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Effect of plant growth-promoting bacteria on the growth and fructan production of *Agave americana* L.

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ABSTRACT

The effect of plant growth-promoting bacteria inoculation on plant growth and the sugar content in *Agave americana* was assessed. The bacterial strains ACO-34A, ACO-40, and ACO-140, isolated from the *A. americana* rhizosphere, were selected for this study to evaluate their phenotypic and genotypic characteristics. The three bacterial strains were evaluated via plant inoculation assays, and *Azospirillum brasilense* Cd served as a control strain. Phylogenetic analysis based on the 16S rRNA gene showed that strains ACO-34A, ACO-40 and ACO-140 were *Rhizobium daejeonense*, *Acinetobacter calcoaceticus* and *Pseudomonas mosselii*, respectively. All of the strains were able to synthesize indole-3-acetic acid (IAA), solubilize phosphate, and had nitrogenase activity. Inoculation using the plant growth-promoting bacteria strains had a significant effect ($p < 0.05$) on plant growth and the sugar content of *A. americana*, showing that these native plant growth-promoting bacteria are a practical, simple, and efficient alternative to promote the growth of agave plants with proper biological characteristics for agroindustrial and biotechnological use and to increase the sugar content in this agave species.

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Introduction

The use of nitrogen-fixing microorganisms and plant growth-promoting bacteria (PGPB) is an important alternative to

replace chemical fertilizers for the cultivation of agricultural plants.

The search for PGPB as well as research on their biological properties are increasing at a rapid pace because efforts are being made to exploit them commercially as inoculants.

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Significant improvement on the growth and yield of crops in response to microbial inoculation has been reported by many workers.^{1–3} Studies confirm that inoculants formulated with PGPB have shown positive effects on the agricultural yield and crop quality.^{2,4} Regarding the effect of PGPB on plant growth, it has been reported that *Glycine max* L. Merrill seedlings inoculated with PGPB (*Pseudomonas* sp. strain AK-1, and *Bacillus* sp. strain SJ-5) demonstrated enhanced plant biomass and that the plants had a higher proline content than control plants.⁵ In another study, the efficiency of *Mesorhizobium*, *Azotobacter*, and *Pseudomonas* on the growth, yield, and disease suppression in chickpea plants (*Cicer arietinum* L.) was evaluated. *Pseudomonas* showed positive IAA production, phosphate solubilization, and antagonistic activities against *Fusarium oxysporum* and *Rhizoctonia solani* compared to other strains.⁶ Martínez-Rodríguez et al.⁷ also reported that cultivable endophytic bacteria from the leaf base of *Agave tequilana* Weber var. Blue have the potential to enhance plant growth.

Scientific evidence supports that the agave genus includes several species of economic, social and cultural importance for people around the world.⁸ Agave plants are greatly relevant to Mexico because this country is considered to be the point of origin of the evolution and diversification of this genus.⁹ Approximately 163 species grow in Mexico, and 123 species are endemic.¹⁰

Agave americana L. has successfully adapted to climatic and edaphic conditions and proliferated in the highlands of Chiapas, Mexico, where it is an important source of natural fibre, medicine, fructans, and traditional alcoholic beverages for the local community. Due to the economic significance of this plant, several commercial plantations have been established in the state of Chiapas to produce sufficient raw materials for agro-industrial use. However, when the plantlets are transplanted to the field, their growth and development is slow, and consequently, 5–7 years are required to obtain mature plants for industrial use.¹¹ An alternative for obtaining mature plants for industrial use is the application of plant growth-promoting bacteria, but it is necessary to assess the possible effects of PGPB on *A. americana* to increase the survival and growth of plantlets. PGPB are rhizosphere bacteria that enhance plant growth by a wide variety of mechanisms, such as phosphate solubilization, siderophore production, biological nitrogen fixation, phytohormone production, antifungal activity, induction of systemic resistance, promotion of beneficial plant-microbe symbioses, and so on.^{12,13}

Many aspects of the microbial community associated with agaves are still unknown and only a manuscript related to¹⁴ suggests that the hypothesis that PGPB inoculation significantly increases the growth and sugar content (mainly inulin) in *A. americana* is true. Therefore, the objective of this study was to evaluate the effect of PGPB inoculation on plant growth and sugar accumulation in *A. americana*.

Materials and methods

Bacterial strains

The bacterial strains ACO-34A, ACO-40, and ACO-140 were chosen subsequent to a study of approximately 235 strains

previously isolated from the rhizosphere of *A. americana*. These three strains were selected based on their capacity for nitrogen fixation, auxin production, P-solubilization and biosynthesis of IAA (Table S1) and were provided by the Instituto Tecnológico de Tuxtla Gutiérrez, while the reference strain *Azospirillum brasilense* Cd was provided by the Centro de Ciencias Genómicas, Cuernavaca, México. All strains were grown in yeast extract-mannitol (YMA) medium¹⁵ at 28 °C and preserved at 4 °C until use.

Phenotypic and genotypic analysis of strains

The cell morphologies of the strains isolated from *A. americana* were examined by light microscopy (Zeiss® PS7, Germany). The Gram reaction was determined using a kit (Merck®, Germany), according to the manufacturer's procedure, and colony morphology was determined with cells grown on YMA plates at 28 °C for 5 days.¹⁶

Bacteriological and physiological characterization of strains ACO-34A, ACO-40, and ACO-140 were performed with isolates from YMA medium. Salt tolerance was evaluated at 28 °C with 0.5, 1.0, 2.0, 3.0 and 5.0% (w/v) NaCl and pH levels of 4.0, 5.0, 9.0 and 11.0. Acid or alkali production was determined on the same medium supplemented with 25 mg mL⁻¹ bromothymol blue as a pH indicator.¹⁶ Antibiotic resistance was tested on YMA plates following the process recommended by Martínez-Romero et al.¹⁷ In addition, the Al and Cu tolerance of the strains were determined on solid YMA medium.¹⁸

16S rRNA gene sequencing and phylogenetic analysis

The strains were grown in 2.0 mL of YMA medium overnight. Total genomic DNA was extracted using a DNA Isolation Kit for Cells and Tissues (Roche®, Switzerland), according to the manufacturer's specifications. PCR was performed with the bacterial universal 16S rRNA primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3'), which amplified products of approximately 1500 bases, and procedures were performed as described by Weisburg et al.¹⁹ The PCR products were purified using the PCR Product Purification System Kit from Roche® and sequenced (Macrogen®, Korea). All sequences were compared with the reference sequences obtained by a BLAST search.²⁰ The sequences were aligned using the CLUSTAL X (2.0) software with the default settings.²¹ Minor modifications in the alignment were made using the BIOEDIT sequence editor. Phylogenetic and molecular evolutionary analyses were performed with MEGA v5.2.²² The phylogenetic tree of the 16S rRNA gene sequences from type strains was constructed by Neighbour-Joining²³ and a Bootstrap analysis with 1000 pseudoreplicates using the Tamura-Nei model.²⁴ The 16S rRNA gene sequence of strains ACO-34A, ACO-40, and ACO-140 were deposited in the GenBank database under the accession numbers KM349967, KM349968, and KM349969, respectively. Additionally, strains ACO-34A, ACO-40, and ACO-140 were deposited in DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), Germany as an open collection under the deposit numbers DSM 101606, DSM 01771 and DSM 01784, respectively.

Measurement of PGPB efficiency

Quantification of IAA production

Bacteria were grown in conical flasks containing 50 mL of YMA medium composed of mannitol (0.25 g L^{-1}), K_2HPO_4 10% (5 mL L^{-1}), MgSO_4 10% (2 mL L^{-1}), NaCl 10% (1 mL L^{-1}), CaCO_3 (1 g L^{-1}) and yeast extract (3 g L^{-1}) at a pH of 6.8, supplemented with 100 mg L^{-1} of L-tryptophan. After incubation at $30 \pm 2^\circ \text{C}$ on a rotary shaker for 48 h, the culture medium was centrifuged at $5000 \times g$ for 10 min, and the supernatant was filtered through a $0.22\text{-}\mu\text{m}$ membrane filter. The IAA levels in the filtered supernatants of each strain were measured by high-performance liquid chromatography (HPLC) using a PerkinElmer model series 200A equipped with a Supelco LiChrosorb RP C_{18} column ($5 \mu\text{m}$; 4.6 by 150 mm). The mobile phase was acetonitrile– $50 \text{ mM KH}_2\text{PO}_4$ (pH 3) (30/70) at a flow rate of 1 mL min^{-1} . Eluates were analyzed with a diode array detector at 220 nm, and IAA was quantified by integrating the area under the peak; authentic IAA (Sigma) was used as a standard. The IAA produced by each strain was measured in triplicate.²⁵

Estimation of phosphate solubilization in broth assay by PGPB

The isolates were individually grown in YM broth medium overnight, and the $\text{OD}_{600 \text{ nm}}$ was adjusted to 1.0. The cells were washed twice in 0.85% sterile Ringers solution before inoculating in National Botanical Research Institute Phosphate (NBRIP) growth medium containing insoluble tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$).²⁶ The pH of the NBRIP medium was adjusted to 7.0 before autoclaving. The strains were inoculated in 20-mL vials containing NBRIP medium and incubated at 30°C on a shaker (150 rpm) for 5 days. After incubation, 5.0 mL of each strain was taken and centrifuged, and the pH of the supernatant was recorded. The available phosphorus content in the culture supernatant as well as the control (supernatant obtained from medium not inoculated with bacteria) was estimated using the vanado-molybdate colorimetric method²⁷ by measuring the absorbance at a wavelength of 420 nm. Each treatment was replicated three times.

Assay for acetylene reduction activity (ARA)

The nitrogen fixation ability of the PGPB strains was determined by the acetylene reduction activity.²⁸ The tubes were sealed with rubber stoppers after inoculation and then incubated for 24 h in NFB medium.²⁹ Ten percent of the air was removed from each tube, and an equal volume of acetylene was injected using a syringe, before incubating the tubes at 30°C for 24 h. A parallel uninoculated control tube was prepared. A 100- μL air sample was taken, and the amount of ethylene produced from acetylene was determined by gas chromatography (Perkin Elmer, USA). The ARA value was reported to as $[\text{ARA nmol C}_2\text{H}_4 \text{ per culture h}^{-1}]$.

Plant inoculation assay

The PGPB strains ACO-34A, ACO-40, and ACO-140 isolated from the *A. americana* rhizosphere and the reference strain *A. brasilense* Cd were evaluated in a biofertilization test. *A. americana* plantlets obtained by micropropagation were planted in polystyrene trays that contained peat as the substrate (the pH

of the peat was adjusted to 6.7 using $35 \text{ g Na}_2\text{CO}_3$ for each 100 g of material) and covered with a polyethylene sheet to maintain the humidity and prevent dehydration. Plants were maintained in a growth chamber at 28°C and 38% relative humidity (RH) with a 14 h light/10 h dark photoperiod. Lighting was provided by fluorescent lamps with $50 \mu\text{E m}^{-2} \text{ s}^{-1}$.³⁰

After two months (60 days after transplantation), the plants were transferred to pots containing peat moistened with free N Fahraeus medium [CaCl_2 , 0.1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.12 g; KH_2PO_4 , 0.1 g; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.15 g; Fe citrate, 0.005 g; Mn, Cu, Zn, B, Mo traces; dist. water, 1000 mL; pH 6.5] as a nutrient source³¹ and placed in a greenhouse at $25 \pm 2^\circ \text{C}$ (natural illumination). The plants were inoculated with 2 mL of a suspension of each PGPB strain at a concentration of $1 \times 10^6 \text{ UFC mL}^{-1}$.^{30,32} Uninoculated plants and others treated with 30 mg of KNO_3 -N per plant served as controls. Four replicates were used per treatment, and the plants were arranged in a completely randomized design. The plants were grown under greenhouse conditions for 90 days. Measurements of the plants, the dry weight, the diameter of the stem, the number of leaves, and the length of the roots were made on the plants during the transplantation phase (m_1) and after 90 days (m_2) in the greenhouse. The data used for statistical analysis were the difference between the m_2 - m_1 measurements.

Extraction and quantification of carbohydrates

The carbohydrates were extracted and quantified from the dried leaves and roots, which were washed with ultrapure water. The samples were crushed and placed in a water bath at 80°C for 40 min, the supernatant was removed by centrifugation at $16,000 \times g$ for 15 min at room temperature, and the precipitate was removed. Samples were adjusted to pH 7.0, filtered through 20- and $45\text{-}\mu\text{m}$ nylon membranes, and stored at 8°C until use. Carbohydrates were identified with a previously described modified method.³³ Briefly, thin layer chromatography was performed using propanol–butanol–water (12:3:4) for the mobile phase and Merck® F254 silica gel plates for the stationary phase. Spots were developed with a solution containing 45% (v/v) aniline (4% v/v in acetone), 45% (v/v) diphenylamine (4% (v/v) in acetone) and 9.1% (v/v) of 85% phosphoric acid. The developing solution was applied to the plates and allowed to dry. The plates were heated to 80°C for 5, 10, and 15 min until a clearly defined colour appeared. The aldoses had a blue-grey colour, and the ketoses and sucrose were red or a mixture of both colours.

For the quantification of carbohydrates, samples were adjusted to pH 7.0 and filtered through 20- and $45\text{-}\mu\text{m}$ membranes before being placed in 2-mL vials. A 10- μL sample of plant extract was injected into a HPLC-IR (Thermo Finnigan®, Ontario, Canada) equipped with a Rezex RCM-monosaccharide Ca^{2+} column, with water as the mobile phase at a temperature of 85°C , a flow rate of 0.3 mL min^{-1} , and a pressure of 300 psi. Inulin from chicory root, fructose (Sigma®, USA), sucrose, and glucose (Baker®, USA) were used as standards.³³

Statistical analysis

All of the data obtained in the tests for inoculation, P-solubilization, IAA production, acetylene reduction activity

(ARA), and carbohydrate quantification using different PGPB strains were statistically analyzed by analysis of variance (ANOVA), and the means were compared by Tukey's test ($p < 0.05$).³⁴

Results and discussion

Investigation of PGPB strains from different plants with the potential to be used as inoculants has increased in recent years, and many are commercially available.^{12,13,35} Taxonomic polyphasic studies are commonly used to analyze bacterial diversity and include morphological, physiological, biochemical, metabolic, and principally, phylogenetic studies.^{36–38} The morphological and physiological characteristics of the bacterial strains isolated from the *A. americana* rhizosphere that have potential as inoculants are shown in Table S2. The strains evaluated were aerobic, Gram-negative, rod-shaped, and grew rapidly in YMA medium. The cells grew at 37 °C and had an acidic reaction. The ACO-40 strain formed circular, lightly opaque yellow colonies from 0.5 to 1.5 mm in diameter with a mucoid appearance. On the other hand, the ACO-140 strain formed circular, creamy beige colonies with regular borders, measured approximately 3–4 mm in diameter, and had no observable pigmentation. Finally, strain ACO-34A was characterized by the formation of circular, semitranslucent creamy colonies that were 1.5–3.0 mm in diameter with a mucoid appearance. These three strains could grow in a pH range from 4.0 to 9.0. As for NaCl tolerance, the ACO-140 strain grew in 5.0% NaCl, but the ACO-34A and ACO-40 strains tolerated no more than 2.0% NaCl. The ACO-34A strain was resistant to ampicillin (100 µg mL⁻¹), carbenicillin (20 µg mL⁻¹), chloramphenicol (100 µg mL⁻¹), and kanamycin (100 µg mL⁻¹). The ACO-40 strain showed a greater sensitivity to the antibiotics assessed. The strains grew in the presence of Al³⁺ (500 µg mL⁻¹) and Cu²⁺ (100 µg mL⁻¹). These results indicated that some of the biological qualities that distinguished these strains included their capacity to grow in a wide pH range, as well as their ability to tolerate different NaCl concentrations and heavy metals, such as Al and Cu. For example, strain ACO-140 managed to grow in 5.0% NaCl. This outcome is of great significance because salinity is one of the harshest environmental factors that prevents a high crop yield, and most agricultural plants are sensitive to a high salt concentration in the soil. Therefore, plant growth promoting bacteria (PGPB) act as one of the most effective tools for the

alleviation of salt stress. *Azospirillum lipoferum* JA4, a genetically tagged strain, was capable of colonizing the roots of wheat seedlings in the presence of a high NaCl concentration.³⁹ Puente et al.⁴⁰ reported that strains of *Azospirillum halopraeferens* and *A. brasilense* survived in seawater and were capable of colonizing the root surfaces of black mangrove seedlings. In light of this, the ACO-140 strain could be an alternative to promote *A. americana* growth under salt stress conditions, as has been previously demonstrated with barley and oats.^{41,42} Additionally, this study demonstrated that these three strains could grow at low pH and tolerate high concentrations of aluminium and copper. PGPB, such as *Pseudomonas fluorescens*, can produce oxalic acid, which also might be a possible mechanism for reducing Al toxicity.⁴³ *Rhizobium* sp. strain BICC 651 produced a threefold higher level of siderophore in the presence of 100 µM Al³⁺, which could be a mechanisms to relieve Al stress,⁴⁴ suggesting that the application of such bacteria may be an efficient approach to alleviate aluminium toxicity and eventually improve soil fertility. Strain ACO-34A was resistant to various antibiotics, which could be important because it could protect the plant against phytopathogens and establish an area in the rhizosphere with a greater capacity to assimilate soil nutrients.⁴⁵

We used a polyphasic approach in this study that combined the phenotypic characteristics of the bacterial strains with a phylogenetic analysis of their 16S rRNA gene sequences to determine the taxonomic status of strains isolated from the *A. americana* rhizosphere (Fig. 1). Strain ACO-40 had a sequence size of 1381 bp, was affiliated with members of the genus *Acinetobacter*, and showed a 99.1% genetic identity with the type strain *Acinetobacter calcoaceticus* NCCB22016^T (Table 1). The 16S rRNA gene sequence of strain ACO-140 was 1387 bp and was classified in the genus *Pseudomonas*, with a 100.0% genetic similarity to *Pseudomonas mosselii* CIP 105259^T.⁴⁶ Finally, the strain ACO-34A sequence was 1333 bp and clustered with members of the genus *Rhizobium*; its 16S rRNA gene sequences had a 96.1% similarity with *Rhizobium daejeonense* L61^T.³⁶

The highest amount of IAA was produced by the *P. mosselii* strain ACO-140 (15.7 mg L⁻¹), followed by the *R. daejeonense* strain ACO-34A and *A. calcoaceticus* strain ACO-40 (Table 2). The reference strain *A. brasilense* Cd also produced a significant amount of IAA (10.4 mg L⁻¹), according to Tukey's test ($p < 0.05$). The capacity of the rhizobial strains to solubilize phosphate was evaluated with a colorimetric method using NBRIP growth medium that contained insoluble tricalcium phosphate. All

Table 1 – Molecular identification of the PGPB strains isolated from *Agave americana* L.

Strain	Closest partial 16S rRNA gene sequence	Accession No.	16S rRNA seq. (bp)	Closest NCBI match/species identity	Reference
ACO-34A	<i>Rhizobium daejeonense</i>	KM349967	1333	<i>R. daejeonense</i> L61 ^T (AY341343) 96.1%	Zhe-Xue et al. ³⁶
ACO-40	<i>Acinetobacter calcoaceticus</i>	KM349968	1381	<i>A. calcoaceticus</i> NCCB22016 ^T (AJ888983) 99.1%	Unpublished
ACO-140	<i>Pseudomonas mosselii</i>	KM349969	1387	<i>P. mosselii</i> CIP 105259 ^T (AF072688) 100%	Dabboussi et al. ⁴⁶

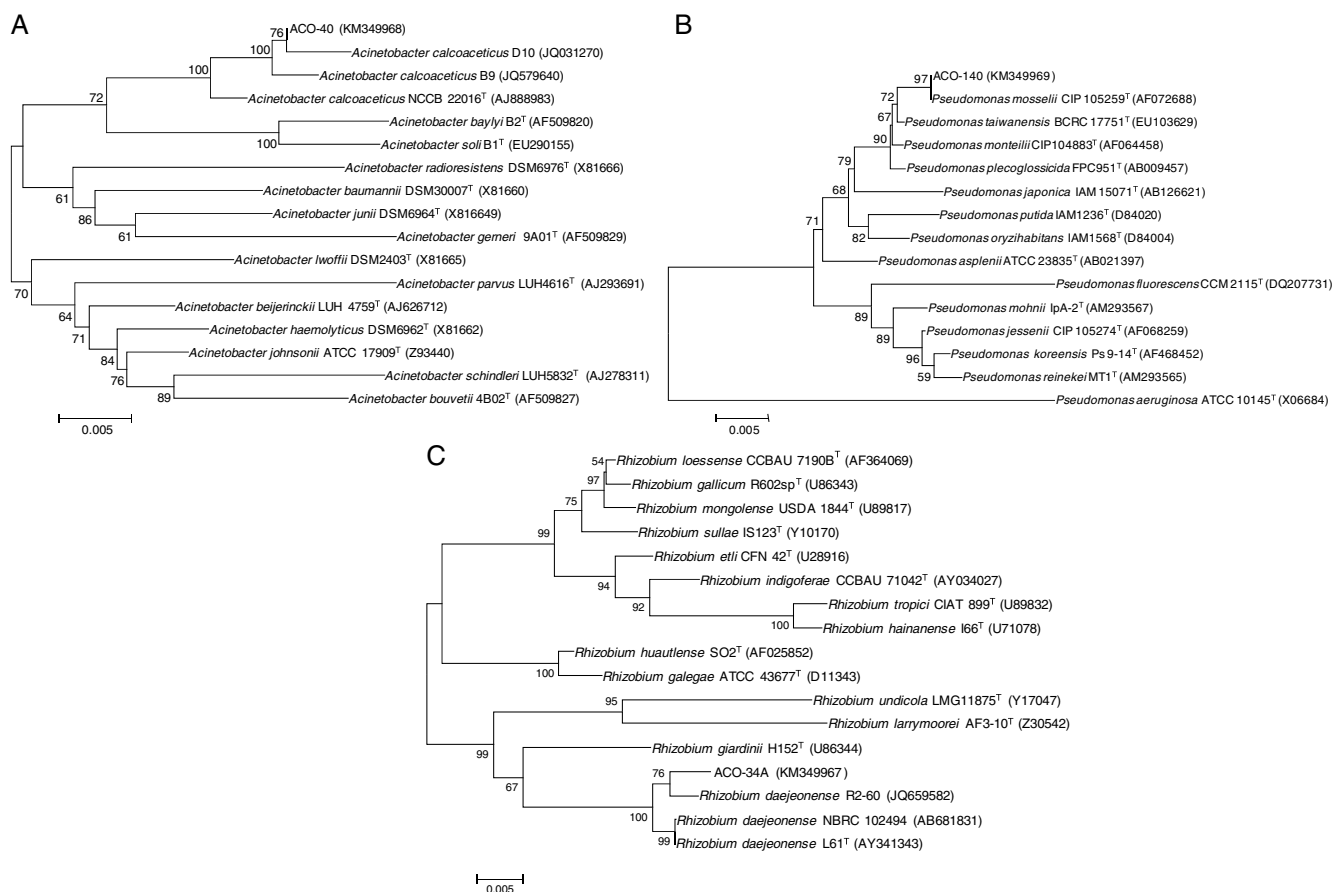


Fig. 1 – Neighbour-joining phylogenetic trees based on 16S rRNA gene sequences. (A) *Acinetobacter calcoaceticus* strain ACO-40 (1381 bp), (B) *Pseudomonas mosselii* strain ACO-140 (1387 bp) and (C) *Rhizobium daejeonense* strain ACO-34A (1333 bp). Only bootstrap values > 50% are shown. Type strains are indicated by the superscript T. The accession numbers for the sequences are indicated within the parentheses. Those generated in this work are shown in bold.

four strains had the capacity to solubilize phosphate after 2–4 days of incubation. Strain ACO-140 exhibited the highest phosphate solubilizing activity (37.8 mg L^{-1}), followed by strain ACO-40 (29.8 mg L^{-1}) and strain ACO-34A (24.7 mg L^{-1}) (Table 2). The pH value of the culture medium decreased from

an initial pH of 6.8 to 4.5 as bacterial growth progressed, suggesting that the bacteria might secrete organic acids to solubilize the insoluble phosphorus.

Recently, endophytic bacteria belonging to the genera *Acinetobacter*, *Bacillus* and *Pseudomonas* with a capacity for

Table 2 – Phosphate solubilization, IAA production, and acetylene reduction activity (ARA) in the PGPB strains isolated from *Agave americana* L.

Treatment	IAA production (mg L^{-1})	P-solubilization (mg L^{-1})	ARA ^b nmol C_2H_4 per culture h^{-1}
<i>Pseudomonas mosselii</i> ACO-140	15.7 A ^a	37.8 A	167.1 B
<i>Acinetobacter calcoaceticus</i> ACO-40	8.5 C	29.8 B	106.1 B
<i>Rhizobium daejeonense</i> ACO-34A	10.3 B	24.7 B	627.4 A
<i>Azospirillum brasilense</i> Cd	10.4 B	24.2 B	821.3 A
MSD ^c ($p < 0.05$)	1.65	7.63	224.39

^a Mean values of four replicates. Means followed by the same letter do not show any significant differences ($p < 0.05$).

^b ARA, acetylene reduction assay ($\mu\text{mol C}_2\text{H}_4$ per culture fresh weigh h^{-1}).

^c MSD, minimum significant difference.

Table 3 – Growth parameters for *Agave americana* plants inoculated with plant growth-promoting bacteria.

Treatment	Plant dry weight (g)	Stem diameter (cm)	Number of leaves	Root length (cm)
<i>Pseudomonas mosselii</i> ACO-140	2.44 B [*]	0.48 A	3.0 A	11.3 CD
<i>Acinetobacter calcoaceticus</i> ACO-40	1.23 D	0.27 CD	2.7 AB	11.3 CD
<i>Rhizobium daejeonense</i> ACO-34A	3.41 A	0.37 B	4.0 A	26.6 A
<i>Azospirillum brasilense</i> Cd	1.65 C	0.34 BC	1.5 BC	16.2 B
KNO ₃ -N	1.07 D	0.23 D	1.5 BC	14.3 BC
Uninoculated	0.76 E	0.18 D	1.2 C	9.8 D
MSD [†] ($p < 0.05$)	0.2975	0.0994	1.4510	3.1339

* Mean values of four replicates. Means followed by the same letter do not show a significant difference ($p < 0.05$).

† MSD, minimum significant difference.

nitrogen fixation, auxin production and phosphate solubilization were isolated from blue agave plants (*A. tequilana*) from Nayarit, Mexico.⁷ *Pseudomonas putida* M5TSA, isolated from an endemic cactus *Mammillaria fraileana* that grows in the Sonoran Desert, solubilized inorganic phosphate and demonstrated rock-weathering capacity.⁴⁷ These pseudomonads are characterized by biochemical mechanisms and specific enzymes that facilitate rock degradation, as well as the biosynthesis of important secondary metabolites, under varied biotic and abiotic stress conditions.⁴⁸

Inoculation of plants with PGPB enhances the assimilation of essential nutrients and plant-associated biological nitrogen fixation.^{13,49} In this study, nitrogenase activity was assessed with the acetylene reduction assay (ARA) to determine the ability of PGPB to fix nitrogen. *A. brasilense* Cd and *R. daejeonense* ACO-34A strains showed maximum nitrogenase activity (821.3 and 627.4 nmol C₂H₄ per culture h⁻¹, respectively) compared to the other strains evaluated (Table 2). Thus, the three strains showed the potential to produce IAA and solubilize phosphate, as well as nitrogen-fixation capacity. These results are important because nitrogen and phosphorus are key elements for the growth and metabolism of agave plants.

Furthermore, inoculation with PGPB had a positive effect on the growth of *A. americana* plants (Table 3). *A. calcoaceticus* strain ACO-40, *P. mosselii* strain ACO-140, and *R. daejeonense* strain ACO-34A all had positive effects on the plant dry weight, stem diameter, number of leaves, and root length compared to uninoculated control plants and to those with added KNO₃. On average, plants inoculated with *R. daejeonense* strain ACO-34A weighed 2.65 g more than uninoculated plants at 90 days post inoculation. The stem diameter of plants inoculated with *P. mosselii* strain ACO-140 differed significantly ($p < 0.05$) from that obtained in the other treatments. Plants treated with the PGPB strains ACO-40, ACO-140, and ACO-34A experienced similar effects for the number of leaves compared to uninoculated plants according to analysis by Tukey's test ($p < 0.05$). Plants inoculated with strain ACO-34A showed a significantly higher root length compared to other treatments ($p < 0.05$). Bashan et al.,⁵⁰ reported that the inoculation of pachycereid cacti species (*Pachycereus pringlei*, *Stenocereus thurberi* and *Lophocereus schottii*) enhanced the establishment and development of the cacti that were inoculated with *A. brasilense* and transplanted into a disturbed urban desert soil. Puente et al.,⁵¹

demonstrated that an association between the giant cardon cactus *P. pringlei* and endophytic bacteria helped the seedlings become established and grow in a soilless environment. It has also been reported that *Enterobacter sakazakii* M2PFe, *Azotobacter vinelandii* M2Per and *P. putida* M5TSA, isolated from the rock-dwelling cactus *M. fraileana*, affected plant growth and the mobilization of elements from rocks.⁵² In this study, similar results were found for *R. daejeonense* strain ACO-34A, which significantly influenced *A. americana* growth, demonstrating its potential as a PGPB due to its capacity for nitrogen fixation, phosphate solubilization, and IAA biosynthesis. PGPB strains such as *A. brasilense* and *R. daejeonense* are biological models that may potentially contribute to the revegetation of eroded soil. Similar results concerning the occurrence and diversity of diazotrophic bacteria in rhizosphere soil and in root and leaf tissues of *Agave sisalana* plants have been reported by Santos et al.,⁵³ as well as a test of their potential for plant growth promotion. Therefore, PGPB strains investigated in this study could be alternative *A. americana* inoculants that would improve its growth and development.

The sugar content in the leaves, stems, and plant roots was measured with a qualitative and quantitative analysis. Thin layer chromatography (Fig. 2) showed that fructan synthesis was different in different plant tissues and that bacterial inoculation influenced the degree of polymerization (PD) of the fructans. Fructose and sucrose were detected in the leaves, stems, and roots of *A. americana* plantlets inoculated with the four bacteria. However, kestose was detected in *A. americana* plantlets inoculated with all of the bacterial strains, except in plantlets inoculated with *A. calcoaceticus* strain ACO-40 (Fig. 2B). Spots corresponding to sucrose were detected with greater intensity in the leaves and the stems compared to roots (Fig. 2B and C). Nystose and other spots corresponding to fructans with PD > 4 were detected in the stems of *A. americana* plantlets inoculated with *P. mosselii* strain ACO-140 (Fig. 2A). Kestose was only detected in plants inoculated with *P. mosselii*. This result may indicate that the induction of kestose and nystose biosynthesis is specific and depends on the inoculated microbial species. Therefore, this phenomenon requires further investigation.

In addition, the inulin concentration varied from 0.23 to 1.09 mg g⁻¹ in the leaves and was higher in plantlets inoculated with *R. daejeonense* strain ACO-34A (Table 4).

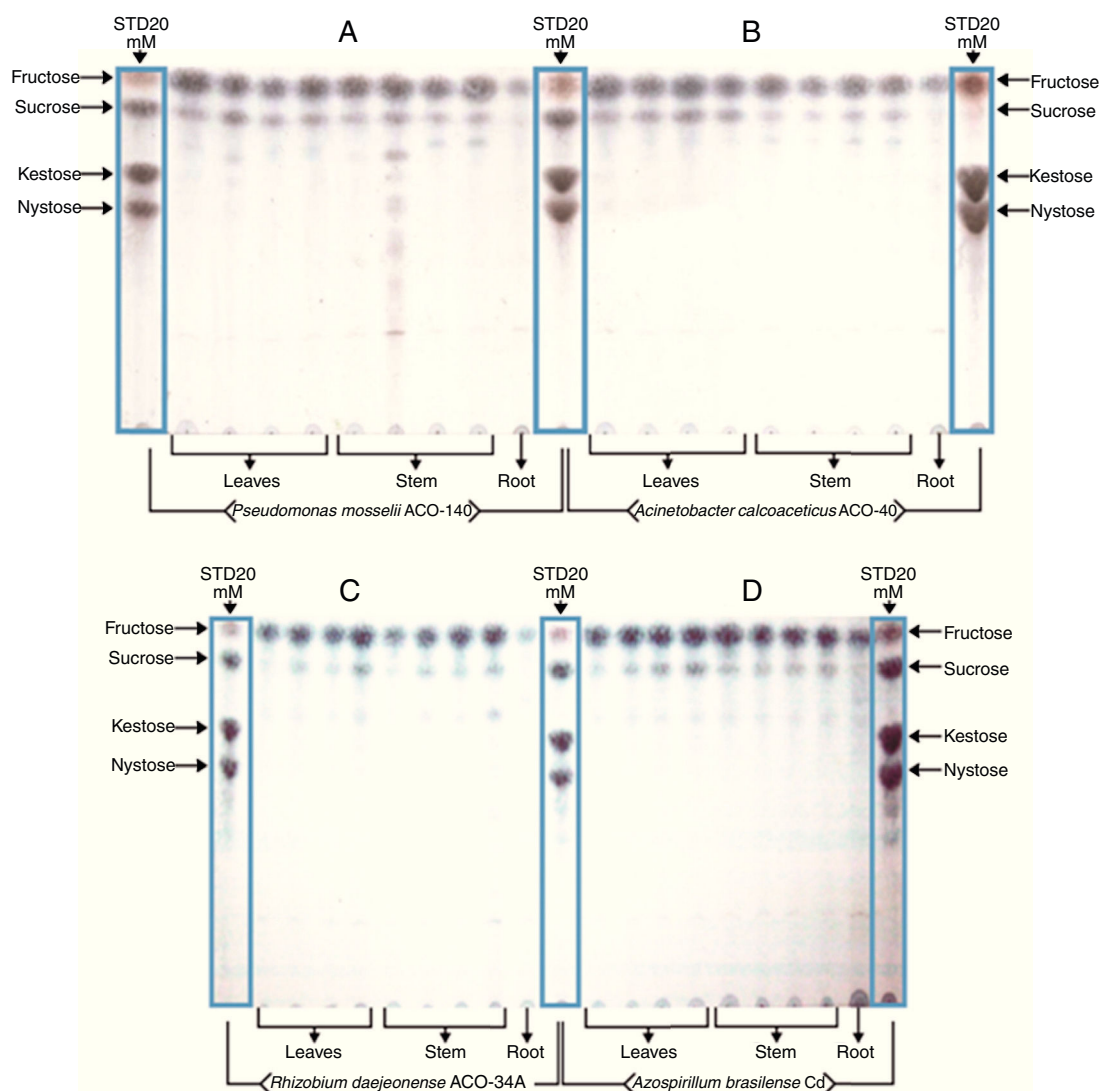


Fig. 2 – Thin layer chromatography (TLC) analysis of fructan exohydrolase and fructan:fructan 1-fructosyltransferases (1-FF1) enzymatic activity in extracts from leaves, stems, and root tissue of in vitro-cultured *Agave americana* plantlets inoculated with (A) *Pseudomonas mosselii* strain ACO-140, (B) *Acinetobacter calcoaceticus* strain ACO-40, (C) *Rhizobium daejeonense* strain ACO-34A, and (D) *Azospirillum brasilense* Cd. The markers represent the mobility of fructose, sucrose, kestose, and nystose.

Nevertheless, Tukey's test showed that the inulin concentration was not significantly different between the uninoculated plants and KNO_3 fertilized plants. This demonstrates the biological potential of the agave species to biosynthesize various types of metabolites such as sugars despite unfavourable growth conditions.⁵⁴ In the roots, the uninoculated plants had a greater inulin concentration than the plants inoculated with the various PGPB.

Significant differences ($p < 0.05$) in the sucrose content of *A. americana* leaves were observed among treatments. Plants inoculated with the PGPB strains had a greater sucrose concentration than the uninoculated plants and those treated with KNO_3 . In the plant roots, no significant difference was observed among treatments for sucrose concentration.

Similarly, the concentration of glucose and fructose in the leaves was higher in plants treated with the *R. daejeonense* strain ACO-34A and *P. mosselii* strain ACO-140, indicated by

Tukey's test ($p < 0.05$). The glucose concentration in the roots was significantly higher in plants inoculated with the *R. daejeonense* strain ACO-34A, and the fructose concentration was higher in plants inoculated with the *A. calcoaceticus* strain ACO-40 compared to the rest of the treatments. These results indicate that fructan synthesis is different in different tissues, and with respect to the inoculated bacteria (Fig. 2). The sugar content in agave plants was different amounts for plants of different physiological ages.⁵⁵ Cedeño¹¹ reported that young *A. tequilana* plants had higher levels of free monosaccharides (glucose, fructose), than adult plants that accumulated fructan from 8 to 12 years. The evaluation of sucrose, fructose and glucose is important because Trevisan et al.⁵⁶ reported that these sugars act on the metabolism of fructans, such as kestose and nystose. The concentration of fructose and glucose in the stems results from the active hydrolysis of fructans by fructanexohydrolase. Sucrose is biosynthesized via crassulacean

Table 4 – Effect of inoculation with plant growth-promoting bacteria on sugar accumulation in leaves and roots of *Agave americana* L.

Treatment	Inulin	Sucrose	Glucose	Fructose	Inulin	Sucrose	Glucose	Fructose
	(mg g ⁻¹) Leaves				(mg g ⁻¹) Root			
<i>Pseudomonas mosselii</i> ACO-140	0.99 A*	1.04 AB	1.97 A	0.561 A	0.35 D	0.17 A	0.02 D	0.031 B
<i>Acinetobacter calcoaceticus</i> ACO-40	0.23 C	1.50 A	0.78 BC	0.054 C	0.84 B	0.16 A	0.19 B	0.040 A
<i>Rhizobium daejeonense</i> ACO-34A	1.09 A	1.59 A	1.43 A	0.856 A	0.76 BC	0.23 A	0.35 A	0.033 B
<i>Azospirillum brasilense</i> Cd	0.28 B	0.77 BC	0.91 BC	0.217 BC	0.43 E	0.15 A	0.08 C	0.032 B
KNO ₃ -N	0.44 AB	0.68 BC	0.63 C	0.131 BC	0.28 E	0.14 A	0.03 D	0.033 B
Uninoculated	0.63 AB	0.75 BC	0.84 BC	0.140 BC	0.93 A	0.27 A	0.08 C	0.012 C
MSD [‡] (<i>p</i> < 0.05)	0.740	0.726	0.628	0.318	0.022	0.146	0.108	0.045

* Mean values of four replicates. Means followed by the same letter do not show significant difference (*p* < 0.05).

‡ MSD, minimum significant difference.

acid metabolism in *A. americana* plants. This disaccharide is a precursor to the synthesis of fructans and is hydrolysed by the action of vacuolar invertase, generating glucose and fructose.^{57,58} The 1-FFT enzyme subsequently converts the fructose moiety to 1-kestose, which is synthesized by the 1-SST enzyme, causing glucose to be released.⁵⁹ 1-kestose is the precursor of all fructans.³³ Detection of neo-kestose indicated 6G-FFT enzyme activity.^{60,61} Fructans with degrees of polymerization DP > 4 that were found in stem samples from plantlets inoculated with the *P. mosselii* strain ACO-140 could be the result of microbial fructosyltransferase activity involved in microbial fructan levan, inulin, or fructo-oligosaccharide biosynthesis.

It is also worth noting that the concentration of inulin and other sugars was increased in plantlets inoculated with the *R. daejeonense* strain ACO-34A and *P. mosselii* strain ACO-140 primarily in the leaves but not in the roots (Table 4). In another study, bacterial endophytes isolated from *A. tequilana* leaves showed the capacity for nitrogen fixation, auxin production, and phosphate solubilization and also increased inulin production.⁷

Conclusions

The *R. daejeonense* strain ACO-34A, *A. calcoaceticus* strain ACO-40, and *P. mosselii* strain ACO-140 showed potential as PGPB due to their phenotypic characteristics as well as their capacity for nitrogen fixation, phosphate solubilization, and IAA biosynthesis. Additional studies are necessary to evaluate the use of these bacteria for commercial application in *A. americana* culture to improve its growth and development and, most importantly, to increase its fructan and inulin contents.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bjmm.2016.04.010](https://doi.org/10.1016/j.bjmm.2016.04.010).

REFERENCES

- Bashan Y, Holguin G, de-Bashan LE. Azospirillum–plant relationships: physiological, molecular, agricultural, and environmental advances (1997–2003). *Can J Microbiol.* 2004;50:521–577. <http://dx.doi.org/10.1139/W04-035>.
- Bashan Y, de-Bashan LE. Bacteria/Plant Growth-Promotion. In: Hillel D, editor. *Encyclopedia of Soils in the Environment*. vol. 1. Oxford, UK: Elsevier; 2005:103–115.
- Lugtenberg BJJ, Kamilova F. Plant-growth-promoting rhizobacteria. *Annu Rev Microbiol.* 2009;63:541–556.
- Zahir ZA, Arshad M, Frankenberger WT. Plant growth promoting bacteria: applications and perspectives in agriculture. *Adv Agron.* 2003;81:97–168.
- Kumari S, Vaishnav A, Jain S, Varma A, Choudhary DK. Bacterial-mediated induction of systemic tolerance to salinity with expression of stress alleviating enzymes in Soybean (*Glycine max* L. Merrill). *J Plant Growth Regul.* 2015;34:558–573.
- Verma JP, Yadav I, Tiwari KN, Jaiswal DK. Evaluation of plant growth promoting activities of microbial strains and their effect on growth and yield of chickpea (*Cicer arietinum* L.) in India. *Soil Biol Biochem.* 2014;70:33–37.
- Martínez-Rodríguez JD, De la Mora-Amutio M, Plascencia-Correa LA, et al. Cultivable endophytic bacteria from leaf bases of *Agave tequilana* and their role as plant growth promoters. *Braz J Microbiol.* 2014;45:1333–1339.

8. Ahumada-Santos YP, Montes-Avila J, Uribe MD, et al. Chemical characterization, antioxidant and antibacterial activities of six Agave species from Sinaloa, Mexico. *Ind Crop Prod*. 2013;49:143–149.
9. García-Mendoza AJ. Distribution of the genus *Agave* (Agavaceae) and its endemic species in Mexico. *Cact Succ J*. 2002;74:177–187.
10. Delgado-Lemus A, Torres I, Blancas J, Casas A. Vulnerability and risk management of *Agave* species in the Tehuacán Valley, México. *J Ethnobiol Ethnomed*. 2014;10:1–15.
11. Cedeño MC. Tequila production. *Crit Rev Biotechnol*. 1995;15:1–11.
12. Bhattacharyya PN, Jha DK. Plant growth-promoting bacteria (PGPB): emergence in agriculture. *World J Microbiol Biotechnol*. 2013;28:1327–1350.
13. Bashan Y, de-Bashan LE, Prabhu SR, Hernandez JP. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant Soil*. 2014;378:1–33.
14. Desgarenes D, Garrido E, Torres-Gomez MJ, Pena-Cabriaes JJ, Partida-Martinez LP. Diazotrophic potential among bacterial communities associated with wild and cultivated *Agave* species. *FEMS Microbiol Ecol*. 2014;90:844–857.
15. Vincent JM. *A Manual for the Practical Study of Root Nodule Bacteria*. Oxford, UK: Blackwell Scientific; 1970:176–189.
16. Toledo I, Lloret L, Martínez-Romero E. *Sinorhizobium americanum* sp. nov., a new *Sinorhizobium* species nodulating native *Acacia* spp. in Mexico. *Syst Appl Microbiol*. 2003;26:54–64.
17. Martínez-Romero E, Segovia L, Mercante FM, Franco AA, Graham P, Pardo MA. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int J Syst Bacteriol*. 1991;41:417–426.
18. Zhang X, Harper R, Karsisto M, Lindström K. Diversity of *Rhizobium* bacteria isolated from the root nodules of leguminous trees. *Int J Syst Bacteriol*. 1991;41:104–113.
19. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal amplication for phylogenetic study. *J Bacteriol*. 1991;73:697–703.
20. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. 1990;215:403–410.
21. Larkin MA, Black-Shields G, Brown NP, et al. Clustal W and Clustal X version 2.0. *Bioinformatics*. 2007;23:2947–2948.
22. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA 5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*. 2011;28:2731–2739.
23. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*. 1987;4:406–425.
24. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol*. 1993;10:512–526.
25. Zakharova EA, Shcherbakov AA, Brudnik VV, Skripko NG, Bulkhin NS, Ignatov VV. Biosynthesis of indole-3-acetic acid in *Azospirillum brasilense* Insights from quantum chemistry. *Eur J Biochem*. 1999;259:572–576.
26. Nautiyal CS. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol Lett*. 1999;170:265–270.
27. Fiske CH, Subbarow Y. A colorimetric determination of phosphorus. *J Biol Chem*. 1925;66:375–400.
28. Burris RH. Nitrogen fixation assay – methods and techniques. *Methods Enzymol*. 1972;24:415–431.
29. Döbereiner J, Marriel I, Nery M. Ecological distribution of *Spirillum lipoferum* Beijerinck. *Can J Microbiol*. 1976;22:1464–1473.
30. Ruíz-Valdiviezo VM, Ventura-Canseco LMC, Castillo Suárez LA, Gutiérrez-Miceli FA, Dendooven L, Rincón-Rosales R. Symbiotic potential and survival of native rhizobia kept on different carriers. *Braz J Microbiol*. 2015;46:735–742.
31. Fahraeus G. The infection of clover root hair by nodule bacteria studied by a single glass slide technique. *J Gen Microbiol*. 1957;16:374–381.
32. Bashan Y. Significance of timing and level of inoculation with rhizosphere bacteria on wheat plants. *Soil Biol Biochem*. 1986;18:297–301.
33. Vizcaino-Rodríguez LA, Rodríguez-Mendiola MA, Mancilla-Margalli NA, Ávila-Miranda ME, Osuna-Castro JA, Arias-Castro C. Biosíntesis in vitro de oligofructanos: inulinas y neoinulinas por fructosiltransferasas de *Agave tequilana* Weber var. Azul y *A. inaequidens* subsp. *Inaequidens* Koch. *Gayana Bot*. 2012;69:66–74.
34. SAS Institute Inc. *SAS/STAT User's Guide*. Version 6.0. 4th ed. Cary, NC: SAS Institute Inc.; 1989:3471–3487.
35. Sanchez AC, Gutierrez RT, Santana RC, Urrutia AR, Michiels J, Vanderleyden J. Effects of co-inoculation of native *Rhizobium* and *Pseudomonas* strains on growth parameters and yield of two contrasting *Phaseolus vulgaris* L. genotypes under Cuban soil conditions. *Eur J Soil Biol*. 2014;62:105–112.
36. Zhe-Xue Q, Hee-Sung B, Jong-Hwan B, Wen-Feng C, Wan-Taek I, Sung-Taik L. *Rhizobium daejeonense* sp. nov. isolated from a cyanide treatment bioreactor. *Int J Syst Evol Microbiol*. 2005;55:2543–2549.
37. Mulet M, Gomila M, Lemaitre B, Lalucat J, García-Valdés E. Taxonomic characterisation of *Pseudomonas* strain L48 and formal proposal of *Pseudomonas entomophila* sp. nov. *Syst Appl Microbiol*. 2012;35:145–149.
38. Rincón-Rosales R, Villalobos-Escobedo JM, Rogel MA, Martínez J, Ormeño-Orrillo E, Martínez-Romero E. *Rhizobium calliandrae* sp. nov., *Rhizobium mayense* sp. nov. and *Rhizobium jaguaris* sp. nov., rhizobial species nodulating the medicinal legume *Calliandra grandiflora*. *Int J Syst Evol Microbiol*. 2013;63:3423–3429.
39. Bacilio M, Rodríguez H, Moreno M, Hernandez JP, Bashan Y. Mitigation of salt stress in wheat seedlings by a *gfp*-tagged *Azospirillum lipoferum*. *Biol Fertil Soils*. 2004;40:188–193.
40. Puente ME, Holguín G, Glick BR, Bashan Y. Root surface colonization of black mangrove seedlings by *Azospirillum halopraeferens* and *Azospirillum brasilense* in seawater. *FEMS Microbiol Ecol*. 1999;29:283–292.
41. Bacilio M, Mendoza A, Bashan Y. Endophytic bacteria of the rock-dwelling cactus *Mammillaria fraileana* affect plant growth and mobilization of elements from rocks. *Environ Exp Bot*. 2012;81:26–36.
42. Chang P, Gerhardt KE, Huang XD, et al. Plant growth-promoting bacteria facilitate the growth of barley and oats in salt-impacted soil: implications for phytoremediation of saline soils. *Int J Phytoremediation*. 2014;16:1133–1147.
43. Appanna VD, Hamel RD, Lévasseur R. The metabolism of aluminum citrate and biosynthesis of oxalic acid in *Pseudomonas fluorescens*. *Curr Microbiol*. 2003;47:32–39.
44. Roy N, Chakrabarty PK. Effect of aluminum on the production of siderophore by *Rhizobium* sp. (*Cicer arietinum*). *Curr Microbiol*. 2000;41:5–10.
45. Beneduzi A, Ambrosini A, Passaglia LMP. Plant Growth-Promoting Bacteria (PGPB): their potential as antagonists and biocontrol agents. *Genet Mol Biol*. 2012;35:1044–1051.
46. Dabboussi F, Hamze M, Singer E, Geoffroy V, Meyer JM, Izard D. *Pseudomonas mosselii* sp. nov., a novel species isolated from clinical specimens. *Int J Syst Evol Microbiol*. 2002;52:363–376.
47. Lopez BR, Bashan Y, Bacilio M. Endophytic bacteria of *Mammillaria fraileana*, an endemic rock-colonizing cactus of the southern Sonoran Desert. *Arch Microbiol*. 2011;193:527–541.

48. Ali SZ, Sandhya V, Rao VL. Isolation and characterization of drought-tolerant ACC deaminase and exopolysaccharide-producing fluorescent *Pseudomonas* sp. *Ann Microbiol.* 2014;64:493–502.
49. Calvo P, Nelson L, Kloepper JW. Agricultural uses of plant biostimulants. *Plant Soil.* 2014;383:3–41.
50. Bashan Y, Rojas A, Puente ME. Improved establishment and development of three cacti species inoculated with *Azospirillum brasilense* transplanted into disturbed urban desert soil. *Can J Microbiol.* 1999;45:441–451.
51. Puente ME, Li CY, Bashan Y. Endophytic bacteria in cacti seeds can improve the development of cactus seedlings. *Environ Exp Bot.* 2009;66:402–408.
52. Lopez BR, Tinoco-Ojanguren C, Bacilio M, Mendoza A, Bashan Y. Endophytic bacteria of the rock-dwelling cactus *Mammillaria fraileana* affect plant growth and mobilization of elements from rocks. *Environ Exp Bot.* 2012;81:26–36.
53. Santos AFD, Martins CYS, Santos PO, et al. Diazotrophic bacteria associated with sisal (*Agave sisalana* Perrine ex Engelm): potential for plant growth promotion. *Plant Soil.* 2014;385:37–48.
54. Gobeille A, Yavitt J, Stalcup P, Valenzuela A. Effects of soil management practices on soil fertility measurements on *Agave tequilana* plantations in Western Central Mexico. *Soil Tillage Res.* 2006;87:80–88.
55. Arrizon J, Morel S, Gschaedler A, Monsan P. Comparison of the water-soluble carbohydrate composition and fructan structures of *Agave tequilana* plants of different ages. *Food Chem.* 2010;122:123–130.
56. Trevisan F, Oliveira VF, Carvalho MAM, Gaspar M. Effects of different carbohydrate sources on fructan metabolism in plants of *Chrysolaena obovata* grown in vitro. *Front Plant Sci.* 2015;6:681, <http://dx.doi.org/10.3389/fpls.2015.00681>.
57. Vargas WA, Pontis HG, Salerno GL. Differential expression of alkaline and neutral invertases in response to environmental stresses: characterization of alkaline isoform as stress leaves. *Planta.* 2007;226:1535–1545.
58. Chen TH, Huang YC, Yang CS, Yang CC, Wang AY, Sung HY. Insights in to the catalytic properties of bamboo vacuolar invertase through mutational analysis of active site residues. *Phytochemistry.* 2009;70:25–31.
59. Lüscher M, Hochstrasser U, Boller T, Wiemken A. Isolation of sucrose:sucrose 1-fructosyltransferase (1-SST) from barley (*Hordeum vulgare*). *New Phytol.* 2000;145:225–232.
60. Fujishima M, Sakai H, Ueno K, et al. Purification and characterization of a fructosyltransferase from onion bulbs and its key role in the synthesis of fructo-oligosaccharides in vivo. *New Phytol.* 2005;165:513–524.
61. Ueno K, Onodera S, Kawakami A, Yoshida M, Shiomi N. Molecular characterization and expression of a cDNA encoding fructan:fructan 6G-fructosyltransferase from asparagus (*Asparagus officinalis*). *New Phytol.* 2005;165:813–824.