

Isolation and characterization of endophytic plant growth-promoting (PGPB) or stress homeostasis-regulating (PSHB) bacteria associated to the halophyte *Prosopis strombulifera*

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Abstract This study was designed to isolate and characterize endophytic bacteria from halophyte *Prosopis strombulifera* grown under extreme salinity and to evaluate in vitro the bacterial mechanisms related to plant growth promotion or stress homeostasis regulation. Isolates obtained from *P. strombulifera* were compared genotypically by BOX-polymerase chain reaction, grouped according to similarity, and identified by amplification and partial sequences of 16S DNAr. Isolates were grown until exponential growth phase to evaluate the atmospheric nitrogen fixation, phosphate solubilization, siderophores, and phytohormones, such as indole-3-acetic acid, zeatin, gibberellic acid and abscisic acid production, as well as antifungal, protease, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. A total of 29 endophytic strains were grouped into seven according to similarity. All bacteria were able to grow and to produce some phytohormone in chemically defined medium with or without addition of a nitrogen source. Only one was

able to produce siderophores, and none of them solubilized phosphate. ACC deaminase activity was positive for six strains. Antifungal and protease activity were confirmed for two of them. In this work, we discuss the possible implications of these bacterial mechanisms on the plant growth promotion or homeostasis regulation in natural conditions.

Keywords *Prosopis strombulifera* · Halophyte · Salinity · Phytohormones · Auxins · Cytokinins · Gibberellins · Abscisic acid · ACC deaminase · Phosphate solubilization · Siderophore production · Nitrogen fixation · *Bacillus* · *Lysinibacillus* · *Pseudomonas* · *Achromobacter* · *Brevibacterium*

Introduction

Environmental stresses such as drought, temperature, salinity, air pollution, heavy metals, pesticides, and soil pH are major limiting factors in crop production because they affect almost all plant functions. Soil salinization is a serious stress condition and also land-degradation problem in arid and semi-arid regions, causing major problem for crop productivity. About 20% of cultivable and a least half of irrigated lands around the world are severely affected by salinity (Rhoades and Loveday 1990). However, in these conditions, there are plant populations successfully adapted and evolutionarily different in their strategy of salt tolerance. *Prosopis* (Leguminosae subfamily Mimosoideae) is a genus with many important arboreal and shrub-like species present in saline zones in America. One such species is the spiny shrub *Prosopis strombulifera* (Lam.) Benth, frequently found in the salinized areas of Argentine

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semi-arid region and extended from the Arizona desert (USA) to Patagonia (Argentina). Reinoso et al. (2004) demonstrated the halophytic character of that species since roots and stems increase their growth in NaCl solutions up to 500 mmol L⁻¹.

Halophytes as well as many tolerant glycophytes plant species have primary and secondary mechanisms to overcome salt stress. In firsts ones, primary mechanisms consist in production of compatible solutes to raise osmotic pressure in the cytoplasm, the exclusion of Na⁺ from the cell or plant tissue, and the isolation of Na⁺ in the vacuole (Blumwald et al. 2000). In some halophytes as well as tolerant glycophytes, a secondary mechanism could be considered the endophytic association between plant and rhizobacteria able to improve the plant growth in abiotic stress conditions, named by Kloepper and Schroth (1978) as plant growth-promoting rhizobacteria (PGPR).

PGPR are free-living soil bacteria that are actually divided into three functional groups: plant growth-promoting bacteria (PGPB), biocontrol-PGPB proposed by Bashan and Holguin (1998) and plant stress homeo-regulating bacteria (PSHB) proposed by Cassán et al. (2009), that can either directly or indirectly facilitate the plant growth in optimal, biotic, or abiotic stress conditions. Indirect plant growth promotion induced by biocontrol-PGPB includes a variety of mechanisms by which the bacteria prevent the phytopathogen deleterious effect on plant growth or development. Direct promotion induced by PGPB may include the plant provision with: fixed nitrogen; phytohormones, such as indol-3-acetic acid (IAA), gibberellic acid (GA₃), and cytokinin such as zeatin (Z); iron, sequestered by bacterial siderophores (Glick et al. 1999) and soluble phosphate (de-Bashan and Bashan 2004). Direct stimulation induced by PSHB may include providing plants with stress-related phytohormones, like abscisic acid (Cohen et al. 2008); plant growth regulators, like cadaverine (Cassán et al. 2009); and catabolism of some molecule related with stress signaling such as bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme reduces plant ethylene level, which is increased by various unfavorable conditions and thus confers resistance to stress (Glick et al. 1998).

From a point of view of salinity condition regulated in plant-associated rhizobacteria, some isolations including genus *Pseudomonas* and *Bacillus* have been shown to have capacity to promote the wheat growth in salinated soils of Uzbekistan (Egamberdieva et al. 2008). Similar results were reported in India in groundnut (*Arachis hypogaeae*) inoculated with *Pseudomonas fluorescens*, according to Saravanakumar and Samiyappan (2007). Bashan et al. (2000) showed that inoculation of the halophyte *Salicornia bigelovii* with a mixture of *Azospirillum halopraeferens*,

Bacillus licheniformis, and other halotolerant rhizobacteria increased the plant growth in seawater conditions. In other interesting work, Mayak et al. (2004) showed that *Achromobacter piechaudii* inoculation in tomato seedlings promoted the plant growth and conferred resistance to salt stress.

The aim of the present study was to isolate and identify endophytic bacteria associated to *P. strombulifera*, to evaluate in vitro its plant growth promotion mechanisms, and to correlate them with the potential effects on the overall physiology and survival of the plant.

Materials and methods

Isolation of endophytic bacteria from *P. strombulifera*

Individuals of *P. strombulifera* were randomly collected according to their natural occurrence in an area of 1 km² from El Berbedero saline located at 33°30'00" S and 66°37'00" W, San Luis, Argentina. Roots containing rhizospheric soil were washed with sterile distilled water, disinfected with 70% ethanol, rinsed, disinfected superficially with 3% sodium hypochlorite, rinsed again to eliminate hypochlorite, and spread on nutrient agar to confirm root surface sterility at 30°C for 5 days. Finally, roots were added with 0.9% NaCl (1:10) and macerated with mortar and pestle. One gram of macerated tissue was placed in a tube containing 9 ml sterile 0.9% NaCl. One milliliter of appropriate (10⁻² to 10⁻⁷) dilution of tissue was plated on Congo Red agar or nitrogen-free base (NFb) media to isolate a free-living diazotrophic bacteria (Döbereiner et al. 1995), Luria agar (LA) to isolate nutritionally demanding bacteria, and yeast extract manitol agar (YEMA) to isolate Rhizobiaceae bacteria (Somasegaran and Hoben 1994). Plates were incubated at 30°C for 2 (RCA, NFb, and LA) or 7 days (YEMA) to isolate bacteria. Morphology and mobility of cultured bacteria were examined by light microscopy. Isolated bacteria were tested for gram coloration with a kit (Britania Laboratories) and for catalase and oxidase activity with disks (Britania Laboratories).

Genotypic characterization and identification

To compare genotypically, isolates obtained from *P. strombulifera* were subjected to a BOX-polymerase chain reaction (PCR) fingerprint evaluation. For this, total DNA amplification fingerprints were performed with BOXA1R primers as previously described by Versaslovic et al. (1994). The primer sequences were as follows: BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3'). PCR mixtures (25 µl) contained: 0.5 µM each primer, 200 µM dNTPs, 3 mM MgCl₂, PCR reaction buffer

(50 mM KCl, 20 mM Tris–HCl, pH 8.0), 1 U Taq DNA polymerase (Promega Corp), and 2 µl of template DNA, previously obtained by heating a freshly isolated bacterial colony in 50 µl of distilled water to 100°C for 15 min. The amplifications were carried out in Thermo Cycler (I Cycler-BioRad). The cycling conditions were as follows: 95°C for 7 min, followed by 35 cycles at 94°C for 1 min, at 52°C for 1 min, at 65°C for 8 min, and at 65°C for 16 min. After the reaction, 10 µl of the PCR reaction was separated in 1.5%-agarose gels containing 1 µg/ml of ethidium bromide and photographed with a Kodak DC290 Digital camera. Isolates which showed the same band patterns were grouped, and from each group was selected a representative strain for subsequent PGPR characterization (see below) and genotypic identification by amplification and partial nucleotide sequences of the ribosomal 16S DNA (DNAr 16S). The partial nucleotide sequences of the 16S rRNA gene (rDNA) were determined by direct sequencing of appropriate PCR products. A DNA region corresponding to nucleotides 20 to 338 of *Escherichia coli* 16S rDNA was amplified from each strain with the universal primers Y1 (59-TGG CTC AGA ACG AAC GCT GGC GGC-39) and Y2 (59-CCC ACT GCT GCC TCC CGT AGG AGT-39) as previously described for proteobacteria (Young et al. 1991). The nucleotide sequence of the PCR products was determined for both strands with an Automatic Laser Fluorescent DNA Sequencer (Pharmacia). Pair-wise comparisons were made using BLAST tool. Partial sequence data for the 16S rRNA genes have been deposited in the European Molecular Biology Laboratory (EMBL)/GenBank/DBJ nucleotide sequence data libraries. Data for strains have been deposited under the following accession numbers: EU683677 (Ps7); EU683678 (Ps8); EU683679 (Ps9); EU683680 (Ps14); EU683681 (Ps19); EU683682 (Ps27); and EU683683 (Ps30).

Evaluation of plant growth promotion, regulation, and biocontrol mechanisms

Each isolate was grown in individual 50 ml flasks containing 10 ml Luria–Bertani (LB) medium at 30°C and 80 rpm shaking, until exponential growth phase ($OD_{600} \sim 1$). For phytohormone determination and quantification, isolates were grown in NFb medium modified by addition of ($g\ l^{-1}$) NH_4Cl (1.25). An aliquot was taken from each pure culture for evaluation of PGPB characteristic (phosphate solubilization, siderophore production, and indol 3-acetic acid, zeatin, and gibberellic acid identification and quantification); PSHB characteristic (1-aminocyclopropane-1-carboxylate deaminase activity and abscisic acid identification and quantification); and biocontrol-PGPB characteristic (antifungal and protease activity).

Phosphate solubilization

Phosphate solubilization was measured by the methods of Katznelson and Bose (1959). Plates containing trypticase soya agar medium supplemented with $Ca_5(PO_4)_3OH$ were inoculated with 1 µl LB pure bacterial culture. Plates were incubated at 30°C and observed daily for 7 days formation of transparent “halos” around each colony. Experiments were performed in triplicate. Positive control was made with *P. fluorescens* strain P1 (provided by the Agriculture Collection Laboratory, IMYZA-INTA; Argentina) in similar culture conditions.

Siderophore production

Siderophore production was determined by the method of Schwyn and Neilands (1987). For this, 1 µl pure bacterial culture grown in LB was inoculated in plates containing agar Chrome Azurol S (CAS). Plates were incubated at 30°C and observed daily for orange color formation around each colony for up to 4 days. Experiments were performed in triplicate. Positive control was made with *P. fluorescens* strain P1 (provided by the Agriculture Collection Laboratory, IMYZA-INTA; Argentina), in similar culture conditions.

Nitrogen fixation

Each isolated strain was inoculated in plates containing NFb medium with or without addition of NH_4Cl as a unique nitrogen source (Döbereiner et al. 1995). Plates were incubated at 28°C for 7 days, and bacterial growth was observed as qualitative evidence of the atmospheric nitrogen fixation. As positive and negative controls, *Azospirillum brasilense* Az39 and *P. fluorescens* R1 were used.

Phytohormone identification and quantification

Bacterial cultures (NFb) in exponential growth phase were separated into several 20 ml fractions, for determination of IAA, abscisic acid (ABA), GA_3 , and zeatin. Fractions were centrifuged at 8,000 rpm for 20 min at 4°C, and supernatants were acidified at pH 2.5 with acetic acid solution (1% v/v). Individual samples were then added with 100 ng of corresponding 2H_6 -ABA (kindly supplied by Prof. Richard P. Pharis, University of Calgary, Canada), 2H_5 -IAA, or 2H_2 - GA_3 (OChemIm, Czech Republic) deuterated internal standard and kept at 4°C for 2 h. No deuterated internal standard was used for Z. Each sample was partitioned four times with the same volume of acetic-acid-saturated ethyl acetate (1%, v/v). After the last

partition, acidic ethyl acetate was evaporated to dryness at 36°C. Dried samples were diluted in 100 µl acetic acid/methanol/water (1:30:70) for ABA determination, acetic acid/acetonitrile/water (1:15:85) for IAA determination, and methanol/water (30:70) for GA₃ and Z determination. They were injected into a reverse-phase C18 high-performance liquid chromatography (HPLC) column (µBondapak, 300×3.9 mm, Waters Associates, Milford, MA, USA) in a Konik 500 (Konik Instruments) system coupled to a UV–Vis Konik 3000 diode-array spectrometer. For each sample, elution was performed at 1 ml min⁻¹ flow rate, and fractions eluting at the retention time corresponding to each pure standard were collected. Z was identified and quantified by HPLC-UV at 254 nm (Tien et al. 1979). IAA, ABA, and GA₃ were identified and quantified by gas chromatography-mass spectrometry with selective ion monitoring (GC-MS-SIM). UV-absorbing fractions at 254, 262, and 220 nm were grouped for IAA, ABA, and GA₃ determination, respectively, then methylated with ethereal diazomethane and silylated with 1:1 pyridine/BSTFA [bis(trimethylsilyl) trifluoroacetamide] plus 1% trimethylchlorosilane (Fluka Chemika, Switzerland) to obtain methyl-trimethylsilyl derivatives of IAA, ABA, and GA₃. Aliquots of each sample were injected directly into a DB1-15N (15 m×0.25 mm, 0.25 µM methyl silicone) capillary column (J&W Scientific) fitted in a Hewlett-Packard 5890 Series II GC with a capillary direct interface to a 5970B Mass Selective Detector. The GC temperature program was 60°C to 195°C at 20°C min⁻¹, then 4°C min⁻¹ to 260°C. Carrier gas (He) flow rate was 1 ml min⁻¹, interface temperature was 280°C, and data acquisition was controlled by a HP 300 Series computer. The amount of free ABA was calculated by comparison of peak areas of the ion at a mass/charge (*m/z*) 196 (molecular ion for [²H₆]ABAMeTMSi) and the ion at *m/z* 190 (molecular ion for [¹H]ABA-MeTMSi) at the corresponding time (Kovats 1958). Similarly, we calculated the amount of free IAA by comparison of peak areas for the parent ion (*m/z*) 194 and (*m/z*) 189 and amount of free GA₃ by comparison of peak areas for parent ion (*m/z*) 506 and (*m/z*) 504. The detection limit for the methodology was established in picogram (pg) range for that experiment. Data from three experiments were analyzed by analysis of variance (ANOVA) followed by post hoc Tuckey test *p*<0.05.

Antifungal activity

Bacterial isolates were screened for in vitro growth inhibition of phytopathogenic fungi *Alternaria* sp. (Cattelan et al. 1999). This fungus genus was chosen because it has been proposed to have worldwide distribution as pathogenic agent and to cause considerable losses under field conditions. Fungal mycelia cultivated for 10 days were used as

control (C). One microliter of each LB pure bacterial culture was inoculated in plates containing MPA (agar potato medium), and a 3-cm-diameter cylinder of mycelia was introduced in the plate center and incubated for 10 days at 25°C. Mycelia growth inhibition was calculated as $I = [(C - T)/C] \times 100$, where *I*=mycelia growth inhibition in percentage, *C*=mycelia diameter in control, and *T*=mycelia diameter in bacteria-inoculated plates.

Protease production

Protease production was determined according to Abo-Abu et al. (2006) with modifications. Plates were inoculated with 1 µl LB pure bacterial culture in halfway points of a Petri dish containing agar milk 3%. Plates were incubated at 28°C and observed daily for formation of transparent haloes around each colony for up to 4 days. Experiments were performed in triplicate.

ACC deaminase activity

ACC deaminase activity was determined by the method of Glick et al. (1995). For this, 1 µl of each LB pure bacterial culture was inoculated into agar plates containing NFb or NFb-ACC modified by addition of 1-aminocyclopropane-1-carboxylate (5.0 g l⁻¹) as unique nitrogen source. Plates were incubated at 28°C and observed daily for colony formation for up to 4 days. Colonies were re-inoculated and incubated in the same experimental conditions. Newly colonies formed in NFb with addition of ACC were considered positive for ACC deaminase activity.

Results

Strain isolation and biochemical and molecular characterization

A total of 29 endophytic strains were isolated from roots of *P. strombulifera*, naturally established in the extreme conditions from “El Bebedero” saline, located in San Luis, Argentina. Cell morphology and topographic gram stain showed a distribution of 68.9% positive and 31.1% negative bacilli. To compare the genotypic redundancy, all isolates were performed for molecular characterization by BOX-PCR fingerprint and consequently divided into seven representative groups. One strain of each group was randomly selected for further molecular and physiological experiments and named with the prefix Ps for *P. strombulifera*. As shown in Table 1, 16S rDNA sequence analysis indicates that strain Ps7 has homology with *Lysinibacillus fusiformis* isolate ASC8; Ps8 with *Bacillus subtilis* strain h-g; Ps9 with *Brevibacterium halotolerans* strain DSM 8802; Ps14 with

Table 1 Identification of putative plant growth-promoting bacterial strains isolated from *P. strombulifera* based on 16S rDNA sequence and EMBL DNA databases

Isolate	Identified as	Isolation number	% Similarity	EMBL accession number	Organism, strain
Ps7	<i>Lysinibacillus fusiformis</i>	3	100	EU253490.1	<i>Lysinibacillus fusiformis</i> isolate ASC8
Ps8	<i>Bacillus subtilis</i>	11	100	EF581127	<i>Bacillus subtilis</i> strain h-g
Ps9	<i>Brevibacterium halotolerans</i>	4	100	AM747812	<i>Brevibacterium halotolerans</i> strain DSM 8802
Ps14	<i>Bacillus licheniformis</i>	1	100	DQ298087	<i>Bacillus licheniformis</i> isolate AD4B
Ps19	<i>Bacillus pumilus</i>	1	100	AM493716	<i>Bacillus pumilus</i>
Ps27	<i>Achromobacter xylosoxidans</i>	5	100	EF396325	<i>Achromobacter xylosoxidans</i> strain 53B
Ps30	<i>Pseudomonas putida</i>	4	100	DQ387442	<i>Pseudomonas putida</i> strain S18

B. licheniformis isolate AD4B; Ps19 with *Bacillus pumilus*; Ps27 with *Achromobacter xylosoxidans* strain 53B; and Ps30 with *Pseudomonas putida* strain S18. All sequences were obtained from EMBL DNA databases. Phylogenetic tree constructed using the partial 16S rDNA sequences of the putative plant-growth promoting isolates and representative bacteria of related taxa is shown in Fig. 1.

Evaluation of direct plant growth promotion mechanisms

The potential PGPB mechanisms of the seven selected strains were evaluated in vitro based on the growth on

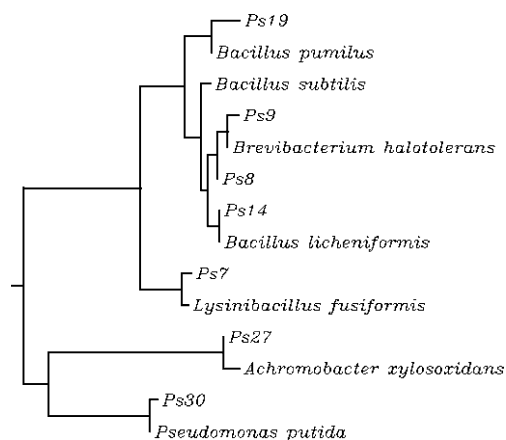


Fig. 1 Phylogenetic tree of partial 16S rDNA sequences of seven putative plant-growth promoting isolates recovered from *Prosopis strombulifera*. The tree was inferred from an alignment of partial 16S rDNA sequences from the local isolates and from selected reference taxa using the EBI (European Bioinformatics Institute) support. Branches are drawn proportionally to DNA distance. The nucleotide sequences used of representative strains were obtained from EMBL under the following accession numbers: EU253490 (*Lysinibacillus fusiformis* isolate ASC8), EF581127 (*Bacillus subtilis* strain h-g), AM747812 (*Brevibacterium halotolerans* strain DSM 8802), DQ298087 (*Bacillus licheniformis* isolate AD4B), AM493716 (*Bacillus pumilus*), EF396325 (*Achromobacter xylosoxidans* strain 53B), DQ387442 (*Pseudomonas putida* strain S18), and for the putative plant growth-promoting bacterial strains: EU683677 (Ps7); 142 EU683678 (Ps8); EU683679 (Ps9); EU683680 (Ps14); EU683681 (Ps19); EU683682 (Ps27); and EU683683 (Ps30)

nitrogen-free medium, phosphate solubilization in agar plate, and siderophore and phytohormone (IAA, Z, and GA₃) production in chemically defined medium. Results are summarized in Table 2. All seven isolates showed capacity to grow in nitrogen-free conditions; however, diazotrophic capacity was evaluated using a qualitative agar plate method, and future experiments should be carried out to confirm, quantify, and compare the capacity of each strain on ¹⁵N enriched liquid medium. Only one, *P. putida* (Ps30), was able to produce siderophores, and none of them solubilized phosphate.

Biosynthesis of indol 3-acetic acid, zeatin, and gibberellic acid showed differences among strains and are summarized in Fig. 2. IAA production was significantly higher for *B. subtilis* (Ps8), *B. pumilus* (Ps19), and *P. putida* (Ps30), which produced 0.5; 0.7, and 2.2 $\mu\text{g ml}^{-1}$, respectively, compared with Ps7, Ps14, and Ps27, which produced less than 0.1 $\mu\text{g ml}^{-1}$, and Ps9 which did not produce the molecule in same experimental conditions.

GA₃ production was significantly higher for *L. fusiformis* (Ps7), *A. xylosoxidans* (Ps27), *B. halotolerans* (Ps9), and *B. licheniformis* (Ps14), which produced 36.5, 50.0, 80.5, and 75.5 $\mu\text{g ml}^{-1}$, respectively, compared to Ps19 which produced 21.3 $\mu\text{g ml}^{-1}$, Ps8 which produced less than 10.0 $\mu\text{g ml}^{-1}$, and Ps30 which did not produce the molecule.

Z production was significantly higher for *B. subtilis* (Ps8) and *P. putida* (Ps30), which produced 25.1 and 22.3 $\mu\text{g ml}^{-1}$, respectively, compared to Ps9 and Ps 19 which produced less than 2.0 $\mu\text{g ml}^{-1}$ and Ps7, Ps14, and Ps27, which did not produce the molecule.

Evaluation of plant homeostasis regulation mechanisms

The potential PSHB mechanisms were evaluated in vitro based on the ACC deaminase activity and abscisic acid production in chemically defined medium, and results are summarized in Table 2.

All seven strains showed capacity to produce ABA in chemically defined medium. ABA production (Fig. 2) was

Table 2 Morphology and biochemical characteristics of eight bacterial strain isolates of *P. strombulifera*

Cepa	Gram staining	Siderophore production	Phosphate solubilization	Nitrogen fixation	ACC deaminase activity	Antifungal activity	Protease production	Phytohormone production
Ps7	BG +	–	–	+	–	–	–	+
Ps8	BG +	–	–	+	+	–	+	+
Ps9	BG +	–	–	+	+	+	+	+
Ps14	BG +	–	–	+	+	–	–	+
Ps19	BG +	–	–	+	+	+	+	+
Ps27	BG –	–	–	+	+	–	–	+
Ps30	BG –	+	–	+	+	–	–	+

BG + gram-positive bacilli, BG– gram-negative bacilli, ACC aminocyclopropane-1 carboxylate, *Phytohormone production* identification of IAA, Z, GA₃, or ABA

significantly higher for *B. subtilis* (Ps8) and *P. putida* (Ps30), which produced 1.8 and 4.2 $\mu\text{g ml}^{-1}$, respectively, compared with *L. fusiformis* (Ps14), which produced 0.3 $\mu\text{g ml}^{-1}$, and all others strains which produced less than 0.2 $\mu\text{g ml}^{-1}$. Bacterial growth in 1-aminocyclopropane-1-carboxylate medium through ACC deaminase activity was positive for all strains except Ps7.

Evaluation of biocontrol activity

The potential biocontrol-PGPB activity was evaluated based on antifungal and protease activity in vitro, and results are summarized in Table 2. *B. halotolerans* (Ps9) and *B. pumilus* (Ps19) inhibited *Alternaria* sp. growth in agar plate more than 50% compared with control treatment

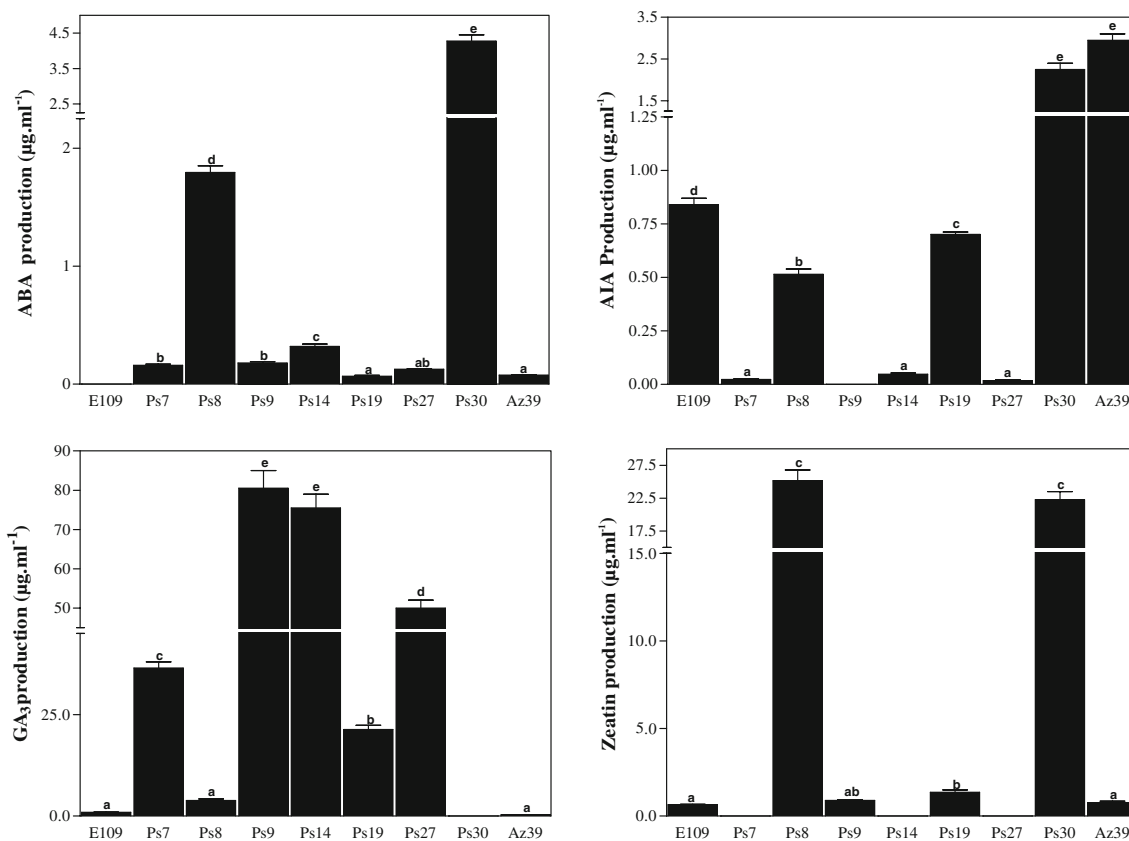


Fig. 2 Identification and quantification of phytohormones by HPLC-UV-GC-MS-SIM of putative plant growth-promoting bacterial strains isolated from *P. strombulifera* obtained from exponential growth phase NFb cultures. Data were analyzed by ANOVA and post hoc Tukey test $p < 0.05$. The phytohormone production was also com-

pared graphically with a previous characterization of *B. japonicum* E109 (Boiero et al. 2007) and *A. brasilense* Az39 (Perrig et al. (2007), obtained in similar experimental conditions. Bars without letters mean that the compound was not detected by the methodology

and, together with *B. pumilus* (Ps8), showed additionally protease activity that could be responsible at least for the bacterial antifungal activity. Despite this, complementary experiments should be carried out with other fungal genera, like *Fusarium*, *Pythium*, *Phytophthora*, or *Sclerotinia*, to confirm the biocontrol capacity of each strain.

Discussion

Soil salinization is the combined result of anthropogenic environmental impact and climatic characteristics. These conditions limit natural vegetation processes, and soil and plant functioning are largely governed by rhizospheric microbial activity that influence the biogeochemical cycles as well as the plant growth and development. In Argentina, there are plant species successfully adapted to saline environments and differentiated in the evolutionary strategy for this tolerance, such as the leguminous shrub *P. strombulifera*. This halophyte has various physiological and biochemical mechanisms that allow optimal growth in saline conditions, and perhaps part of its adaptive success would depend at least on its ability to establish and maintain effective associations with plant growth-promoting endophytic or rhizospheric bacteria. In this regard, there are many publications relating the plant salinity-tolerance model (Reinoso et al. 2004; Llanes et al. 2005); however, there are no published works about the identity and physiological capacities of the endophytic or rhizospheric bacteria associated with this halophyte in natural saline conditions. Our work is the first report about the isolation and molecular characterization of *P. strombulifera* endophytic bacteria, as well as PGPB, biocontrol-PGPB, and PSHB mechanism evaluation.

From roots of *P. strombulifera* were isolated 29 endophytic strains, in which cell morphology as well as gram staining showed a 68.9% frequency of positive rod shape, spore-forming bacilli identical to *Bacillus* sp. and *Brevibacterium* sp. In the case of *Bacillus* sp., it was obtained in 80% of gram-positive isolations and comprises one of the most common soil bacteria groups. Because of their spore-forming ability, strains have high tolerance to adverse ecological conditions, and this fact could determined the high number of isolations obtained from *P. strombulifera* roots growing in extreme salinity. Genotypic identification by 16S rDNA sequencing showed a prevalence of 18.75% for *L. fusiformis* (Ps7), 68.75% for *B. subtilis* (Ps8), 6.25% for *B. licheniformis* (Ps14), and 6.25% for *B. pumilus* (Ps19).

Twenty percent of positive bacilli obtained corresponded to *B. halotolerans*. *Brevibacterium* genus was established by Breed (1953), with *Brevibacterium linens* as the type species, for a number of gram-positive, short, unbranching,

rod-shaped bacteria. *Brevibacteria* exist in a number of different habitats, especially in those having a high salt concentration. Most members of this family grow well in the presence of 8% NaCl, and many strains also grow in 15% NaCl (Collins, 2006). This fact could determine the isolation from the saline habitat colonized by *P. strombulifera*.

Substantial percentage of isolations (31.1%) was gram-negative bacilli, and genotypic identification showed a prevalence of 55.5% for *A. xylosoxidans* (Ps27) and 44.5% for *P. putida* (Ps30). *A. xylosoxidans* is a gram-negative, non-spore-forming and motile straight rod which has been previously considered as a promoter of plant growth due to the presence of various physiological and biochemical mechanisms, as the improvement of NO₃⁻ uptake by roots or ethylene level depletion via expression of putative ACC deaminase (Blaha et al. 2005). Additionally, Tilak et al. (2005) suggest that *A. xylosoxidans* has natural ability to tolerate salinity because it was identified in a bacterial community isolated from the rhizosphere of salt-affected rice. In the same way, Forchetti et al. (2007) mentioned that *Achromobacter* sp. was the only isolated bacteria obtained from sunflower rhizosphere under drought conditions, indicating better tolerance to water stress.

P. putida is a gram-negative, rod-shaped, non-spore-forming, typically motile bacterium with one or more polar flagella. This nonpathogenic species is a free-living saprophytic organism in soil where they play an important role in decomposition, biodegradation, carbon and nitrogen cycles, and promotion of plant growth due to the presence of various physiological and biochemical mechanisms such as ACC deaminase activity (Grichko and Glick, 2001) or indole 3-acetic acid production (Prikyl et al. 1985). In this regard, it has increased root and shoot elongation in canola, lettuce, and tomato as well as crop yields in potato, radishes, rice, sugar beet, tomato, lettuce, apple, citrus, beans, ornamental plants, and wheat (Kloepper et al. 1988).

Plant growth-promoting mechanisms identified in endophytic strains isolated from *P. strombulifera* roots are summarized in Table 2. None of the strains isolated demonstrated the ability to solubilize phosphates in chemically defined medium, and in this regard, some authors have previously reported this capability in other strains of the same species identified in this work (Rodríguez and Fraga 1999).

Siderophore production in CAS medium (Table 2) was only proved for *P. putida* (Ps30). Cox et al. (1981) reported that plant-associated *P. fluorescens* and *P. putida* produce small phenolate siderophores derived from salicylic acid and cysteine called pyochelins and pyoverdins. Direct use of *P. putida* siderophores by plants has been demonstrated in many species, including dicot legumes such as peanut or monocots such as sorghum (Bar-Ness et al. 1992).

All isolates were able to grow in nitrogen-free culture medium, and this capability could be attributed to the acquisition of atmospheric nitrogen by biological fixation. Recently, Forchetti et al. (2007) isolated endophytic *Achromobacter xiloxidans* and *B. pumilus* from sunflower (*Helianthus annuus* L.) roots, and both two strains were capable to grow in chemically defined medium without nitrogen source through the biological nitrogen fixation. In similar experiments, free-living nitrogen-fixing *L. fusiformis* strain was isolated from rhizosphere of different crops in Chungbuk and exhibited the highest nitrogenase activity in controlled experimental conditions (Park et al. 2005). Identification of nitrogen-fixing heterotrophic bacteria isolated from rice fields in the Yangtze River Plain by Xie et al. (2006) showed the existence of *nif* genes in three strains of *B. licheniformis* and one of *B. pumilus*. There are no references about the nitrogen fixation in *B. subtilis* or *Brevibacterium halotolerant* strains.

In this work, production of phytohormones such as IAA, Z, GA₃, and ABA, was proved in all seven isolated strains; however, each microorganism showed intrinsic characteristics about the type and quantity of compound produced in our experimental conditions. *A. brasilense* Az39 (Perrig et al. 2007) and *Bradyrhizobium japonicum* E109 (Boiero et al. 2007) were used as control PGPR strains and showed a particular phytohormone production pattern, in which IAA was the mostly abundant phytohormone present in the culture medium, compared to *Prosopis* isolated strains (Fig. 2), in which IAA, Z, and ABA production were equally high in the same strains (Ps8 and Ps30).

P. putida produced 2.25 $\mu\text{g ml}^{-1}$ IAA, 22.31 $\mu\text{g ml}^{-1}$ Z, and 4.27 $\mu\text{g ml}^{-1}$ ABA in chemically defined medium, and these were the highest concentrations produced (Fig. 2). Mehnaz and Lazarovits (2006) showed that *P. putida* produced IAA in defined medium. According to Glick et al. (1999), the primary way is common in many PGPB, and an indole-3-pyruvate *decarboxylase* enzyme converts tryptophan to indole-3-acetic acid via indole-3-pyruvic acid. Bioassay experiments of canola seeds and mung bean cuttings inoculation with *P. putida* strain GR12-2 resulted in a significant increase of lateral root development and adventitious roots, respectively, compared to uninoculated controls (Mayak et al. 1999). There is a single report about cytokinin production by *P. putida* (Nieto and Frankenberger 1989). In this paper, bacteria produced zeatin-like compounds (zeatin riboside, dihydrozeatin riboside, *t*- and *c*-zeatin) in nitrate culture medium. This is the first report, using an unequivocal methodology, about the abscisic acid production by *P. putida* in defined culture medium, and this capacity could be considered as a plant homeostasis regulator mechanism.

A. xylosoxidans produced 0.017 $\mu\text{g ml}^{-1}$ IAA, 50 $\mu\text{g ml}^{-1}$ GA₃, and 0.127 $\mu\text{g ml}^{-1}$ ABA in chemically defined medium (Fig. 2). Ma et al. (2008) showed that *A.*

xylosoxidans Ax10 produced nearly to 5.0 $\mu\text{g ml}^{-1}$ IAA in defined culture medium, and this fact could be related to improvement of root growth and copper phytoextraction by *Brassica juncea* plants. Forchetti et al. (2007) showed that *A. xylosoxidans* SF2, isolated from sunflower roots, produced 0.002 $\mu\text{g ml}^{-1}$ ABA in minimal medium. This is the first report using an unequivocal methodology about gibberellic acid production by *A. xylosoxidans* in chemically defined medium.

L. fusiformis produced 0.02 $\mu\text{g ml}^{-1}$ IAA, 36.5 $\mu\text{g ml}^{-1}$ GA₃, and 0.15 $\mu\text{g ml}^{-1}$ ABA in chemically defined medium (Fig. 2). Park et al. (2005) showed that *L. fusiformis* PM5 and PM24 produced 100 $\mu\text{g ml}^{-1}$ IAA in defined medium, and this was considered as promising potential mechanism for developing plant growth in inoculation conditions. This is the first report using an unequivocal methodology about the gibberellic acid and abscisic acid production by *L. fusiformis* in chemically defined medium.

B. licheniformis produced 0.05 $\mu\text{g ml}^{-1}$ IAA, 75.5 $\mu\text{g ml}^{-1}$ GA₃, and 0.32 $\mu\text{g ml}^{-1}$ ABA in chemically defined medium (Fig. 2). Gutiérrez-Mañero et al. (2001) showed that *B. licheniformis* isolated from rhizosphere of alder (*Alnus glutinosa* [L.] Gaertn), produced physiologically active gibberellins GA₁ (0.13 $\mu\text{g ml}^{-1}$), GA₃ (0.05 $\mu\text{g ml}^{-1}$), and GA₄ in addition to precursor GA₂₀ and the isomers 3-epi-GA₁ and iso-GA₃. Additionally, dwarf phenotype induced in alder seedlings by paclobutrazol was effectively reversed by extracts from bacterial culture media or exogenous application of GA₃. This is the first report using an unequivocal methodology about indole-3-acetic acid and abscisic acid production by *B. licheniformis* in chemically defined medium.

B. pumilus produced all phytohormones evaluated, 0.70 $\mu\text{g ml}^{-1}$ IAA, 1.36 $\mu\text{g ml}^{-1}$ Z, 21.3 $\mu\text{g ml}^{-1}$ GA₃, and 0.06 $\mu\text{g ml}^{-1}$ ABA in chemically defined medium (Fig. 2). Like *B. licheniformis*, Gutiérrez-Mañero et al. (2001) showed that *B. pumilus* isolated from alder produced similar quantities of physiologically active gibberellins, and bacterial culture media could reverse the dwarf phenotype in paclobutrazol-treated seedlings. Forchetti et al. (2007) showed that *B. pumilus* SF3 and SF4, isolated from sunflower roots, produced 3.4 and 10.6 pmol ml^{-1} ABA, respectively, in chemically defined medium. Recently, Kang et al. (2006) proved that *B. pumilus* SE34 secreted high levels of indole-3-acetic acid in tryptophan-amended medium in stationary phase. This is the first report about zeatin production by *B. pumilus* in chemically defined medium.

B. subtilis produced all phytohormones evaluated, 0.44 $\mu\text{g ml}^{-1}$ IAA, 25.1 $\mu\text{g ml}^{-1}$ Z, 3.85 $\mu\text{g ml}^{-1}$ GA₃, and 1.79 $\mu\text{g ml}^{-1}$ ABA in chemically defined medium (Fig. 2). There are few publications about gibberellin

production by *B. subtilis*, all published in the decade between 1960 and 1970. According to Katznelson et al. (1965), bacteria produced gibberellin-like substances which were identified by bioassay method. *B. subtilis* strains CM1 and CM5, isolated from cowdung microflora, produced 0.09–0.37 mg Γ^{-1} IAA in Nutrient Broth (NB) medium (Swain et al. 2007). In other work published by Zaidi et al. (2006), a nickel (Ni)-tolerant *B. subtilis* strain SJ-101 was characterized because it could facilitate Ni accumulation in the Indian mustard plant (*B. juncea* [L]. Czern and Coss) var. Pusa Bold (DIR-50) through IAA production, estimated in 50 $\mu\text{g ml}^{-1}$ in specific culture media. Recently, Arkhipova et al. (2005) evaluated cytokinin production by *B. subtilis* growing in culture media and inoculated lettuce plants using specific antibodies method. Zeatin riboside (ZR) was shown to be the main cytokinin present, with 0.8–1.2 μg equivalent ml^{-1} ZR. Also, inoculation of lettuce plants with bacteria increased the cytokinin content and plant shoots and root weight by approximately 30% compared with controls. This is the first report with accurate methodology about gibberellic acid production by *B. subtilis* in chemically defined medium.

B. halotolerans produced 0.89 $\mu\text{g ml}^{-1}$ Z, 90.0 $\mu\text{g ml}^{-1}$ GA₃, and 0.18 $\mu\text{g ml}^{-1}$ ABA in chemically defined medium. This is the first report using an unequivocal methodology about gibberellic acid, abscisic acid, and zeatin production by *B. halotolerans* in chemically defined medium.

ACC deaminase activity in agar plate was positive for *B. subtilis* (Ps8), *B. halotolerans* (9), *B. licheniformis* (Ps14), *B. pumilus* (Ps19), *A. xylosoxidans* (Ps27), and *P. putida* (Ps30), and this is the first report about ACC deaminase capacity described in *B. subtilis*, *B. halotolerans*, and *B. licheniformis*. There are many reports about ACC deaminase activity in *P. putida*. Shah et al. (1998) isolated from rhizosphere of bean, corn, and clover, the strains UW1, UW2, and UW3 of *P. putida*, which possessed the ACC deaminase capacity. About *Achromobacter* genus, Mayak et al. (2004) informed that *A. piechaudii* ARV8 contain ACC deaminase activity and thus should be able to lower ethylene production in inoculated host plants. Recently, Ma et al. (2008) showed that *A. xylosoxidans* Ax10 utilized ACC as a sole N source in Dworkin and Foster (1958) salts minimal medium, and this fact could be related to improvement of root growth and copper phytoextraction by *B. juncea* plants. In other work, Belimov et al. (2001) reported the isolation of *B. pumilus*, *P. putida*, and 13 other strains containing ACC deaminase from the rhizoplane of pea (*Pisum sativum* L.) and Indian mustard (*B. juncea* L.) grown in sewage sludge contaminated with heavy metals. Hall et al. (1996) proposed a model in which plant growth could be promoted by PGPB through ACC deaminase activity, and since then, many works were published in this topic. Then, Glick et al. (1998) proposed that ACC

deaminase activity in PGPB could reduce two to four-folds ethylene level, which is increased by several types of stress, thus conferring resistance to stress in inoculated plants. In this sense, *P. fluorescens* strain TDK1 possessing ACC deaminase activity enhanced the saline resistance in groundnut plants, which in turn resulted in increased yield (Saravanakumar and Samiyappan 2007). Growth of canola plants inoculated with *P. putida* showing ACC deaminase activity, was promoted in the presence of inhibitory levels of salt (Cheng et al. 2007). In other report, seven PGPB strains, including ACC deaminase *A. piechaudii*, were isolated from soil samples taken from the Arava region (Israel) by Mayak et al. (2004). Authors found that PGPB populating dry salty environments can increase resistance to salt stress in tomato and suggest that the bacterium acts to alleviate the salt suppression of photosynthesis.

Results of our work suggest that *P. strombulifera* is naturally associated with a variety of endophytic microorganisms, which have different physiological and biochemical capabilities. In regard to the direct mechanisms, phytohormone production (IAA, Z, or GA₃) was confirmed in many isolates, and we speculate that the bacterial production of these molecules could benefit the plant through induction of some changes in its morphology, especially in roots, or increasing its length to absorb more water or its length to explore the soil in depth. In regard to the homeostasis regulation mechanisms, the ABA production or ACC deaminase activity were confirmed in many isolates, and the current literature has identified a strong relationship between that rhizobacterial capabilities and colonized plant tolerance to salt stress (Cohen et al. 2008; Glick et al. 1998). Preliminary experiments of our group (unpublished data) showed that the germination and early growth of *P. strombulifera* are promoted in inoculated seedlings growing under normal or saline conditions. Considering that, we could speculate about an “endophytic consensus” generated between some strains that colonize the same plant, in which everyone expresses one or more mechanisms to jointly determine a global response to promote plant growth or regulate its homeostasis under abiotic stress conditions.

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