Plant Growth Promoting Bacteria Isolated From a Mexican Natural Ecosystem Induce Water Stress Resistance in Maize and Sorghum Plants

Juan G Colli Mull1, Gustavo A de la Riva de la Riva1,*, Coczy D Vargas-Sámano1, Gisselle Pérez-Machado2 and Guillermín Agüero-Chapin3,4
1Departamento de Biología, Instituto Tecnológico Superior de Irapuato (ITESI), Carretera Irapuato-Silao Km 12.5, Colonia El Copal, Irapuato, Guanajuato, México
2Departamento de Fisiología, Facultad de Medicina y Odontología, Universidad de Valencia, Ave. Blasco Ibáñez 46010, Valencia, España
3CIMAR/CIMARR, Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos, s/n, Porto 4450-208, Portugal
4Biology Department, Faculty of Sciences, University of Porto, Rua do Campo Alegre, s/n, Porto 4169-007, Portugal

Abstract

The negative environmental impact due to the excessive use of chemical products on agricultural crops is mitigated by using plant growth-promoting microorganisms, including bacteria and beneficial fungi associated to plant roots. Microorganisms could play a significant role in this respect due to their genetic diversity, ubiquity, interaction with crop plants and properties of tolerance to extremities. Therefore the microbial biodiversity and its effect on soil quality, soil nutrient cycling, plant growth promotion is considered a hub of attention for bio-prospection studies as an alternative to favor sustainable agriculture. The biodiversity of bacteria was deciphered from different sites in “Sierra Gorda” highlands, considered Reserve of Biosphere, in the downland region of central Mexico, known as Bajo. Culturable bacteria were isolated from soil rhizosphere samples and biochemically characterized due their indole-acetic acid (IAA) synthesis and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, as well as siderophore and polyhydroxybutyrate productions, cellulase and chitinase activity and phosphate mobilization. Bacteria were identified by the amplification of the 16S rRNA gene and BLAST analysis. Three strains, Pseudomonas variovencis XIU1297 and Luteibacter sp. XIU1292, Acinetobacter inoffii XIU12138 were selected to test their effects on the growth of Maize and Sorghum under water stress at greenhouse conditions. The results show differential growth promotion effect of those bacteria in maize and sorghum. The bacteria were selected to conform a consortium suitable to use in agriculture as biofertilizer.

Keywords: Soil bacteria; Plant growth; Water stress

Introduction

It’s known that microbiota displays multiple properties influencing in plant development, soil productivity, biomass production and conservation of whole ecosystems [1-3]. Microorganisms play important roles in different biogeochemical cycles and in the mobilization, cycling and transformation of inorganic and organic chemical soil compounds. Soil microorganisms influence in structure fertility, plant health and nutrition [4-9]. Microbial biodiversity play an important role in conservation and restoration of ecosystems and it’s also a source of microorganisms for biotechnology developments. In the soil, bacteria are the most abundant microorganisms particularly in the rhizosphere, where they are able to colonize plant roots. They get multiply and are the most abundant microorganisms particularly in the rizosphere, interactions with bacterial communities, its biodiversity and the relative abundances of individual bacterial taxa have been examined extensively in many ecosystems over the past 30 years. However, few studies have been focused on biodiversity and growth rates of specific taxa in natural soil microbial communities. It has been known for some time that the soil hosts a large number of bacteria (often around 10^8 to 10^9 cells per gram of soil) but the number of culturable bacterial cells in soil is generally only about 1% of the total number of cells present [13].

The most abundant bacterial genera in soil reported are, Azospirillum ssp., Alcaligenes ssp., Arthrobacter ssp., Acinetobacter ssp., Bacillus ssp., Burkholderia ssp., Erwinia ssp., Pseudomonas ssp., Rhizobium ssp., Rhodococcus ssp. and Streptomyces ssp. [14]. For the maintenance of bacterial communities in the soil, their members should have different related biochemical activities for nutrient cycling, biological control and plant growth promotion. Bacterial communities can be classified according to their effects on plants through the way they interact with roots. Some are pathogens whereas other triggers beneficial effects on plants such as, nitrogen fixing, auxins gibberellins and cytokinins [15-18]. Others produce siderophores which are essential for aiding in the biocontrol of pathogens, promoting antifungal or supporting activities in the control of the deleterious effects of pathogens by producing inhibitory substances, excluding them from the roots by competition or by inducing systemic resistance [19-22]. Plant Growth Promoting Bacteria (PGPB) comprises free-living and those that form specific symbiotic relationships with plants (e.g. Rhizobia spp. and Frankia spp.).

PGPB species of Pseudomonas and Bacillus sp. can produce phytohormones or growth regulators inducing the formation of a greater amount of fine roots in crops and consequently, increase absorptive surface of plant roots for uptake of water and nutrients [24]. PGPB release siderophores, low-molecular-weight compounds with high Fe^3+ chelating affinity that may scavenge iron by formation of soluble Fe^3+ complexes by active transport mechanisms of bacteria and plants. In addition acquisition of iron is also important for some pathogenic bacteria and therefore PGPB successfully compete for iron contributing to their reduction [25]. Significant increases on growth and yield of important crops in response to inoculation with PGPB have been reported [15]. Azospirillum, Pseudomonas

*Corresponding author: Gustavo A. de la Riva de la Riva. Departamento de Biología, Instituto Tecnológico Superior de Irapuato (ITESI), Carretera Irapuato-Silao Km 12.5, Colonia El Copal, Irapuato, Guanajuato, México. Phone and fax: 51-462-606-799-00, e-mail: gudelariva@itesi.edu.mx

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and Azotobacter strains could influence germination and seedling growth [26,27]. It has been shown that wheat yield increased up to 30% after inoculation with Azotobacter sp. and up to 43% with Bacillus inoculation. Strains of Pseudomonas putida and Pseudomonas fluorescens could increase root and shoot elongation in canola, wheat and potato [28-31]. The download region of Central Mexico, known as Bajío, is an important region for biodiversity due to its location in the confluence of Nearctic and Subtropical biogeographic regions. The intensive mining and agricultural and industrial activities impacted the ecology of the region, the use of fertilizer and other agrochemicals displaced the original micro biota. We started an extensive program of microbial prospecting of Natural Protected Areas (NPAs) in the state of Guanajuato, Mexico. The state of Guanajuato has twenty-four NPAs categorized according to their biodiversity, territorial extension, economic and social development; those that fall in the mountains of Sierra Gorda are classified as biosphere reserves, zones of sustainable management, natural management zones and ecological park. For each zone, the management form may differ, but all are destined for ecological restoration and sustainable development. These areas are well preserved showing a high microbial diversity, which is very important for our bio prospecting program that includes the study of microbial diversity and their potentiality to develop useful applications for soil, forest and ecological restoration providing positive implications in agriculture sustainability. In this work, we isolated cultivable microorganisms from soil and disclosed characteristics interesting for agricultural purposes such as growth stimulating factors, siderophore production, chitinase and cellulase activities, and polymer production. The isolated bacteria showing activities related to pest and pathogen biocontrol and plant growth promotion were biochemically and taxonomically characterized. Three strains, Pseudomonas variovencis XiU1297, Luteibacter sp. XiU1292 and Acinetobacter iwoffii XiU12138, were selected to test their effects on the growth of Maize and Sorghum under water stress and evaluated at greenhouse conditions. The results show positive but differential effects of those bacteria for each assayed crop.

Materials and Methods

Soil samples and isolation

Soil samples were taken from rhizosphere associated to Quercus ssp. forest at Natural Protected Area "Charco Azul", at 2,191 m upper the sea level (Xichú, Guanajuato, Mexico) in the Sierra Gorda Biosphere Reserve. Soil samples were collected from four replicates plots. The samples were taken within 3 km radius from the center of the base camp (21°18’50.5"N 100°06’39.2"W). Soil samples were kept on ice and transferred to the laboratory and primary suspensions were extracted by adding 9 ml of sterile 1X Phosphate Buffer Saline (PBS buffer: 8g/L NaCl, 0.2 g/L KCL, 0.2 g/L KH PO4, 1.15 g/L Na2HPO4, pH 7.4) to 1g of each sample. The resulting suspensions were agitated during 48 hours at 100 rpm at room temperature. Serial dilutions up to 1:106 of each primary suspension were prepared and 1 mL was plated on solid LB medium. These plates were then incubated at 30°C for 16-72 h before the selection, classification and the phenotype/genotype identification process. Bacterial colonies were picked up and cultivated in 5 mL LB medium, grown for two days at 100 rpm agitation and room temperature and conserved in glycerol. Each isolate was registered in the Institutional Tissue and Culture Collection (ITCC).

Culture media

Bacterial strains were isolated and cultured in liquid or solid LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar).

Mineral Media MM9 medium (6.8 g/L Na HPO4, 0.3 g/L KH PO4, 0.5 g/L NaCl, 1 g/L NH4Cl and 0.2% glucose) was used to grow bacterial strains for chitinase, cellulase and siderophore assays and CAS-blue agar was also used in siderophore test [32]. The production of poly-hydroxibutirate by bacterial isolates were tested using media M-PHB 1 (20 g/L sucrose, 2 g/L (NH4)2SO4, 1 g/L KH PO4, 0.2 g/L MgSO4.7H2O, 0.02 g/L CaCl2.2H2O, FeSO4.7H2O 0.01 g/L, agar 15 g/L, pH 7.0) and M-PHB 2 (glucose 10 g/L, MgSO4.7H2O 0.2 g/L, CaCl2.2H2O 0.01 g/L, FeSO4.7H2O 0.01 g/L, EDTA 0.01 g/L, KH PO4 0.6 g/L, K HPO4 0.9 g/L, pH 7.0). Sambouraud Dextraux Agar (SDA) medium (4% dextrose, 1% peptone and 2% agar), was used to growth test fungi Fusarium verticillioide, Fusarium solanii and Rizoctonia solani.

Gram staining

The differentiation of bacteria into either the gram-positive or the gram-negative group we follow the procedure described by farmer [33].

Qualitative and quantitative indole-acetic acid production

Screening of bacterial isolates for indole acetic acid (IAA) was done qualitative and quantitative. The ability of the strains to produce indole acetic acid (IAA) was tested according to the qualitative method reported by Sawar and Kremer [34]. Microorganism incubation was carried out for 72 h at 150 rpm in complete darkness in the culture medium indicated. The 150 μL of culture were distributed into 96-well plate following by addition of 100 μL of Sarkanowsky reagent (12 g/L FeCl3 in 7.9 M H2SO4) with a 1:1 relation between the reagent and the supernatant from microorganism culture. The reaction lasted for 30 min in darkness to yield a pink colored product. Indolic compounds concentration was analyzed by spectrophotometry at 520 nm. Serial dilutions prepared from Indole-3-acetic acid sodium salt (Sigma, USA) and used as IAA standard. The quantitative assays of IAA were carrying for selected strains using the same described conditions but with presence tryptophan at 0, 50, 150, 300, 500 and 1000 μg/mL. IAA concentration was measured in the spectrophotometer at 520 nm and quantified using an IAA standard. The experiments were done in triplicates and data were expressed as the mean value ± standard error [35].

DNA extraction and 16s rRNA amplification

DNA was extracted from isolates by using a Microbial DNA extraction kit (MO BIO Laboratories Inc., Carlsbad, CA, USA). The fragments of 16s rRNA gene were amplified by using the primers RP1 (cgcaattgctgacacAGAGTTGATCCTGAC) and FDI (cgcaattgctgacacAGAGTTGATCCTGAC) [36]. The PCR conditions were determined as follows: pre-denaturing step at 95°C for 2 min, denaturing at 94°C for 50 s, annealing at 57°C for 50 s, polymerization at 75°C for 2 min and post PCR at 75°C for 10 min and 35 cycles. PCR products were analyzed on 1% agarose gel and sequenced. Bacteria were identified by BLAST sequenced analysis of the amplified 16s rRNA gene fragments.

ACC deaminase activity

ACC deaminase activity was evaluated by measuring of α-ketobutyrate and ammonia production when the ACC is cleavage by ACC deaminase [37,38]. The standard curve were obtained by serial dilutions of α-ketobutyrate at 0, 0.01, 0.05, 0.1, 0.2, 0.5, 0.75 and 1 mM concentrations in 100 mM Tris-HCl pH 8.5 (total Volume 500 μL), mixed in each case with 400 μL of 0.56 N HCl and 150 μL DNF solution (0.1 g 2,4-dinitrophenylhydrazine, Sigma, USA; in 100 mL of 2 N HCl) followed by addition of one mL of 2 N NaOH. Finally absorbance was determined at 540 nm. The different strains were grown...
in 5 ml of LB medium at 28°C until they reached stationary phase. The bacterial cells were collected by centrifugation, washed twice with 0.1 M Tris-HCl (pH 7.5), suspended in 2 ml of modified M9 minimal medium supplemented with 3 mM final concentration of ACC without PEG for non-stress condition and with PEG 6000 (-0.30 MPa) for drought stress condition and then incubated 28°C with shaking for additional 36-72 h. The induced bacterial cells were collected by centrifugation (3000 rpm for 5 min), twice washed with 0.1 M Tris-HCl (pH 7.5) followed by centrifugation (3000 rpm for 5 min and finally resuspended in 200 μl of 0.1 M Tris-HCl (pH 8.5). The resuspended cells were labilized by adding 5% toluene (v/v) and mixed at the highest vortex speed for 30 s. As positive control we used 50 μl of cell suspension with 5 μl of 0.3 M ACC and as negative control we used 50 μl of labilized bacterial cell suspension without ACC, incubated at 28°C for 30 min. The blank included 50 μl of 0.1 M Tris-HCl (pH 8.5) with 5 μl of 0.3 M ACC. The samples were then mixed with 500 μl of 0.56 N HCl and the debris was removed by centrifugation at 12,000 g for 5 min. A 500 μl aliquot of the supernatant was transferred to a glass test tube and mixed with 400 μl of 0.56 N HCl and 150 μl of DNF solution (0.1 g 2,4-dinitrophenylhydrazine in 100 ml of 2 N HCl); and the mixture was incubated at 28°C for 30 min. 1 ml of 2 N NaOH was added to the sample before determining absorbance at 540 nm.

**Siderophores production analysis**

Siderophore production was analyzed on chrome azurol S agar plates. Bacteria were grown for 2 weeks at room temperature on plates and siderophore producing organisms were able to extract iron from the blue Fe(III)-CAS complex forming an orange depletion zone in the medium. The chromeazur (CAS) agar assay was used according to the modifications introduced by Silva-Stenico et al. [40]. Positive colonies were identified by the formation of a clear halo around them, showing a visual change in color from dark blue to orange. In addition, antifungal activity was tested by diffusion method against Fusarium verticillioides, Fusarium solani and Rhizoctonia solani on Sabouraud Dextrose Agar (SDA) medium [41]. Firstly the test fungi were grown in SDA medium until sporulation, diluted suspension of spore were taken and suspended in 10 ml of sterile 1X PBS and number of CFU was counted. Then, 100 μl, of a 10^7 CFU/ml were spread on SDA plates. Wells (diameter 10 mm) were made into SDA plates and filled with 100 μl of bacterial culture at concentration of 10^6 CFU/ml. LB liquid media with 100 μl nystatin solution (100 μg/ml) was used as a positive control. The antifungal activity was evident when growth inhibition zone against test fungi appeared.

**Cellulase and chitinase activities**

For cellulase activity, we used carboxy-methyl-cellulose (CMC) in solid minimal media containing 0.01% MgSO_4·7H_2O, 0.01% KHPO_4, 0.05% yeast extract, 0.7% (NH_4)_2SO_4, 0.1% NaCl and 1.5% bacteriological agar. CMC degradation by the isolates by covering the Petri dishes with Congo Red dye, as described by Teather and Wood [42]. CMC degradation was indicated by a clear zone around the colonies. For Chitinase activity, the strains were cultivated in medium contained of 1% chitin, 0.01% Congo Red, 0.01% MgSO_4·7H_2O, 0.01% KHPO_4, 0.05% yeast extract, 0.7% (NH_4)_2SO_4, 0.1% NaCl and 1.5% bacteriological agar and were incubated at 37°C for five days. The isolates causing a clear zone were considered positive to this test [43].

**Phosphate-solubilizing assay**

The ability of isolates to solubilize phosphate was assessed qualitatively using potato-dextrose yeast agar (PDA, pH 7.0) supplemented with freshly precipitated 50 ml 10% KPO_4 plus and 100 ml 10% CaCl_2 per liter of PDA medium. Each bacterial culture was streaked in the center of PDA-Calcium phosphate plate and then incubated during two weeks at 27°C. The clearing zone surrounding the developed bacterial colony shows phosphate solubilization [22].

**Polyhydroxybutyrate (PHB) detection**

Polyhydroxybutyrate (PHB) production was detected in bacterial isolates by growing it on Petri dishes containing Media 1 or Media 2 [44]. Isolates of axenic colonies were randomly picked and cultured in solid MM9 supplemented with 5 g glucose/L and 0.5 mg Nile blue (Sigma, St. Louis, MO, USA). Petri dishes were incubated for 4-5 days at 30°C. The orange fluorescence observed under UV (λ = 312 nm) was used to detect PHB positive colonies in contrast with barely visible fluorescence of PHB-negative colonies. Standard were prepared from pure PHB (Sigma, USA) by modified method at concentrations ranging from 1 to 10 μg/ml [45,46]. Comparing the fluorescence intensity in the halo generated by each isolates we grouped it in tree groups according to biopolymer production by named low (lower 3 ng/ml), middle (between 3 and 6 μg/ml) or high (between 6 to 10 μg/ml).

**Effect of bacterial strains on plant growth in maize and sorghum**

Bacterial inoculum was prepared by growing cells in nutrient broth at 30°C, 120 rpm until the end of the exponential growth phase. Bacteria were then harvested by centrifugation (5000 rpm/min for 10 min), washed and suspended in PBS to 10^6 CFU/ml. Germinated seed were inoculated with 1 ml of bacterial suspension and control seeds with 1 ml PBS into plastic pots filled with 200 g sterilized soil at final bacterial concentration of 5 × 10^5 CFU/g soil (1 ml of 10^6 CFU/ml200 g soil). The inoculated seedlings were cultured under greenhouse conditions with 14 h/day photoperiod at 28°C during the day, 20°C during the night and 60% relative humidity. All pot experiments contained five seedlings and five replicas were performed per pot. The plants were harvested after six weeks of inoculation and dry and fresh plant biomass were measured and compared to control plants (NC).

**Effect of bacterial strains on plant growth under water stress conditions**

We followed the method described by Mayak et al. to evaluate the effect of bacterial isolates on different plants crops maintained under greenhouse conditions [47]. Plants were cultivated in soil conditions similar to agricultural lands of Bajo region at greenhouse and were subjected to water stress at week 3 after germination. The irrigation was reassumed at the beginning of week 4. The effects of bacterial treatments were measured after 3, 4, 5 and 6 weeks by calculating the relative water content (RWC). RWC was measured by comparing fresh weight (FW), fully turgid weight (FTW) and dry weight (DW). Firstly, FW was measured in collected plants; then they were conserved in humid camera at 25°C in the dark during 48 h to measure the FTW. Ones we determined FW and FTW, all the plants were dried during
Prevalence of the bacterial inoculants in plant rhizosphere and bulk soil

Besides the effect of inoculated bacterial strains on sorghum and maize under water stress conditions, we also explore the prevalence and dynamic of the inoculated strains. Rhizospheric and bulk soil samples were collected at the start day, and after 3, 4, 5 and 6 weeks. For each treatment and sampling time the roots (three plants per repl), were analyzed to determine the population dynamic of the three bacterial strains during the water stress experiment. To quantify the number of UFC in soil and rhizosphere of both crops, three replicates per treatment were independently analyzed. For rhizosphere, adhering soil was removed and treated. The rest of the soil was well mixed and used for determination of bacterial persistence in bulk soil. For rhizosphere bacterial isolation, roots were washed with sterile tap water before microbial cells were extracted as follows: the roots were cut into pieces of approximately 1 cm length and carefully mixed. Five gram of roots was placed in sterile flask and shaker for 30 s at high speed after adding 15 ml of sterile 0.3% NaCl. This step was repeated three times [48]. Aliquots of the each rhizosphere microbial cell suspension were immediately processed to determine CFU counts by plating serial dilutions onto LB agar media supplemented with antibiotic allowing the growth of inoculated bacteria but no other soil bacteria. The used antibiotics were selected previously as follows: as antibiotic allowing the growth of inoculated bacteria but no other soil bacteria.

Statistical Analysis

The effect of the selected bacterial strains on plant growth was measured in green house at two conditions: normal irrigation scheme and water stress conditions. Water stress experiments were evaluated by the calculation of RWC. The evaluation of selected bacterial strains on maize, sorghum was carried out in greenhouse experiments. Data were analyzed by analysis of variance (ANOVA, p-level <0.05). The statistical analysis and graphic construction were performed using Minitab 16 (Minitab Inc. Minitab Acad. Software Center, Pennsylvania).

### Results

#### Bacterial isolation

The soil samples used in this study was collected in selected prospective zone of “Charco Azul” NPA (Xichú, Guanajuato, Mexico) located in “Sierra Gorda Reserve of Biosphere” and bacterial colonies were grown, picked and cultured. About 3.5 log⁰ CFU/g were found in soil samples. Because of a large number of isolates, the strains were previously characterized by their morphological appearance (white, opaque of translucent and rough, granular or wispy colonies) and by gram assays and the three strains included in this study resulted in gram-positive. Massive qualitative determination of indole-acetic acid production has been used to select the most promising bacterial strains and three of them were selected for further characterization.

#### Determination of indole-acetic acid production

The three selected strains displayed remarkable high level of IAA synthesis in the absence and presence of L-tryptophan. In the absence of tryptophan the three strains showed similar IAA production between 0.915 μg/ml to 0.972 μg/ml. In the presence of 0, 50, 100, 150, 200, 500 and 1000 μg/ml of up to 7 times in IAA production were observed, with compared with the level obtained in absence of tryptophan but no differences between bacterial strain were observed (Table 1).

#### Identification of selected bacterial isolates

We identified the isolates by partially sequencing of the PCR amplified 16s rRNA gene followed by comparison against the NCBI sequence database using the BLASTN algorithm [49]. We compared the sequences of amplified 16s fragment of selected strain with the 16S sequences from other strains isolated from the same place to observe if they have common phylogeny (Figure 1). The three isolates were classified as Pseudomonas variovencis strain X11297, Luteibacter sp. Strain X11292 and Acinetobacter iwofii Xi12138.

### ACC deaminase activity

The three selected isolates were screened for ACC deaminase based on the enrichment method, where ACC play the role of sole nitrogen source. The ACC deaminase enzyme activity was assayed under both non-stress and drought stress conditions by quantifying the amount of α-ketobutyrate produced during the deamination of ACC by the enzyme ACC deaminase. The strains grew well on MM9 medium with either ACC or ammonium sulfate serving as the sole nitrogen source. To simulate drought stress condition the MM9 medium supplemented with PEG 6000. The highest amount of ACC deaminase activity (4.97–5.35 μM/mg protein/h of α-ketobutyrate) was obtained under non-stress condition whereas the lowest activity (3.17 to 3.35 μM/mg protein/h of α-ketobutyrate) was obtained under induced drought stress condition (Table 2). The same levels of ACC deaminase activity with where obtained for all strains, but well correlated differences between non-stressed and drought stress conditions have been observed.

### Table 1: Quantitative determination of IAA production at different tryptophan concentrations

<table>
<thead>
<tr>
<th>Serial Number</th>
<th>Bacterial Strain</th>
<th>IAA production (μg/ml) at different tryptophan concentrations (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>X11292</td>
<td>Luteibacter sp.</td>
<td>0.458</td>
</tr>
<tr>
<td>X11297</td>
<td>Pseudomonas variovencis</td>
<td>0.522</td>
</tr>
<tr>
<td>X112138</td>
<td>Acinetobacter iwofii</td>
<td>0.475</td>
</tr>
</tbody>
</table>

The values represent the mean of three independent experiments, n: 3, σ: Standard Deviation
The three strains have been assayed for other important metabolic activities: synthesis of siderophore, cellulase, chitinase, phosphate mobilization and biopolymer (PHB) production. The results of preliminary qualitative tests showed that *Luteibacter* sp. (XIU1292) had high level of siderophore production, as well as cellulase activity but low chitinase, phosphate mobilization and PHB polymer production. *Pseudomonas vranovencis* (XIU1297) exhibited highest level of IAA, siderophore, phosphate mobilization and PHB production a medium level of chitinase activity and lowest cellulase activity. Finally, *Acinetobacter iwoffii* (XIU12138) presented high IAA production and chitinase activities and lower levels in the rest of assays (Table 3).

**Plant growth promotion and water stress resistance conferring by selected strains in maize and sorghum**

We evaluate the plant-growth and water stress resistance of three selected bacterial isolates on maize and sorghum. We selected these three bacteria for their high level of IAA production, but also for the other biochemical activities important for plant growth and health. Experimental results showed that bacteria strains promote growth in...
Figure 2: Effect of bacterial strains on roots development (A) and stems and leaves (B) in maize plants under water stress, evaluated by fresh and dry weight. Treatments: T1- No bacterial treatment, T2- Luteibacter sp. XIU1292, T3- Pseudomonas vranovensis XIU1297, T4- Acinetobacter iwoffii XIU12138, T5- Daily irrigated plant without bacterial treatment. Water irrigation was discontinued at the end of week 3 and restarted at the end of week 4.

Figure 3: Effect of bacterial strains on roots development (A) and stems and leaves (B) in sorghum plants under water stress, evaluated by fresh and dry weight. Treatments: T1- No bacterial treatment, T2- Luteibacter sp. XIU1292, T3- Pseudomonas vranovensis XIU1297, T4- Acinetobacter iwoffii XIU12138, T5- Daily irrigated plant without bacterial treatment. Water irrigation was discontinued at the end of week 3 and restarted at the end of week 4.
both plant species although all three shows relatively the same level of IAA production. In case of maize, the best growth-promotion was achieved by using \textit{P. variophoca} XiU1297, followed by \textit{Luteibacter sp} XiU1292 and \textit{A. iwoffii} XiU12138 (Figure 2). For sorghum, the highest growth-promotions were obtained by \textit{Luteibacter sp} XiU1292 followed by \textit{P. variophoca} XiU1297 and \textit{A. iwoffii} XiU12138 (Figure 3). These results are well correlated with the water stress resistance and recovery patterns observed in the assayed plants as well as in RWC. In both crops, the highest RWC values were obtained for root developments regarding the achieved for stems and leaves.

### Prevalence of the bacterial inoculants

We explore the dynamic prevalence of the inoculated strains during the water stress experiment. Previously we selected antibiotics allowing the positive selection of each isolated from the background as follows: \textit{Luteibacter sp} XiU1292 (Tetracycline 50 μg/ml), \textit{P. variophoca} XiU1297 (Cephalxin 30 μg/ml) and \textit{A. iwoffii} XiU12138 (Kanamycin 30 μg/ml). The used antibiotics allowed us to control other bacteria in the soil derived from the non-sterile experimental condition in greenhouse. Rhizosphere and bulk soil samples were collected at the started day, and at the weeks 3, 4, 5 and 6, after inoculation of plants from both crops with the PGPB. We quantify the number of UFC in soil and rhizosphere of both crops as described. The ability of isolates to colonize the rhizosphere of maize and sorghum as well as persistence in bulk soil was determined by plating and growing onto LB agar media supplemented with corresponding antibiotic. The inoculants density (CFU counts/g RDM) was calculated logarithmically (Log10) in each step for both crops (Table 4). We observed that during the first two weeks the prevalence of bacterial CFU in bulk soil drops to less than 5 × 10^4 CFU per gram of substrate, compared with initial concentration of 5 × 10^5 UFC/g soil. When water supply was suspended this tendency continues until water irrigation was restored at week four. Then, the prevalence continues stable until the end of experiment. In with rhizosphere, we failed to detect bacterial inoculants in weeks 2. The inoculated bacteria were found in rhizosphere since the Week 3rd until the week 4th. Then the bacterial prevalence was stabilized during the rest of experimental period.

#### Discussion

Soil is a dynamic ecological niche hosting living microorganisms that play an important role in processes essential for supporting plant development and the whole ecosystem. Most soils contain an enormous diversity of microorganisms: bacteria, actinomycetes, fungi, algae and protozoa. It has been reported that a typical gram of soil contains approximately ∼9 × 10^9 bacteria, 4 × 10^8 actinomycetes, 2 × 10^3 fungi, 3 × 10^3 algae, 5 × 10^3 protozoa and 3 × 10^4 nematodes although the numbers and types of these organisms and their dynamic vary greatly depending on the soil and climatic conditions [50]. The soil biota are also essential in decomposition and nutrient cycling processes, mineral and phosphorous mobilization, nitrogen fixation, plant adaptation to biotic and abiotic stress and growth promotion. The diversity of microbiota and the knowledge of its components, biochemical activities, biotic and abiotic interactions represent an opportunity to use soil microbial communities to increase crop productivity. From the bioprospected area Charco Azul, bacterial strains were isolated, morphologically characterized and integrated to Institutional Tissue and Culture Collection (ITCC). The majority of bacterial isolates were collected from rhizosphere, the small zone adjacent to plant roots where major microbial activity is concentrated and from aggregates of organic matter found in the bulk soil. The comparison of 16S genes among selected bacterial strains isolated from Charco Azul show the phylogeny relationship. The three bacterial strains (\textit{L. sp} strain XiU1292, \textit{P. variophoca} strain XiU1297 and \textit{A. iwoffii} strain XiU12138) were selected for evaluation on maize and sorghum plants showing that they produce plant growth regulators and have a consistent prevalence in the soil conditions of

<table>
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<th>A. Maize</th>
<th>Bacterial Strain Location</th>
<th>Week 2 (10^4)</th>
<th>σ (10^4)</th>
<th>Week 3 (10^4)</th>
<th>σ (10^4)</th>
<th>Week 4 (10^4)</th>
<th>σ (10^4)</th>
<th>Week 5 (10^4)</th>
<th>σ (10^4)</th>
<th>Week 6 (10^4)</th>
<th>σ (10^4)</th>
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<tr>
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σ: Standard Deviation. CFU/g RDM: Colony forming units per gram of root dry weight, p ≤ 0.05
the Bajío. They exhibit similar growth patterns during their cultivation in both solid and liquid LB medium (data not shown). These similarities were desirable to study their beneficial effect on maize and sorghum plants together with their plant growth promotion effects, resistance to water stress and specific plant-bacteria interactions. Some differences among these strains were found in other activities, those differences could influence in the effects observed on plant growth. The IAA production determined on these three bacterial isolates showed no remarkable differences in IAA production with is without the presence of tryptophan. We observed similar tryptophan depend pattern in IAA production as reported previously by other authors which is relevant to mention since the ability of PGPB to produce IAA in the rhizosphere greatly depend on presence and abundance of precursors [47,51,52]. Ethylene, a plant hormone which is found in all higher plants, is an important modulator of plant growth and development and it acts as a key factor to adequate response of the plants to a wide range of stresses [53]. The role of ethylene in physiological plant development is evident in different tissue developments such as roots, stems, leaves, flowers and fruits [30]. In case of legumes this hormone modulates rhizobia nodulations rooting of cuttings and plant’s interaction with beneficial fungi including mycorrhizal symbiosis [54-56]. Ethylene synthesis is affected by a number of different factors including temperature, light, gravity, nutrition, the general hormonal correlation in plant and wild range of abiotic and biotic stresses [57]. Generation of ethylene is liked by the consumption of 1-aminocyclopropane-1-carboxylate (ACC), a precursor of ethylene. The measure 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity is crucial to evaluate the bacterial trait involved in plant growth. ACC deaminase is responsible for the cleavage of the plant ethylene precursor, ACC, into ammonia and α-ketobutyrate decreasing ACC levels and ethylene levels in plants. Comparing ACC deaminase activity of the three stains we observed similar pattern (Table 2), at the same level as reported by other authors [52]. This activity drop when plants are in drought stress condition. It has been suggested that reduction in ethylene production may improve plant cells by increasing membrane fluidity [52,58] and by activating the expression of some abiotic-stress related genes that participate in conferring resistance during abiotic stress as water stress [59,60]. Although the IAA production is considered as the most important criterion to identified PGPB, other factors must be taken in account to explain the beneficial effect of bacteria on plant development, such as plant-microorganism interactions, the prevalence of applied bacterial strains in soil and the reduction of ethylene production [30,50]. Other traits that have been studied included synthesis of siderophore, cellulase, chitinase, phosphate mobilization and biopolymer (PHB) production (Table 3). These aspects are directly related to plant growth since they influence plant development and health. In case of siderophore production, highest production was detected for strain L. sp. XiU1292 and P. vranovencis XiU1297 followed by the A. iwoffii XiU12138. All three strains were confronted to fungi Fusarium verticillioides, Fusarium solani and Rizoctonia solani, important pathogens of maize and sorghum and the three bacteria exhibited antifungal activity evidenced by growth inhibition zone against test fungi. We cannot distinguish a consistent differences between growth inhibition zone produced by this strain respect to the other two although in vitro assays distinguished lower siderophore production in case A. iwoffii XiU12138. Other antifungal factors may be present also in PGPB, like glucanase and chitinase activities or secondary metabolites synthesis. Combining siderophore and chitinase activities we found that A. iwoffii XiU12138 showed highest chitinase activity but lowest siderophore synthesis compared with L. sp. XiU1292, that showed high siderophore but low chitinase activities and P. vranovencis XiU1297, that showed high siderophore but medium chitinase activities. However more detailed studies, including secondary metabolism synthesis, are required to define this matter. The antifungal activity in PGPB is important particularly because of its incidence in the plant health and production yields. Bacterial strains, which expressed siderophore, chitinase and glucanase activities, should be active against wide range of fungi, having glucanases and chitin in their cell wall composition. Phosphorous is one of the most important elements in plant nutrition, phosphate solubilization or mobilization is usually linked with Calcium. There are two main types of phosphorus in soil, organic and inorganic phosphates, largely present in insoluble forms that impact negatively in plant nutrition since insoluble forms could not be used for plant nutrition. Organic phosphates are an important but also immobilized form of phosphorous accounting 20-80% of total soil phosphorous [61]. To make this important compounds available to plants, phosphate mobilization trait is an important characteristic to evaluate in all PGPB. In our case P. vranovencis XiU1297 showed highest phosphate mobilization activity “in vitro” than the other two stains that showed a low activity. The ability of PGPB to solubilize precipitated phosphates and to enhance phosphate availability to maize, sorghum and other crops represents another possible mechanism of plant growth promotion under field conditions. Infection and invasion of roots by bacteria requires degradation of plant cell walls, which might be an active process involving plant polymer degrading enzymes. Cellulase, as well as other activities, like pectinases, is often produced by phytopathogenic bacteria such as P. solanacearum and E. chrysanthi which hydrolyses cellulose and pectin present in plant cell wall, allowing the pathogens to penetrate the plant tissues. In our experience cellulase and pectinases are actively secreted to the bulk soil helping to recycle plant debris such as fallen leaves, flowers and fruits [62]. In this study cellulase and pectinase activities of strains didn’t affect the growth or health of the seedlings. Since our bacterial prevalence studies we consider that as soon as bacterial strains colonize the plant rhizosphere or become endophytic plant exudates as well as other members of endophytic or rhizospheric microbiota suppress the production of such enzymes. In our case a colonization processes were observed in both crops for all the three inoculated bacterial and the results of plant-PGPB experiment were well correlated with the prevalence of bacterial strains in both rhizosphere and bulk soil (Table 4). The biopolymer (PHB) production is anther trait that was also evaluated. The highest level was detected in case of P. vranovencis XiU1297. Synthesis of biopolymer by PGPB is frequently demerited as an important plant growth promotion because it is considered undirected factor. Many species of beneficial bacteria form micro colonies or biofilm. Bacterial surface components, such as PHB, in association with bacterial signals can be important in the process of biofilm formation and also in functionally and structurally supporting the diverse arrays in rhizospheric bacterial communities as well as helping its cohesion, bacterial activity and survival by forming bacterial aggregates [63]. The environment occupied by soil bacteria range from rhizosphere, a small layer rich in nutrient and plenty of bacteria exhibiting a wide range of biochemical activities, to the bulk soil, deficient in nitrogen, phosphates and water, among other important nutrients [64,65]. Another important aspect of biopolymers producing by PGPB is its role in bioaugmentation and bioremediation of impacted soils. Many industrial and agricultural activities during five hundred years, but especially in the last forty years, have caused the significant increase in the concentration toxic residues and pollutants in environments in the Bajío regions and in the rest of the world. Together with mining,
industrial, agriculture and other contaminants, there are also natural residues from volcanic geological origins of this region. Biopolymer producing PGPB can help the structure of microbiota in rhizosphere, drought resistance and augmentation in presence of soil contaminants [66,67]. Maize and sorghum plants were cultivate in soil conditions similar to agricultural lands of Bajío region but at greenhouse to manage the water supplied regime. The three bacterial strains added to these crops showed remarkable effect on their growth and water stress resistance, as it was previously reported [59,50]. However both plant species showed different responses to the treatments. In case of maize (Figure 2) better growth-promotion was obtained with *P. variovencis* XiU1297, followed by *L. sp* XiU1292 and *A. iwoffii* XiU12138. In case of sorghum (Figure 3), the highest growth-promotion was obtained with *L. sp* XiU1292, followed by *P. variovencis* XiU1297 and *A. iwoffii* XiU12138. These results correlated with the water stress resistance observed in assayed plants and the same tendency was observed when the RWC was determined (Figure 4). Our results are congruent with the observation reported in tomato plants [26]. Treated plants exposed to water stress condition showed enhanced resistance and recovery. The values of fresh and dry weights as well as the RWCs also explain such effects. The differences between the values of weights and RWC determined for roots and aerial part of the plant (stems and leaves) indicate a predominant root-specific effect by PGPB. However we also observed that PGPB can stimulate differentially plant growth depending on compatibility of plant-microorganism interactions. To explore the prevalence of each bacterial strain in maize and sorghum the recovery of bacteria from rhizosphere and bulk soil was done (Table 4). During the first two weeks, just after suspension of water supply, the UFC in bulk soil drops to less than 5 × 10^4 UFC per gram of soil, compared with initial concentration of 5 × 10^5 UFC/g soil (1 mL of 10^8 CFU/ml; 200 g soil=5 × 10^5 UFC/g soil). The adaptation of bacteria to new conditions could be the factor explained the initial drop in UFC.

After induced drought stress the decreasing tendency continues but stabilization becomes when the water supply was restored at week 4. Bacterial prevalence continues stable to the end of experiment but some differences were observed between both plant species, revealing preferences in plant-bacteria interactions. Similar tendency was obtained when we analyzed bacterial colonization of maize and sorghum rhizosphere. We failed in the detection on bacterial inoculants in week 2 and just at week 3 after inoculation we began to recover inoculated bacteria in rhizosphere, probably because the process of plant-bacteria interaction and rhizosphere colonization was in the initial steps. The relative amounts of bacteria in rhizosphere were well correlated with the differences found in bulk soil. The dynamic of the bacterial prevalence changed since drought stress was induced at week 2. The prevalence of rhizospheric bacterial didn’t show any decrease and continue growing after stabilization at week 4, a tendency that continue during the rest of experiment. These results drive us to think that rhizosphere bacteria better survive the abiotic stress than those remaining in bulk soil and the presence of PGPB help the plant to support the water stress conditions. Plants under stress conditions are able to incorporate the soil nutrients and maintaining their health and physiological conditions when PGPB
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activities and environmental behaviors to conform different bacterial characteristics of plants and the generation of specific changes in the plant-bacterial interaction, bacterial viability and prevalence in soil, the promotion of specific gene expression and the production of certain compounds influencing in plant behavior [69]. The soil characteristics influence to the microorganisms in the rhizosphere ant its activity [13]. Since bioprospected area is a temperate pine and oak forest (16°C average annual temperature), humid (1200 mm annual rainfall) and the organic matter content are about 40%. We need to evaluate the behavior and the effect of bacterial strains under soil and climatic conditions where the strains are projected to apply. We used maize and sorghum plants according to those existing in a semi-warm semi-humid region (700 mm annual rainfall), chernozem (black lands) soil type. Concerning the water stress resistance, it has been reported that bacterial RNA chaperones confer abiotic stress tolerance on plants and improved the grain yield in maize under water limited conditions [70-72].

Conclusion

Diversity of factors acts in the global effect of PGPB to plant development and this reveal the need of direct evaluation in crops before the bacterial application. The mechanism underlying the stimulation of overall plant performance mediated by PGPB in different situations is still an active research field due to its complexity. The selected strains differentially promoted plant development in maize and sorghum. *P. vranovencis* XU1297 acts better in maize while the bacteria L. sp. XU1292 does it better in sorghum. These abilities complement each other and conceive these strains suitable to integrate a bacterial consortium for application in agriculture together with other bacterial isolates. The absence of antagonism among bacterial strains selected as the consortium components were already essayed within these and other PGPB-bacteria, complementing different biological activities and environmental behaviors to conform different bacterial consortium suitable to use in different crops. The conformation of beneficial microbial consortia and its application contributes to the development of sustainable agriculture.

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