Environmental Microbiology

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

Neyser De La Torre-Ruiz\textsuperscript{a}, Víctor Manuel Ruiz-Valdiviezo\textsuperscript{b}, Clara Ivette Rincón-Molina\textsuperscript{b}, Martha Rodríguez-Mendiola\textsuperscript{a}, Carlos Arias-Castro\textsuperscript{a}, Federico Antonio Gutiérrez-Miceli\textsuperscript{b}, Héctor Palomeque-Dominguez\textsuperscript{b}, Reiner Rincón-Rosas\textsuperscript{b,}\textsuperscript{∗}

\textsuperscript{a} Plant Biotechnology, DEPI Instituto Tecnológico de Tlajomulco, Carretera a San Miguel Cuyutlán, Tlajomulco de Zúñiga, Jalisco, Mexico
\textsuperscript{b} Laboratory of Biotechnology, Instituto Tecnológico de Tuxtla Gutiérrez, Tuxtla Gutiérrez, Mexico

\textbf{A R T I C L E  I N F O}

Article history:
Received 4 June 2015
Accepted 11 February 2016
Available online xxx
Associate Editor: Iêda de Carvalho Mendes

Keywords:
Agave
Inoculation
Bacteria
Inulin
16S rRNA

\textbf{A B S T R A C T}

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.

© 2016 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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 PGBP 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.

\footnotesize{\textsuperscript{∗} Corresponding author. E-mail: reriro61@hotmail.com (R. Rincón-Rosas).}

\textsuperscript{1517-8382/© 2016 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).}

Please cite this article in press as: De La Torre-Ruiz N, et al. Effect of plant growth-promoting bacteria on the growth and fructan production of Agave americana L. Braz J Microbiol. (2016), http://dx.doi.org/10.1016/j.bjm.2016.04.010}
Significant improvement on the growth and yield of crops in response to microbial inoculation has been reported by many workers.\textsuperscript{1-5} Studies confirm that inoculants formulated with PGPB have shown positive effects on the agricultural yield and crop quality.\textsuperscript{6-8} 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.\textsuperscript{9} 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.\textsuperscript{10} Martínez-Rodríguez et al.\textsuperscript{11} 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.\textsuperscript{12} 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.\textsuperscript{13} Approximately 163 species grow in Mexico, and 123 species are endemic.\textsuperscript{14} 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.\textsuperscript{15} 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.\textsuperscript{16-20}

Many aspects of the microbial community associated with agaves are still unknown and only a manuscript related to\textsuperscript{21} 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) medium\textsuperscript{22} 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\textsuperscript{23}, PS7, Germany). The Gram reaction was determined using a kit (Merck\textsuperscript{24}, Germany), according to the manufacturer’s procedure, and colony morphology was determined with cells grown on YMA plates at 28 °C for 5 days.\textsuperscript{25} 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.\textsuperscript{26} Antibiotic resistance was tested on YMA plates following the process recommended by Martínez-Romero et al.\textsuperscript{27}. In addition, the Al and Cu tolerance of the strains were determined on solid YMA medium.\textsuperscript{28}

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\textsuperscript{29}, Switzerland), according to the manufacturer’s specifications. PCR was performed with the bacterial universal 16S rRNA primers fD1 (5′-AGAGTTTGTATCCTGGCTCAG-3′) and rD1 (5′-AAGAGGTTGATCAGCC-3′), which amplified products of approximately 1500 bases, and procedures were performed as described by Weisburg et al.\textsuperscript{19}. The PCR products were purified using the PCR Product Purification System Kit from Roche\textsuperscript{30} and sequenced (Macrogen\textsuperscript{31}, Korea). All sequences were compared with the reference sequences obtained by a BLAST search.\textsuperscript{20} The sequences were aligned using the CLUSTAL X (2.0) software with the default settings.\textsuperscript{32} Minor modifications in the alignment were made using the BIOEDIT sequence editor. Phylogenetic and molecular evolutionary analyses were performed with MEGA v5.2.\textsuperscript{33} The phylogenetic tree of the 16S rRNA gene sequences from type strains was constructed by Neighbor-Joining\textsuperscript{34} and a Bootstrap analysis with 1000 pseudoreplicates using the Tamura-Nei model.\textsuperscript{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), K₂HPO₄ 10% (5 mL L⁻¹), MgSO₄ 10% (2 mL L⁻¹), NaCl 10% (1 mL L⁻¹), CaCO₃ (1 g L⁻¹) and yeast extract (3 g L⁻¹) at a pH of 6.8, supplemented with 100 mL L⁻¹ of l-tryptophan. After incubation at 30 ± 2 °C on a rotary shaker for 48 h, the culture medium was centrifuged at 5000 x g for 10 min, and the supernatant was filtered through a 0.22-μm 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 C₁₈ column (5 μm; 4.6 by 150 mm). The mobile phase was acetonitrile–50 mM KH₂PO₄ (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 OD₅₆₀ 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 (Ca₃(PO₄)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 [ARA nmol C₂H₄ 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. brasiliense Cdbl 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 g Na₂CO₃ 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 μE m⁻² s⁻¹.³⁰

After two months (60 days after transplantation), the plants were transferred to pots containing peat moistened with free N Fahraeus medium [CaCl₂, 0.1 g; MgSO₄·7H₂O, 0.12 g; KH₂PO₄, 0.1 g; Na₂HPO₄·2H₂O, 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 ± 2 °C (natural illumination). The plants were inoculated with 2 mL of a suspension of each PGPB strain at a concentration of 1 × 10⁶ UFC mL⁻¹.³²,³³ Uninoculated plants and others treated with 30 mg of KNO₃-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 (m₁) and after 90 days (m₂) in the greenhouse. The data used for statistical analysis were the difference between the m₂–m₁ 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 × g for 15 min at room temperature, and the precipitate was removed. Samples were adjusted to pH 7.0, filtered through 20- and 45-μm nylon membranes, and stored at 4 °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-μm 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 Ca²⁺ 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

Please cite this article in press as: De La Torre-Ruiz N, et al. Effect of plant growth-promoting bacteria on the growth and fructan production of Aegle americana L. Braz J Microbiol. (2016), http://dx.doi.org/10.1016/j.bjm.2016.04.010
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.\textsuperscript{12,13,35} Taxonomic polyphasic studies are commonly used to analyze bacterial diversity and include morphological, physiological, biochemical, metabolic, and principally, phylogenetic studies.\textsuperscript{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\textsuperscript{-1}), carbenicillin (20 µg mL\textsuperscript{-1}), chloramphenicol (100 µg mL\textsuperscript{-1}), and kanamycin (100 µg mL\textsuperscript{-1}). The ACO-40 strain showed a greater sensitivity to the antibiotics assessed. The strains grew in the presence of Al\textsuperscript{3+} (500 µg mL\textsuperscript{-1}) and Cu\textsuperscript{2+} (100 µg mL\textsuperscript{-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.\textsuperscript{39} Puente et al.\textsuperscript{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.\textsuperscript{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.\textsuperscript{43} Rhizobium sp. strain BICC 651 produced a threefold higher level of siderophore in the presence of 100 µM Al\textsuperscript{3+}, which could be a mechanisms to relieve Al stress,\textsuperscript{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.\textsuperscript{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 NCBC22016\textsuperscript{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\textsuperscript{T}.\textsuperscript{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\textsuperscript{T}.\textsuperscript{36}

The highest amount of IAA was produced by the P. mosselii strain ACO-140 (15.7 mg L\textsuperscript{-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\textsuperscript{-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  | Rhizobium daejeonense                  | KM349967      | 1333              | R. daejeonense L61\textsuperscript{T} (AY341343) 96.1% | Zhe-Xue et al.\textsuperscript{46} |
| ACO-40   | Acinetobacter calcoaceticus            | KM349968      | 1381              | A. calcoaceticus NCBC22016\textsuperscript{T} (AJ888983) 99.1% | Unpublished |
| ACO-140  | Pseudomonas mosselii                   | KM349969      | 1387              | P. mosselii CIP 105259\textsuperscript{T} (AF072688) 100% | Dabboussi et al.\textsuperscript{46} |
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) | ARA² (nmol C₂H₄ per culture h⁻¹) |
|----------------------------|-------------------------|---------------------------|---------------------------------|
| Pseudomonas mosselii      | 15.7 A¹                   | 37.8 A                    | 167.1 B                          |
| ACO-140                   | 8.5 C                    | 29.8 B                    | 106.1 B                          |
| Acinetobacter calcoaceticus | 10.3 B                  | 24.7 B                    | 627.4 A                          |
| ACO-40                    | 10.4 B                  | 24.2 B                    | 821.3 A                          |
| Rhizobium dacejonense     | 16.5 C                  | 7.63 A                    | 224.39                           |
| ACO-34A                   | 16.5 C                  | 7.63 A                    | 224.39                           |
| Azospirillum brasilense Cd | 16.5 C                  | 7.63 A                    | 224.39                           |

¹ Mean values of four replicates. Means followed by the same letter do not show any significant differences (p < 0.05).

² ARA, acetylene reduction assay (µmol C₂H₄ per culture fresh weight h⁻¹).

³ 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) |
|---------------------|----------------------|--------------------|------------------|------------------|
| Pseudomonas mossellii ACO-140 | 2.44 B               | 0.48 A             | 3.0 A            | 11.3 CD          |
| Acinetobacter calcoaceticus ACO-40 | 1.23 D               | 0.27 CD            | 2.7 A             | 11.3 CD          |
| Rhizobium daejeonense ACO-34A | 3.41 A               | 0.37 B             | 4.0 A            | 26.6 A           |
| Azospirillum brasilense Cd | 1.65 C               | 0.34 BC            | 1.5 BC            | 16.2 B           |
| KNO3-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*                | 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. Ratios of these pseudomonads were characterized by biochemical mechanisms and specific rock degradation, as well as the biosynthesis of important secondary metabolites, under varied biotic and abiotic stress conditions.

Inoculation of plants with PGPB enhances the assimilation of essential nutrients and plant-associated biological nitrogen fixation. 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). This, 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. mossell 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. mossell 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. reported that the inoculation of pachycerecid cacti species (Pachycereus pringlei, Stenocereus thurberi and Lophocephus 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. 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 sakazukii M2PFe, Azotobacter vinelandii M2Per and P. putida M3TA, isolated from the rock-dwelling cactus M. fraileana, affected plant growth and the mobilization of elements from rocks. 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., 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. mossell strain ACO-140 (Fig. 2A). Kestose was only detected in plants inoculated with P. mossell. 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).
Nevertheless, Tukey’s test showed that the inulin concentration was not significantly different between the uninoculated plants and KNO₃ fertilized plants. This demonstrates the biological potential of the agave species to biosynthesize various types of metabolites such as sugars despite unfavourable growth conditions. 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 KNO₃. 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. daejonense strain ACO-34A and P. mosselli 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. daejonense 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. Cedeño 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. reported that these sugars act on the metabolism of fructans, such as kestose and nystose. The concentration of fructose and glucose in the

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 A. americana plantlets inoculated with (A) Pseudomonas mosselli strain ACO-140, (B) Acinetobacter calcoaceticus strain ACO-40, (C) Rhizobium daejonense strain ACO-34A, and (D) Azospirillum brasiliense Cd. The markers represent the mobility of fructose, sucrose, kestose, and nystose.
Table 4 – Effect of inoculation with plant growth-promoting bacteria on sugar accumulation in leaves and roots of Agave americana L.

| Treatment                     | Inulin (mg g⁻¹ Leaves) | Sucrese (mg g⁻¹ Leaves) | Glucose (mg g⁻¹ Leaves) | Fructose (mg g⁻¹ Leaves) | Inulin (mg g⁻¹ Root) | Sucrese (mg g⁻¹ Root) | Glucose (mg g⁻¹ Root) | Fructose (mg g⁻¹ Root) |
|-------------------------------|-------------------------|-------------------------|-------------------------|--------------------------|----------------------|----------------------|----------------------|-----------------------|
| Pseudomonas mosselli 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               |
| Acinetobacter calcoaceticus 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               |
| Rhizobium daejeonense 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               |
| Azospirillum brasilense 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               |
| KNO₃-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 (p < 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.

Acknowledgements

We thank the Laboratory of Ecology Genomics (CCG-UNAM) for their technical assistance. The project was funded by Tecnológico Nacional de México (TNM, Mexico) (5665.15-P and 5663.15-P) from the project ‘Infraestructura 251805’ from the Consejo Nacional de Ciencia y Tecnología (CONACyT, Mexico).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjm.2016.04.010.

REFERENCES

1. 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.
2. 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.
3. Lugtenberg BJ, Kamlova F. Plant-growth-promoting rhizobacteria. Annu Rev Microbiol. 2009;63:541–556.
4. Zahir ZA, Arshad M, Frankenberger WT. Plant growth promoting bacteria: applications and perspectives in agriculture. Adv Agron. 2003;81:97–168.
5. 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.
6. 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.
7. Martinez-Rodriguez JD, De la Mora-Amutio M, Plassencia-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.
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 Engel): 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. Arrizón 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, Fontis HC, 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 YY, Sung HY. Insights into 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: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.