adverse conditions (Nocker et al. 2012). In this work *Bacillus* spp. showed EPS production. This has also been reported by other authors (Chowdhury et al. 2011; Yuan et al. 2011).

All strains showed the ability to produce IAA in the presence of L-tryptophan as a precursor. However, some strains showed a slight synthesis variation, and even strains belonging to the same genus (*Bacillus*) produced different amounts of IAA in liquid medium. Several bacterial species have been recognized as IAA producers, like *Pseudomonas* sp., *Bacillus* sp., *Azotobacter* sp. and others (Ahmad et al. 2008). This can be due to the fact that IAA production can be influenced by culture conditions, stage of growth and substrate availability (Mirza et al. 2001). IAA production is an important feature because it is responsible for the regulation of several cellular and plant growth processes (Lambrecht et al. 2000). It was possible to observe a high frequency of IAA production by members of the Enterobacteriaceae family and this has also been reported by Souchie et al. (2007), where they verified concentrations equivalent to 7.1 mg mL^{-1}. Ahmad et al. (2008) have observed the production of this hormone by *Bacillus* sp., with the production of 7.03 μg mL^{-1}. Despite its importance, if produced in high concentrations it can inhibit the growth of some microorganisms, like it has been observed for some yeasts (Prusty et al. 2004).

The highest levels of Ca–P solubilization were observed for members of the Enterobacteriaceae family. This has also been demonstrated by other report, in which *Enterobacter* sp. obtained from soils with low P concentration exhibited high levels of phosphate solubilization (568–642 μg mL^{-1}) (Kumar et al. 2010). Phosphorus, although abundant in soils, both in organic and inorganic forms, is the second plant growth-limiting nutrient due to the easy binding capacity to Ca, Fe and Al, depending on soil pH, what causes its precipitation (Gyaneshwar et al. 2002). Soils from semi-arid region of Brazilian Northeast tend to be slightly acid or alkaline (Pereira and Faria 1998), being P associated to Ca. Thus, the choice of NBRIP medium with $Ca_3(PO_4)_2$. Phosphate solubilization can occur through the release of organic acids or pH reduction (Chen et al. 2006). Alternatively, the production of exopolysaccharide with Ca-binding ability has also been demonstrated as an important factor for phosphate dissolution, acting concomitantly with organic acids (Yi et al. 2008). There are several reports over phosphate solubilization exhibited by a variety of species: *Azotobacter* sp., *Pseudomonas* sp., *Bacillus* sp., *Burkholderia* sp. (Ahmad et al. 2008; Oliveira et al. 2009).

The production of volatile compounds is reported for several microorganisms and they can act as growth-promoting or inhibiting agents (Kai et al. 2009). There are several volatiles described to date (Xu et al. 2004) and HCN is one of them (Blumer and Haas 2000). The inhibition of fungal pathogens' growth by volatiles produced by bacteria or fungi is well reported (Zou et al. 2007). Ammonia production is another plant growth-promoting feature responsible for the indirect plant growth promotion through pathogens' control (Minaxi et al. 2012). *Bacillus* species seem to be ammonia producers. Joseph et al. (2007) detected ammonia production in 95% of *Bacillus* sp., followed by 94.2% of *Pseudomonas* sp., 74.2% of *Rhizobium* sp. and 45% of *Azotobacter* sp. The microorganisms with cellulase activity besides their ecological role in the litter degradation, can also play an important role in the suppression of diseases by the inhibition of fungal growth (Sindhu and Dadarwal 2001) like oomycetes such as *Pythium* sp. which have cellulose as a majority component of their cellular wall.

In general, the isolates belonging to the Enterobacteriaceae and Bacillaceae families shown in this study demonstrated some good *in vitro* plant growth promotion abilities. However, it is possible to observe that high scores for some analyzed traits did not match with high scores for other features, highlighting the difficulty of obtaining one single "super" bacterium with all the desired characteristics. As stated by Dias et al. (2009) plant growth promotion in the environment is not driven by one single species, so the choice of bacteria with different growth promotion abilities in order to provide a consortium that could be further applied for several purposes becomes important.

The inoculation of *Bacillus* spp. and *Pantoea* sp. in *Z. mays* L. seedlings showed significant increases in leaf area, stalk length and shoot dry biomass under water stress, however these effects are not clearly correlated to the production of IAA, phosphate solubilization and other mechanisms. Araújo and Guerreiro (2010) observed that bacteria that promoted maize growth were not necessarily those that produced more IAA. Increase in growth, yield and nutrient absorption by plants may occur through the expression of one or more plant growth-promoting characteristics. In this way, the *in vitro* selection of PGPR with multiple traits and their greenhouse evaluation under controlled conditions is important (Rana et al. 2011).

This work indicated the potential of *Bacillus* spp. for maize growth promotion under water stress. Besides this is the first report to describe cacti-associated bacteria from Brazilian semi-arid region with plant growth-promoting abilities.

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