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Research Article

Plant growth-promoting rhizobacteria associated to candelilla rhizosphere (*Euphorbia antisyphilitica*) and its effects on *Arabidopsis thaliana* seedlings

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Abstract

In the communities of Sierra Mojada and Viesca, Coahuila, Mexico of Coahuila desert, two rhizosphere samplings of candelilla (*Euphorbia antisyphilitica* Zucc) were collected to isolate, characterize, and identifying plant growth-promoting rhizobacteria (PGPR); 165 rhizobacteria were tested *in vitro* with *Arabidopsis thaliana* seedlings to evaluate their potential as plant growth promoters, and obtaining 21 strains with best results in the variables of the number of secondary roots and fresh weight concerning the uninoculated control. Their salinity tolerance was evaluated at concentrations from 0.85 M, 1.7 M and 2.55 M of NaCl. Biochemical tests were accomplishing such as siderophores production, phosphates solubilization, production of Indole-3-acetic acid (IAA), and the activity of the ACC deaminase enzyme. The results obtained from 21 strains selected, high activities were obtained in organic substances like a siderophores since they developed a translucent orange halo around their growth; four rhizobacteria developed a clear halo around the bacterial growth with a thickness between 1.487 mm ± 0.667 mm and 5.267 mm ± 0.704 mm in phosphates solubilization; in the production of Indole-3-acetic acid (IAA), the bacterial strains showed the presence of this phytohormone, with values from 4.444 μg mL^{-1} to 19.286 μg mL^{-1}; and according to the activity of the ACC deaminase enzyme, values from 0.424 to 1.306 μmol α-KB/h/mg Pr were showed. 16S rRNA sequencing was carried out and genus identified were *Bacillus*, *Staphylococcus, Acinetobacter, Cronobacter* and *Siccibacter.* The results obtained show the potential of the isolated rhizobacteria as growth promoters and the increase in the biomass of the *Arabidopsis thaliana* seedlings is evident. This is a first indication to proceed to carry out tests in different phenological stages in crops of agricultural importance.

Keywords: halophilic rhizobateria; Indole-3-acetic acid; phosphates solubilization; salinity; siderophores

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Introduction

The rhizosphere is the area determined between the roots of the plant and the soil that surrounds it (Dijkstra *et al.*, 2014), which is subdivided into three areas: Ectorhizosphere, rhizoplane, and endorhizosphere (Johansson *et al.*, 2004). The microorganisms that inhabit the rhizosphere act in the nutrient cycles of the soil-plant system (Singh and Mukerji, 2006). The bacterial populations of this microenvironment are mainly favored by the organic compounds produced by the plant such as organic acids, amino acids, sugars, phenolic acids, flavonoids, enzymes, among others, as well as by the presence of nutrients, pH, and soil texture (Stafforsuch, 2005; Singh and Mukerji, 2006; Raaijmakers *et al.*, 2009). The microorganisms attached to the root establish interactions with plants in two main ways: those that form a symbiotic relationship (*Rhizobium*-Leguminous) and those of free life, which can be in the soil, on or inside the tissues of the plant (Kloepper *et al.*, 1988; Frommel *et al.*, 1991). The free-living bacteria that benefit plant development and even intervene as biological control of phytopathogens are commonly known as plant growth-promoting rhizobacteria (PGPR) (Palacio-Rodríguez *et al.*, 2016).

Rhizobacteria have various mechanisms to promote plant growth, including an increase in nutrient mobilization, nitrogen fixation, improved nutrient absorption, and biological control of pathogens (Bais *et al.*, 2006; De-Bashan *et al.*, 2007; Dias *et al.*, 2009; Hariprasad and Niranjana, 2009; Altomare and Tringovska, 2011). Also, they synthesize growth-regulating substances such as gibberellins, cytokinins, and auxins, they stimulate the density and length of root hairs, thereby increasing the absorption capacity of water and nutrients, which results in greater growth and adaptation to conditions of drought, acidity, and alkalinity (Sandhya *et al.*, 2010; Saraf *et al.*, 2011). Some rhizobacteria have antagonistic results on other microorganisms and induce systemic resistance in plants (Sharma *et al.*, 2007).

Currently, the agricultural sector in dry arid zones suffers from serious abiotic factors such as salinity, which is exacerbated by the high applications of chemical agro-inputs by farmers. Malik *et al.* (2018), explains that an option to chemical fertilization is the use of biofertilizers from beneficial microorganisms. However, these microorganisms must have the ability to benefit plants under saline conditions and high temperatures because they are conditions that prevail in agriculture in arid-saline environments.

The central area of Coahuila desert is a region located in the central north-western part of Mexico country. It has an area of 61,706 km^2. The prevailing edaphoclimatic conditions are extreme and its vegetation is widely adapted to them (temperatures above 50 °C in summer and below -4 °C in winter; rainfall below 100 mm per year) and soils low in organic matter (<0.3%). Among the main plants that can be observed in its soils with a high concentration of salts (> 20 gr / kg of dry soil) are candelilla, lechiguilla, sotol, ocotillo, and various cactis (CONAFOR, 2009). Some of this kind of plants in others deserts, showed the presence of beneficial microorganisms which are play an important role providing nutrients to this kind of plants. According the above, the aim of this research was to isolate, characterize, and identify strains of rhizobacteria (ecto and endo) that are present in the rhizosphere of candelilla (*Euphorbia antisyphilitica*) and evaluate their potential to promote growth in *Arabidopsis thaliana* seedlings, studying their capacity to growth under salinity conditions (0.85 M, 1.7 M and 2.55 M) of NaCl and biochemical tests such as siderophores production, phosphates solubilization, production of Indole-3-acetic acid (IAA), and the activity of the ACC deaminase enzyme.

Materials and Methods

Collection of samples and isolation of bacteria from endorizosphere and ectorizosphere

The rhizosphere and root samples were collected in two study areas of Coahuila desert, the first area in the Sierra Mojada (Ejido San Jose de Madero, Coahuila) with coordinates 27° 0' 54" N and 103° 34' 33" W and the second in Sierra Viesca (Rancho Penjamo, Coahuila) with coordinates 25° 10' 41" N and 102° 39' 12" W (Figure 1).

On each sampling sites, root and soil samples were collected 10 cm away from the stem of five wild plants of *E. antisyphilitica* in flowering stage. Five subsamples were taken for each plant (500 g of soil and roots) and at a depth of 10, 20 and 40 cm. Candelilla plants were selected for their robustness (0.80 ± 10 cm wide), height (0.90 ± 10 cm) and color (deep opaque green). The collected material was deposited in dark plastic bags, labeled with their corresponding collection data and stored in a thermal container with ice that had a temperature of 4 ± 1 °C for transferring to the Microbial Ecology Laboratory of the Faculty of Biological Sciences – Universidad Juarez del Estado de Durango (UJED).

For the isolation of the rhizobacteria from the endorhizosphere, 1 g of the root was weighed, washed with 70% ethanol, and rinsed with 0.5X sterile PBS solution, then it was macerated with a sterile mortar with 10 mL of 0.5X PBS solution.

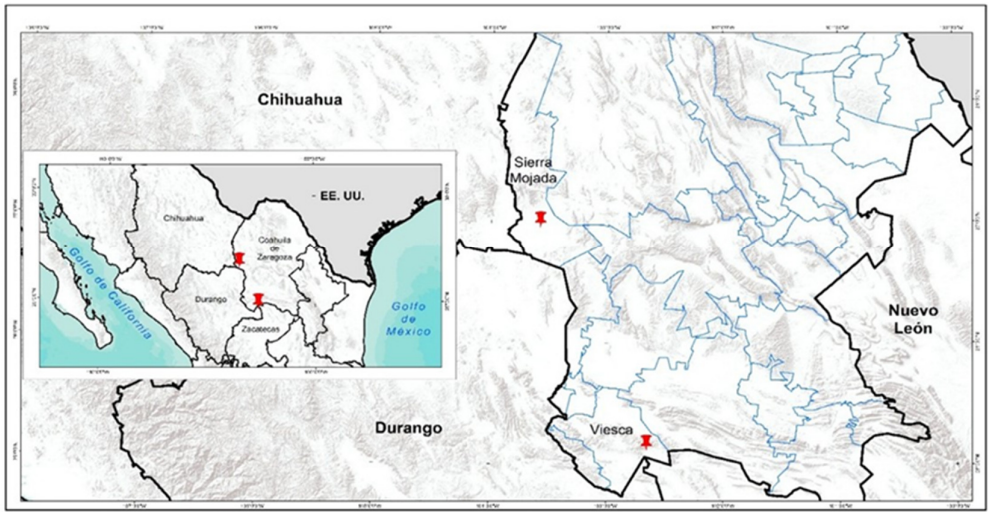

Figure 1. Geographic localization of the sampling sites in Sierra Mojada and Viesca, state of Coahuila, Mexico

For the isolation of the rhizobacteria from the ectorhizosphere, 1 g of rhizospheric soil adhered to root system was suspended in 10 mL of sterile 0.5X PBS solution and placed in the shaking incubator for 5 min at 200 rpm.

Aliquots (100 µL) of serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}) were inoculated from both procedures (endorhizosphere and ectorhizosphere) in Petri dishes with culture media: Free Nitrogen (NFb) for nitrogen-fixing bacteria (Dobereiner and Day, 1975), Luria Bertani (LB) is a generalist medium (Bertani 1951), and Chrome Azurol S (CAS) for siderophore-producing bacteria (Schwyn and Neilands, 1987). Then, all Petri dishes were incubated at 30 °C for 5 days. Finally, with the support of a sterile loop, the colonies were taken separately and conserved in 700 µL of bacterial culture with 300 µL of glycerol for their conservation and they were stored at -70 °C. Afterwards, each bacterial isolate was isolated and differentiated bacterial strains according to colonial morphology. The bacterial strains isolated in this study were named with codes that

represent the medium where they were isolated (NFb, LB, CAS), the area of the rhizosphere where they were isolated (endorhizosphere and ectorhizosphere) and the strain number (1, 2, 3, etc.) (Example: NFbEndo3).

Also, the soil was analyzed; it consisted of detecting in ppm (mg·kg soil): N-NO_3, P, Ca^{+2}, Mg^{+2}, Na^{+}, C.I.C. in mEq·100 mg, % Exchangeable Cations: Ca^{+2}, Mg^{+2}, Na^{+} and K^{+} were based on Aguilar et al. (2013).

Evaluation of the growth promoting capacity of rhizobacteria in Arabidopsis thaliana

Seeds of *Arabidopsis thaliana* (Col-0) firstly were disinfected in an Eppendorf tube with 20% sodium hypochlorite, stirring for 5 m in the vortex, then they were rinsed with sterile distilled water four times and seeds were placed at 4 °C for 24 h for stratification. Subsequently, 45 seeds were placed in Petri dishes with MS 0.2X medium; Petri dishes were placed in a bioclimatic chamber (Torrey@ RV26TVC26) with photoperiods of 16 h light and 8 h of darkness at 25 °C. Then after 4 days the germinated *A. thaliana* seedlings were inoculated and placed at a rate of 7 seedlings per Petri dish.

The inoculation consisted in firstly rhizobacteria isolated and selected from the endo and ectorhizosphere of the candelilla were independently developed in the liquid medium from which they were isolated (NFb, LB and CAS). The incubation conditions were continuous shaking (120 rpm) and a temperature at 30 °C (Reinhold *et al.,* 1987). Between 14 and 24 h (logarithmic phase) elapsed, the concentration of the culture of each bacterium was determined, using the following procedure: 1 mL of the culture was poured into a cell for spectrophotometer (master spectrum FISHER SCIENTIFIC 415), taking the reading of absorbance at a wavelength of 540 nm against a medium control (NFb, LB and CAS without bacteria). The bacterial was diluted until obtaining an absorbance of 1.00 unit, which corresponds to a suspension of 2.5 x 10^8 CFU mL^{-1}. To each of the cultures (Table 1) amount of germinated seed (radicle emerged in 1 gr) (390 ± 7) of *A. thaliana* were added in a 50 mL Kitazato flask, they were subjected to vacuum at 100 mm Hg for 1 m (Carrillo *et al.*, 1988). Root growth measurements were made every 48 h for 12 days; each of the experiments were performed in quadruplicate; each Petri dishes contained seven seedlings, throwing 588 experimental units. The variables evaluated were lateral root number (secondary roots) and fresh weight. The number of secondary roots was counted using an AllScience stereoscope 12 days after sowed. For the fresh weight the plant was removed from the petri dish with tweezers and weighed on an Ohaus brand analytical balance (Lopez-Bucio *et al.*, 2007).

Salinity tolerance experiments, the phosphates solubilization, production of IAA, siderophores and ACC deaminasa activity

The bacterial strains that were characterized by their ability for plant growth-promoting were exposed to different concentrations of NaCl to see their ability to develop. LB solid medium supplemented with different concentrations of NaCl 0.85 M, 1.7 M, and 2.55 M was used; the rhizobacteria were streaked in LB with NaCl and incubated at 30 °C, after three days the development of the bacterial strains was observed, the growth diameter reading was taken to evaluate the tests with a millimeter vernier (Mitutoyo America Corporation). Also was considered in the presence of colony a classification: (+++) maximum development; (++) medium development; (+) minimum development; (-) no development (Palacio-Rodríguez *et al.*, 2016).

The phosphates solubilization were evaluated through the solid medium Pikovskaya; according to what was reported by Goldstein (1986). The evaluation of the production of Indole-3-acetic acid (IAA) of the rhizobacteria was carried out according to what is reported, by Bric *et al.* (1991). The siderophores production was determined by the method Schwyn and Neilands (1987) using Chromium Azurol S (CAS) medium. The activity of the ACC deaminase enzyme was determined according to the method described by Penrose and Glick (2003).

Molecular Identification

The identification of PGPR was carried out by DNA extraction using the CTAB technique, according to the method of Doyle and Doyle (1990), then the partial amplification of the 16S rRNA gene was carried out

by means of PCR using the oligonucleotides 27F 5′AGAGTTTGATCMTGGCTCAG 3′ and 1492R 5′GGTTACCTTGTTACGACTT 3`, the PCR product was sent to McLAB in San Francisco, CA, USA for sequencing. The sequences obtained were subjected to comparison using BLAST (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the taxonomy of bacterial strains (Weisburg *et al.*, 1991; Jha *et al.*, 2011).

Statistical analysis

The development parameters (secondary roots and fresh weight) of the seedlings were analyzed in a random experimental design, a statistical analysis of ANOVA was carried out followed by a test of Tukey's T (HSD), with a P value = 0.05. Analyzes were performed using Graph Pad Prism 6 program.

Results

Candelilla rhizobacteria isolation and soil analysis

For the area in Sierra Mojada, Ejido San Jose de Madero, Coahuila, a total of 77 bacterial strains were isolated (Figure 2a), and in the second area Sierra Viesca, Rancho Penjamo, Coahuila, a total of 88 bacterial strains were isolated (Figure 2b). In Table 1, we can observe that the area of the Sierra Mojada *vs* the Viesca area, showed numerical differences in the microorganisms isolated from the CAS medium (ecto and endorhizosphere), numerically favoring the area of Viesca. Another appreciation was with those developed on the LB medium, where at the ectorhizophere level, the Sierra Mojada area stood out numerically; the opposite occurred in the NFb medium where the Viesca area the values of ectorizospheric microorganisms were higher than those of the Sierra Mojada area (Figure 2ab). To another hand, considering morphologically identical colonies from the 165 isolates, some of them showed morphological duplication, reducing to 21 strains (Table 1).

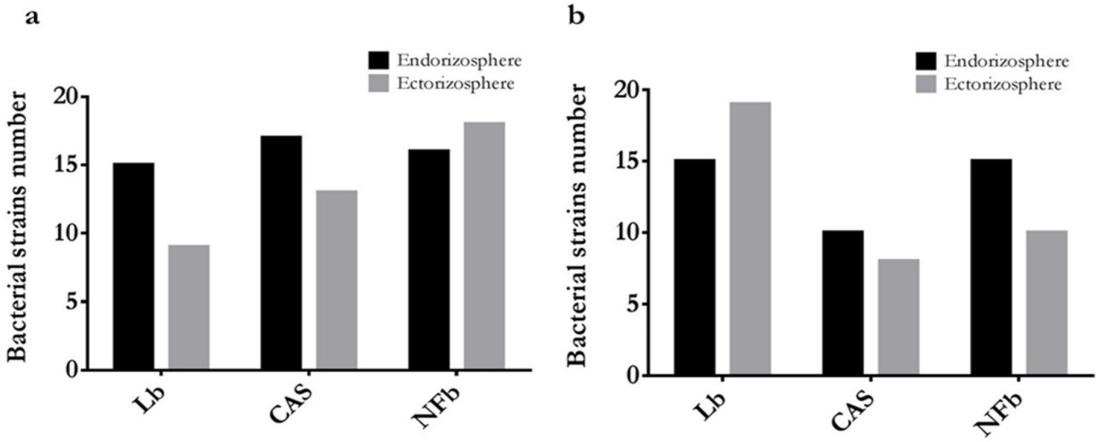

Figure 2. Culture media used and the number of strains isolated in the study area: (a) Sierra Mojada, (b) Sierra Viesca

Table 1. Colonial morphology of bacteria isolated from the candelilla rhizosphere

Medium	Zone	Number	Form	Elevation	Edge	Colour	Surface	Density	Consistency	Reflected light	Transmit. light	Gram stain
San Jose												
Lb	Ecto	8	circle	flat	whole	beige	opaque	opaque	butyrose	sparkly	opaque	positive
NFb	Endo	16	circle	flat	whole	yellow	smooth	opaque	gentle	mate	opaque	negative
Viesca												
Lb	Endo	2	irregular	flat	whole	beige	smooth	opaque	butyrose	mate	translucent	negative
Lb	Endo	6	fusiform	flat	whole	beige	smooth	opaque	butyrose	mate	opaque	positive
Lb	Endo	7	irregular	flat	lobed	beige	rough	opaque	butyrose	mate	opaque	positive
Lb	Endo	13	rhizoid	elevated	lobed	beige	opaque	opaque	butyrose	mate	opaque	positive
Lb	Endo	15	filamentous	flat	filamentous	beige	rough	opaque	butyrose	mate	opaque	positive
CAS	Endo	4	irregular	flat	whole	beige	smooth	translucent	mucoid	mate	opaque	negative
CAS	Endo	7	fusiform	flat	whole	yellow	smooth	translucent	mucoid	mate	translucent	positive
CAS	Endo	10	pinpoint	flat	whole	yellow	smooth	translucent	mucoid	sparkly	translucent	positive
CAS	Endo	17	fusiform	flat	whole	beige	smooth	translucent	butyrose	sparkly	translucent	negative
NFb	Endo	12	irregular	flat	curly	transparent	sparkly	translucent	butyrose	sparkly	translucent	negative
Lb	Ecto	1	irregular	flat	curly	beige	smooth	translucent	gentle	mate	opaque	negative
CAS	Ecto	2	irregular	flat	whole	yellow	sparkly	translucent	butyrose	sparkly	translucent	positive
CAS	Ecto	4	pinpoint	flat	whole	beige	sparkly	translucent	butyrose	sparkly	translucent	positive
CAS	Ecto	11	fusiform	flat	curly	beige	sparkly	opaque	gentle	sparkly	opaque	negative
CAS	Ecto	12	fusiform	flat	curly	yellow	mate	opaque	dry	mate	opaque	positive
CAS	Ecto	13	pinpoint	flat	whole	transparent	sparkly	translucent	dry	sparkly	translucent	positive
NFb	Ecto	1	circular	flat	whole	other	smooth	translucent	dry	sparkly	translucent	negative
NFb	Ecto	8	pinpoint	flat	whole	white	smooth	translucent	dry	sparkly	translucent	negative
NFb	Ecto	18	fusiform	flat	whole	beige	smooth	opaque	butyrose	mate	opaque	negative

In relation to soil analyses in the two areas sampled, results showed that candelilla plants develops in soils with a sandy loam texture (sand: 62.13 ±3.22; silt 15.33 ± 2.47; clay: 22.54 ± 2.09); EC. Milimmho s.sc = 8.02 ± 1.23; saturation = 31.9 ± 3.02 g kg^{-1}; field capacity = 13.11±2.99 g kg^{-1}; O.M. 0.041 ± 0.010 g kg^{-1}; $N\text{-}NO_3$ = 0.05 ± 0.01 mg kg^{-1}; P = 2.09 ± 0.11 mg kg^{-1}; permeability K^+ ($cm \cdot h$) = high to excessive 14.9 ± 1.01; and exchangeable cations: Ca^{+2} = 263 ± 33.12 g kg^{-1}; Na^+ = 176 ± 30.282 kg^{-1}). These physical-chemical characteristics indicate a halotolerance of candelilla plants according to C.E. obtained in the analyzes and a low content of O.M. present in both areas.

Effect of inoculation with candelilla rhizobacteria on the growth of Arabidopsis thaliana seedlings

Strains isolated from Sierra Mojada, Ejido San Jose de Madero, Coahuila

In this test, two bacterial isolates (LbEcto8 and NFbEndo16) from 77 endo and ectorizosphere were positive to show results in variables evaluated, considering their respective control without inoculation. In lateral root number variable at 12 days after sowing (das), those treatments of seed germinated of *A. thaliana* and inoculated with LbEcto8 and NFbEndo16 rhizobacteria´s showed a significant difference (Tukey, P≤0.05) *vs* treatment non inoculated. The treatment with the LbEcto8 strain obtained an average of 27 secondary roots while the NFbEndo16 strain obtained an average of 22 for the number of secondary roots; 13 secondary roots was to the control without rhizobacteria. According to fresh weight variable, similar development with lateral root number was observed in this variable. The results indicated 77.35 mg in fresh weight, and 80.55 mg and fresh weight respectively to LbEcto8 and NFbEndo16 rhizobacteria, compared with the control without inoculation which obtained an average of 75.48 mg of fresh weight (Figure 3). The seedlings inoculated with the remaining bacterial isolates, among the observed effects were a rotting, turning first brown and then black.

Figure 4 show the effect of inoculation in the number of lateral roots and root fresh weight on which it is observed their abundance compared to the non-inoculated control plants.

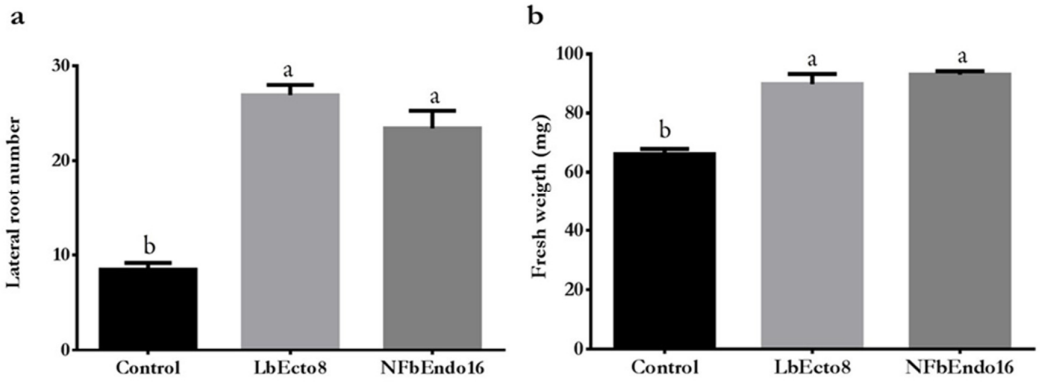

Figure 3. (a)Effect of the inoculation of rhizobacteria (LbEcto8 and NFbEndo16 strain) from Sierra Mojada in *A. thaliana* seedlings, in root growth at 12 days after inoculation; a) lateral root number, (b) fresh weight
Different letters indicate a significant difference (P≤0.05) between inoculated seedlings and the uninoculated control.

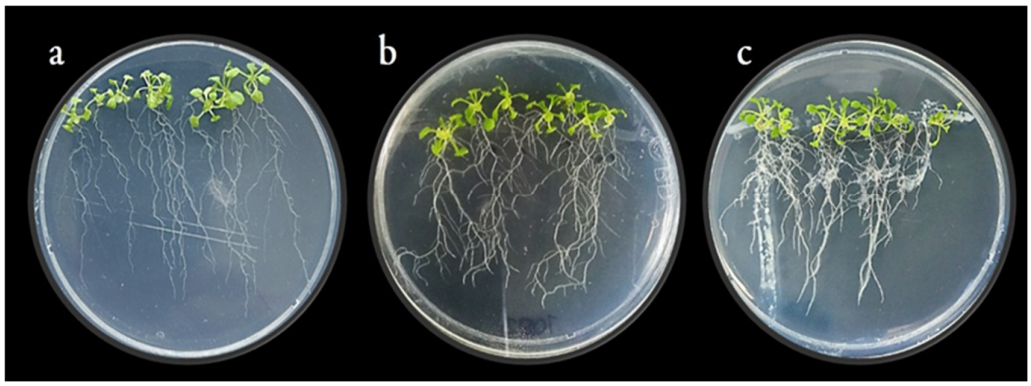

Figure 4. Effect of inoculation of rhizobacteria from Sierra Mojada, Ejido San Jose de Madero, Coahuila, on the growth of *A. thaliana* seedlings 12 days after inoculation in Petri dishes on 0.2× MS medium agar
a) Control, b) LBEcto8 and c) NFbEndo16.

Strains isolated from Sierra Viesca, Rancho Penjamo, Coahuila

In lateral root number and fresh weight variable´s carried out for this sampling zone, 19 rhizobacteria were tested (LbEndo2, LbEndo6, LbEndo7, LbEndo13, LbEndo15, CASEndo4, CASEndo7, CASEndo10, CASEndo17, NFbEndo12, LbEecto1, CASEcto2, CASEcto4, CASEcto11, CASEcto12, CASEcto13, NFbEcto1, NFbEcto8, NFbEcto18) (Figure 5 and 6). The results indicate in the lateral roots number a significant difference (P≤0.05), compared with the uninoculated control; a similar behaviour was to fresh weight variable.

In this comparison, the rhizobacteria inoculated in *A. thaliana* seedlings, which showed a significant difference (Tukey, P≤0.05) with respect to the uninoculated control, which in the variable of number of secondary roots obtained an average of 9, were the strains CASEcto2 with 22, CASEcto4, CASEndo10 and CASEcto12 with 19 secondary roots on average. For the fresh weight variable, the uninoculated control obtained an average weight of 76.1 mg and the rhizobacteria that showed a significant difference (Tukey, P≤0.05) were NFbEcto18 and CASEcto12 with 130.0 mg and 180.9 mg of fresh weight respectively (Figures 5 and 6).

In other form of representation, Figure 7, showed the control is surpassed by LbEndo7, CASEndo4, NFbEndo12, CASEcto4, CASEcto13, NFbEcto1 and NFbEcto18 treatments. However, same Figure 7 show us the control stands out those treatments based on the rhizobacteria CASEcto11 and NFbEcto8.

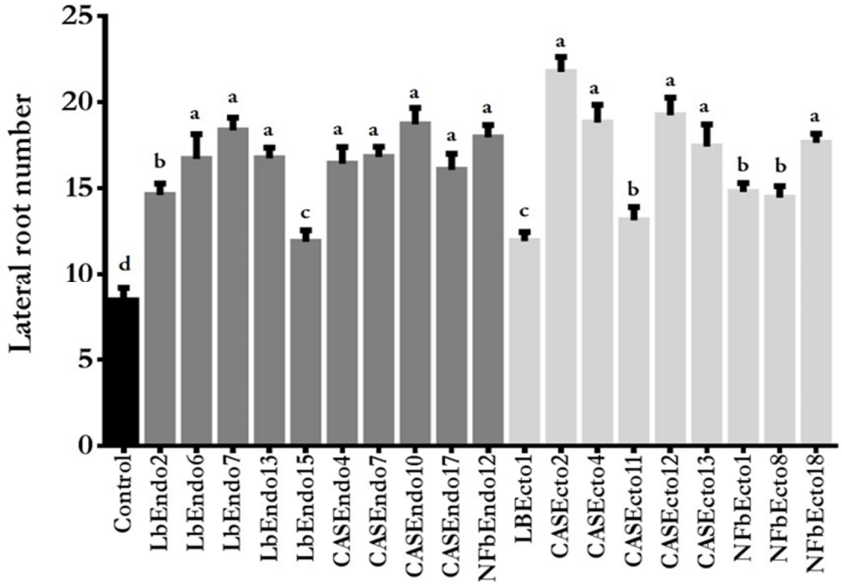

Figure 5. Effect of the inoculation of rhizobacteria in *A. thaliana* seedlings, where it is observed that the strains LbEndo6, LbEndo7, LbEndo13, CASEndo4, CASEndo7, CASEndo10, CASEndo17, NFbEndo12, CASEcto2, CASEcto4, CASEcto12, CASEcto13, NFbEcto18, increased the number of secondary roots of the plant 12 days after inoculation.
Different letters indicate a significant difference (P≤0.05) between inoculated seedlings and the uninoculated control.

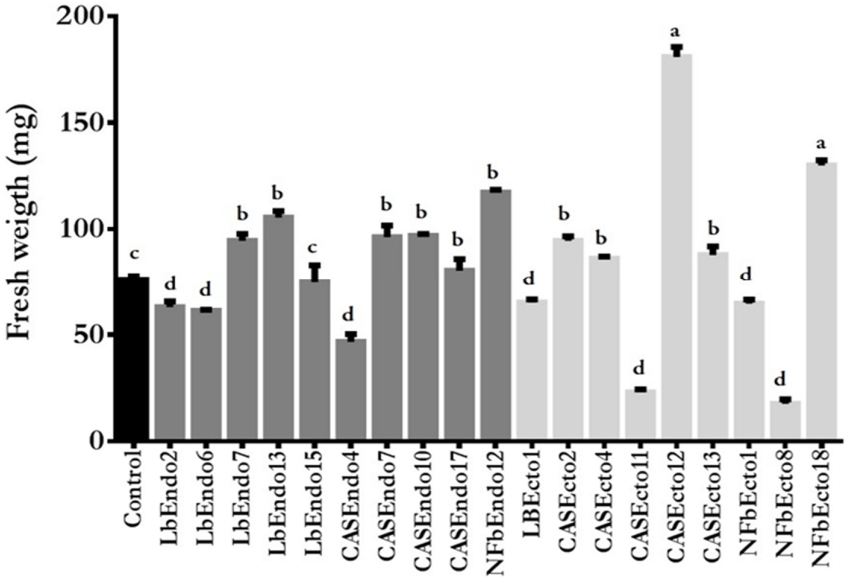

Figure 6. Effect of the inoculation of the rhizobacteria in *A. thaliana* seedlings, on fresh weight (mg) variable
Different letters indicate a significant difference (P≤0.05) between inoculated seedlings and the uninoculated control.

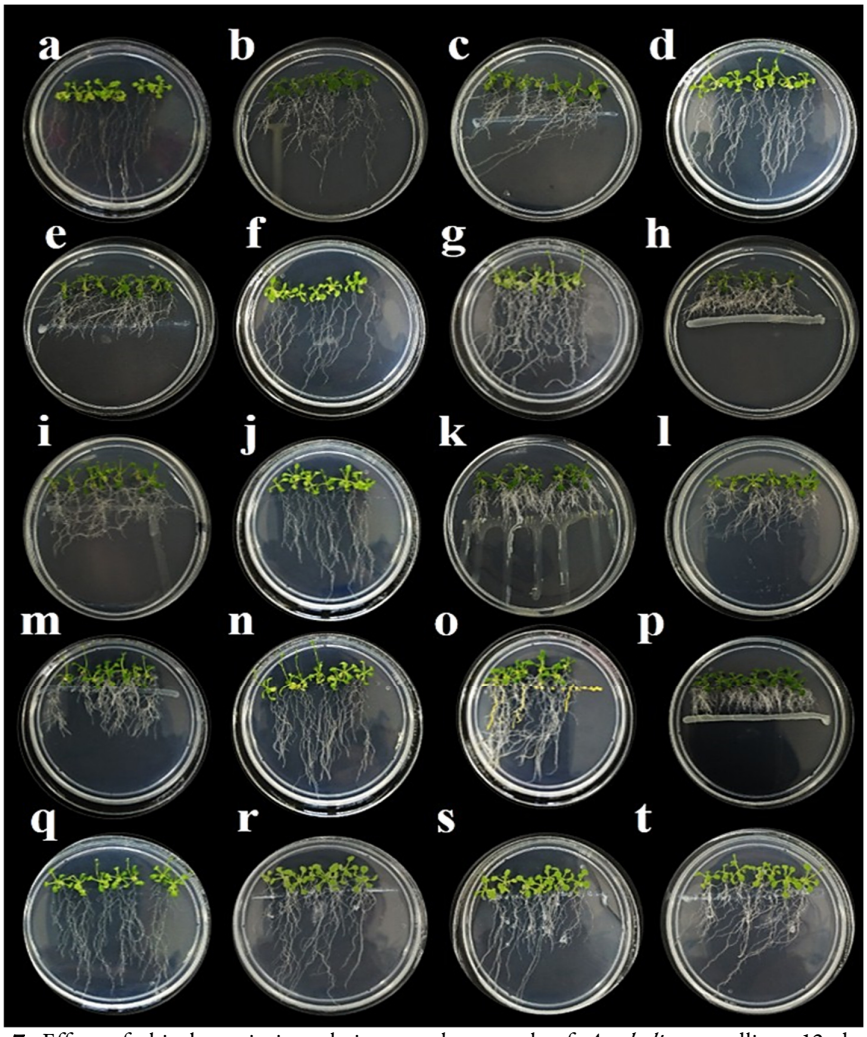

Figure 7. Effect of rhizobacteria inoculation on the growth of *A. thaliana* seedlings 12 days after inoculation in Petri dishes on 0.2× MS medium agar. a) Uninoculated control, b) LbEndo2, c) LbEndo6, d) LbEndo7, e) LbEndo13, f) LbEndo15, g) CASEndo4, h) CASEndo7, i) CASEndo10, j) CASEndo17, k) NFbEndo12, l) LbEecto1, m) CASEcto2, n) CASEcto4, o) CASEcto11, p) CASEcto12, q) CASEcto13, r) NFbEcto1, s) NFbEcto8, t) NFbEcto18

Tolerance of rhizobacteria to different concentrations of NaCl

The rhizobacteria selected from the two areas sampled (LbEndo2, LbEndo6, LbEndo7, LbEndo13, LbEndo15, CASEndo4, CASEndo7, CASEndo10, CASEndo17, NFbEndo12, NFbEndo16, LbEecto1, LbEcto8, CASEcto2, CASEcto4, CASEcto11, CASEcto12, CASEcto13, NFbEcto1, NFbEcto8, NFbEcto18), tested under salinity conditions of NaCl (0.85 M, 1.7 M and 2.55 M), all isolates had the ability to grow optimally in LB medium at 1.7 M, however, the rhizobacteria LbEcto8, CASEcto4, CASEcto13, CASEndo10 and CASEndo7 showed greater development up to the concentration at 2.55 M of NaCl (Table 2). All isolated had the opportunity to growth at 0.85M (5%) of NaCl with a maximum development. Our results showed that isolated bacterial could get in the classification as some halophilic bacteria (2% to 5% NaCl), moderately halophilic (5% to 20% NaCl), and extreme halophilic (20% to 30% NaCl), depending on their salt requirement according to Oren (2008), Kanekar *et al.* (2011) and Faraj *et al.* (2016).

Table 2. Growth bacterial under different salinity concentrations of NaCl of rhizobacteria (endorhizosphere/ectorhizosphere) of *E. antisyphilitica* from Sierra Mojada, Ejido San Jose de Madero, Coahuila and Sierra Viesca, Rancho Penjamo, Coahuila

Rhizobacteria ID	Salinity tolerance			
	0.85M (5%)	1.7M (10%)	2.55M (15%)	3.4M (20%)
LbEndo2	++	+	-	-
LbEndo6	+++	-	-	-
LbEndo7	+++	++	+	-
LbEndo13	+++	+++	+	-
LbEndo15	++	+	+	-
CASEndo4	++	+	+	-
CASEndo7	+++	+++	+++	-
CASEndo10	+++	+++	+++	-
CASEndo17	+	+	-	-
NFbEndo12	+++	+	+	-
NFbEndo16	+	+	+	-
LbEcto1	+++	+	+	-
LBEcto8	+++	+++	+	-
CASEcto2	+++	+	+	-
CASEcto4	+++	+++	+++	+
CASEcto11	+	+	+	-
CASEcto12	+++	+	+	-
CASEcto13	+++	+++	+++	+
NFbEcto1	+++	++	+	-
NFbEcto8	+++	+	+	-
NFbEcto18	+++	++	+	-

(+++) maximum development; (++) medium development; (+) minimum development; (-) no development

Phosphate solubilization

For the determination of phosphate solubilization, it was observed that the rhizobacteria isolated from the candelilla root CASEcto2, CASEcto4, CASEcto13 and LBEcto8 (Figure 8), showed growth potential in Pikovskaya solid medium, determined by the formation of a clear halo around the bacterial growth with a thickness of 1.487 mm ± 0.667mm, 2.133 mm ± 0.399 mm, 2.933 mm ± 0.530 mm, 5.267 mm ± 0.704 mm, respectively (Table 3). This result represents 19% of microorganisms selected and isolated in this study.

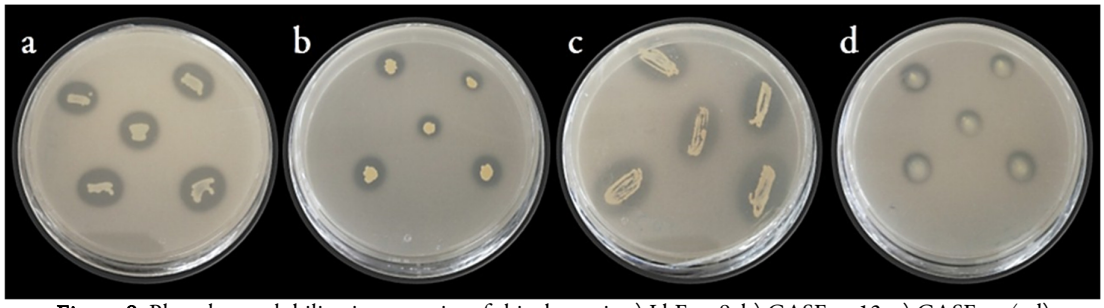

Figure 8. Phosphate solubilization capacity of rhizobacteria a) LbEcto8, b) CASEcto13, c) CASEcto4, d) CASEcto2

Indole-3-acetic acid production (IAA)

The results showed that in the production of IAA in all the selected bacterial strains showed the presence of this phytohormone, with values from 3.175 μg mL^{-1} to 22.619 μg mL^{-1} (Table 3).

Table 3. Results of the biochemical characterization of isolated rhizobacteria

Rhizobacteria ID	Phosphate solubilization (mm)	IAA production (µg mL^{-1})	Siderophores production	ACC deaminase (µmol α-KB/h/mg Pr)
LbEndo2	-	13.016	-	0.614
LbEndo6	-	13.889	-	0.747
LbEndo7	-	16.772	+	1.082
LbEndo13	-	12.778	+	1.154
LbEndo15	-	4.630	+	1.306
CASEndo4	-	17.460	+	0.518
CASEndo7	1.333 ± 0.488	15.000	+	0.748
CASEndo10	1.400 ± 0.507	11.984	-	0.157
CASEndo17	-	3.175	+	0.424
NFbEndo12	-	22.619	+	0.720
NFbEndo16	-	5.397	+	0.665
LbEcto1	-	12.937	+	0.388
LBEcto8	5.267 ± 0.704	14.127	+	0.757
CASEcto2	1.487 ± 0.667	17.249	+	0.657
CASEcto4	2.133 ± 0.399	6.429	+	0.457
CASEcto11	-	11.455	+	0.550
CASEcto12	2.667 ± 0.900	11.905	+	0.344
CASEcto13	2.933 ± 0.530	5.026	+	0.685
NFbEcto1	-	13.862	+	0.537
NFbEcto8	-	3.757	+	0.902
NFbEcto18	-	14.630	+	0.878

Arithmetic means ± standard deviation of triplicate experiments

(+) Development (-) No development

Siderophores production

In the assay carried out to demonstrate the production of siderophores; all the rhizobacteria isolated from the candelilla root (LbEndo2, LbEndo6, LbEndo7, LbEndo13, LbEndo15, CASEndo4, CASEndo7, CASEndo10, CASEndo17, NFbEndo12, NFbEndo16, LbEecto1, LbEcto8, CASEcto2, CASEcto4, CASEcto11, CASEcto12, CASEcto13, NFbEcto1, NFbEcto8, NFbEcto18 showed a translucent orange halo around their colony, suggesting, that these isolates have the ability to produce these low molecular weight organic substances.

ACC deaminase activity

The results showed that in the ACC deaminase activity all of the selected bacterial strains showed its presence, ranging from 0.157 to 1.306 µmol α-KB/h/mg Pr (Table 3), standing out that isolated LbEndo7, LbEndo13 and the LbEndo15 with values above of 1 µmol α-KB / h / mg Pr, while the least productive was CASEndo10.

Molecular identification

The characterization using the 16S ribosomal gene shows that, from the isolated bacterial microorganisms, and based on the biological properties, the microbial isolated corresponded to those indicated in Table 4, being *Bacillus* the most frequent of the genera.

Table 4. Molecular identification by gen 16Sr of the rhizobacteria isolated from candelilla
ID = identification, bp= base pairs

ID	Taxon	bp	Identity	Acces Number
LbEndo2	*Bacillus paramycoides*	1403	99	NR_157734.1
LbEndo6	*Bacillus mojavensis*	466	99	NR_118290.1
LbEndo7	*Staphylococcus epidermidis*	1396	99	NR_113957.1
LbEndo13	*Bacillus subtilis*	1366	95	NR_104873.1
LbEndo15	*Bacillus pseudomycoides*	1379	99	NR_113991.1
CASEndo4	*Bacillus pacificus*	1365	93	NR_157733.1
CASEndo10	*Bacillus cereus*	1398	99	NR_074540.1
CASEndo17	*Bacillus subtilis*	1379	97	NR_027552.1
NFbEndo12	*Cronobacter muytjensii*	724	98	NR_118088.1
NFbEndo16	*Staphylococcus epidermidis*	1398	99	NR_113957.1
LbEcto1	*Siccibacter colletis*	1360	99	NR_134807.1
LbEcto8	*Acinetobacter lactucae*	242	80	NR_152082.1
CASEcto2	*Siccibacter colletis*	1371	99	NR_134807.1
CASEcto4	*Staphylococcus epidermidis*	1402	99	NR_036904.1
CASEcto11	*Acinetobacter lwoffii*	1344	97	NR_113346.1
CASEcto12	*Mixta gaviniae*	641	99	NR_117305.1
CASEcto13	*Staphylococcus epidermidis*	1377	95	NR_113957.1
NFbEcto1	*Bacillus subtilis*	657	99	NR_104873.1
NFbEcto8	*Siccibacter colletis*	1371	99	NR_134807.1
NFbEcto18	*Acinetobacter johnsonii*	353	82	NR_164627.1

Discussion

In the present study, 21 rhizobacteria isolated from the candelilla rhizosphere were characterized, with the ability to stimulate growth in *Arabidopsis thaliana*. Our results agree with those study´s conducted by Palacio-Rodriguez *et al.* (2017) and Ramos-Acosta *et al.* (2015) where the beneficial microbial are presented in plants from Chihuahuan Desert at Coahuila State. Palacio-Rodriguez *et al.* (2017) from the rhizosphere of *Distichlis spicata* isolated 75 bacterial strains, 31 of wich were isolated from the endorizosphere and 44 from the ectorizosphere, using culture media Lb, KB, Jensen and NFb and Ramos-Acosta *et al.* (2015) in mesquite rhizosphere, 64 bacterial strains were isolated (16 from the endorizosphere and 28 from the ectorizosphere and 19 from the mesquite rhizoplane with the culture media (CRYEMA, KB and LB).

An evident *in vitro* phyto-stimulation was observed in *A. thaliana* seedlings after 12 days of inoculation. The root architecture was modified through changes in the growth of the main root, as well as the increased formation of lateral roots and root hairs, this could translate into a benefit of great importance for the acquisition of water resources and nutrients for the plant. In a study conducted by Estrada-González *et al.* (2017) seeds of *A. thaliana* were inoculated with PGPR to see their response, and a root growth promotion was obtained between 48% and 82% compared to the control without inoculation.

In our study, five rhizobacteria (LbEcto8, CASEcto4, CASEcto13, CASEndo10, CASEndo7) belonging to the moderately halophilic group were identified since they can tolerate a wide range of salt concentrations, evidenced by their growth in different concentrations of NaCl. In accordance with the above and knowing the classification of halophilic microorganisms with respect to their salt requirement, it is possible to confirm that the strains isolated in this study belong to the group of moderately halophilic bacteria, since they can tolerate a wide range of salt concentrations.

It is possible to use this type of halophilic bacteria, isolated from saline environments, as a possible option for the bioremediation of salinized soils for agricultural production. Palacio-Rodriguez *et al.* (2017) isolated

PGPR from the rhizosphere of *D. spicata* from hypersaline rhizospheric soils (EC of 10.12 mS cm^{-1}), these rhizobacteria were subjected to a salinity tolerance and had a resistance to a concentration of 5%, 10% and 15% NaCl, a same salt concentration used in our study, where LbEcto8, CASEcto4 and CASEcto13 rhizobacteria were isolated. One of the most obvious effects of rhizobacteria in our study was observed in the increase in root mass, mainly in the number of secondary roots, which provides plants with a larger area for the absorption of water and nutrients. This is because PGPR can enhance plant growth by a wide variety of mechanisms like phosphate solubilization, siderophore production, biological nitrogen fixation, rhizosphere engineering, production of 1-Aminocyclopropane-1-carboxylate deaminase (ACC), phytohormone production, exhibiting antifungal activity, production of volatile organic compounds (VOCs) and induction of systemic resistance, etc. (Bhattacharyya and Jha, 2012). As in this investigation, Ramos-Acosta *et al.* (2015) determined the solubilization of phosphates, it was observed that the rhizobacteria isolated from the mesquite root of La Poza Salada; EctoLB15 and RizoNFb2, showed growth potential in solid Pikovskaya medium, determined by the formation of a clear halo around the bacterial growth.

Angulo *et al.* (2014) reported PGPR with the ability to solubilize phosphates with a halo diameter between 2 ± 0.2 and 1.07 ± 0.1 mm. At the same study, a test of indoleacetic acid (IAA) production of rhizobacteria isolated from the root of *Eucalyptus nitens* reported the presence of this phytohormone with values from 4.60 μg mL^{-1} to 28.09 μg mL^{-1}. The evaluation by Gonzalez-Mancilla *et al.* (2017) indicated that the isolated strains of PGPR produced between 21 μg mL^{-1} and 26 μg mL^{-1} of IAA, where it is also mentioned that the production of auxins by the strains is conditioned by the type and age of the associated culture, in addition to the bacterium species and genus.

Indoleacetic acid produced by PGPR plays an important role in the plant-microorganism interaction, benefiting the promotion of root and foliar growth of plants as can be seen in the results obtained in the growth promotion test of this research; in this variable was standing out that isolate grown in NFb medium and that was obtained from the candelilla rhizosphere from Viesca region. In second position are the microorganisms named CASEndo4 and CASEcto2. The least producers were CASEndo17 and the NFbEcto8.

The results reported by Ramos-Acosta *et al.* (2015) in the trial to demonstrate the production of siderophores in CAS culture medium, six PGPR isolated from the rhizosphere of mesquite, showed the formation of a translucent orange halo around their growth, which allows confirming that these bacterial strains have the ability to produce siderophores. It has been shown that siderophores can, by themselves, act as efficient activators of induced systemic resistance systems in plants (Ran *et al.*, 2005; Meziane *et al.*, 2005; Bakker *et al.*, 2007). The ability of siderophores to act as pathogen suppressors depends on the plant, the phytopathogen to be controlled and the composition of the soil, the bacteria and the affinity of the siderophore for iron (Glick, 1995; Dellagi *et al.,* 2009).

Recent studies have shown that PGPRs containing ACC deaminase induce the production of longer roots, increasing the absorption of water (Zahir *et al.*, 2008; Esquivel-Cote *et al.*, 2013). Ribaudor *et al.* (2013) mentions that from the study of the relationship between the presence of ACC deaminase activity in PGPR and the inhibitory effect of ethylene on it, a possible role of the enzyme in the PGPR/PGPR interaction mechanism has been proposed plant. At the same time, Palacio-Rodriguez et al. (2017) report an activity of the ACC deaminase enzyme of 91 μmol α-KB / h / mg Pr of a PGPR isolated from the rhizosphere of *D. spicata.*

Regarding the rhizobacteria identified, the *Bacillus* has stood out for the promoting effect it exerts on plant growth. *Bacillus megaterium* and *Bacillus subtilis* are the most studied to determine the solubilization of phosphates, which is one of the mechanisms that PGPR use to promote growth (Tejera *et al.*, 2011) In the research carried out by Nawangsih *et al.* (2010) they were able to isolate *Staphylococus epidermidis* from healthy tomato stems and when testing it as a control agent against the *Ralstonia solanacearum* its effect was positive, they mention that *Staphylococus epidermidis* had not been reported as a bacterium endophyte tomato. Few reports of *Staphylococcus epidermidis* mention its ability to promote plant growth.

Conclusions

The rhizosphere of candelilla (*Euphorbia antisyphilitica)* that develops naturally in the Coahuila desert, specifically in the Sierra Mojada and Sierra Viesca, has a large reservoir of PGPR; in the present study 165 bacterial strains were isolated, from which 21 excelled in growth promotion, which was evident in the increase in root mass, mainly in the number of roots secondary in *A. thaliana*.

The rhizobacteria LbEcto8, CASEcto4, CASEcto13, CASEndo10 and CASEndo7 showed the ability to resist high concentrations of salt (15%) for which it is possible to confirm that the strains isolated in this study belong to the group of moderately halophilic bacteria.

The solubilization capacities of phosphates, production of IAA and siderophores that the isolated rhizobacteria presented, indicate that they can be used as growth promoters and for biological control in a crop of agronomic interest in future investigations.

The molecular identification of the most efficient rhizobacteria in plant growth promotion was achieved, finding bacteria of the genera *Bacillus* sp., *Staphylococcus* sp., *Acinetobacter* sp., *Cronobacter* sp. *and Siccibacter* sp. This is a first indication to proceed to carry out tests in different phenological stages in crops of agricultural importance.

Authors' Contributions

Conceptualization: MTSR, JAOV, JSM; Methodology: MTSR, JSM, EORP; Validation: JAOV, JSM, EORP; Formal analysis: MFH, PPR; Investigation: MTSR; Data curation: EORP, PYC, PPR; Funding acquisition: JAOV, JSM; Project administration: MTSR; Writing: MTSR, EORP; Review and editing: JAOV, JSM, EORP. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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