

## Enterobacteria isolated from an agricultural soil of Argentina promote plant growth and biocontrol activity of plant pathogens

López, Silvina M. Y.<sup>1,4</sup>; Graciela N. Pastorino<sup>2,5</sup>; Ismael Malbran<sup>3,4</sup>; Pedro A. Balatti<sup>1,2,4</sup>

<sup>1</sup>Comisión de Investigaciones Científicas de la provincia de Buenos Aires (CICBA), Argentina <sup>2</sup>Cátedra de Microbiología Agrícola, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Argentina; <sup>3</sup>Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. <sup>4</sup>Centro de Investigaciones de Fitopatología (CIDEFI), Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Comisión de Investigaciones Científicas de la provincia de Buenos Aires, Argentina; <sup>5</sup>gnpastorino@gmail.com

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Bacteria promote growth by different mechanisms like phosphate (Pi) solubilization, Indol Acetic Acid (IAA) synthesis and siderophores production. The purpose of this study was to isolate bacteria that promote the growth of plants and may also act as antagonistic organisms of plant pathogens. Pi solubilizing microorganisms that were isolated from the soils of Tres Arroyos, Buenos Aires; were also able to synthesize IAA and produce siderophores. The ability of these bacteria to solubilize Pi was directly related with the synthesis of organic acids that lowered the pH and was not related with phosphatase activity. The ability of the organisms to solubilize Pi was indirectly related with the amount of soluble Pi present in the media. Though Pi solubilizing microorganisms are mainly associated with the rhizoplane exudates, in this case did not induce Pi solubilization. In addition to promote plant growth, these bacteria proved to be antagonistic of plant pathogens such as *Fusarium graminearum* and *F. solani*.

**Palabras clave:** PGPB (Plant Growth Promoting Bacteria), solubilización de Pi, sideróforos, AIA, antagonista

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Las bacterias que promueven el crecimiento vegetal lo hacen por diferentes mecanismos como la solubilización de fósforo, la síntesis de ácido indolacético y la producción de sideróforos. El objetivo de este trabajo fue aislar bacterias que promuevan el crecimiento de plantas y que también puedan actuar como organismos antagónicos de los hongos fitopatógenos. Los microorganismos fueron aislados de suelos de Tres Arroyos, pcia de Bs As y se encontraron bacterias solubilizadoras de fosfato inorgánico, que también sintetizan ácido indol acético y sideróforos. La capacidad de estas bacterias para solubilizar fosfatos (Pi) podría estar directamente relacionada con la síntesis de ácidos orgánicos que disminuyen el pH y no guardan relación con la actividad de la fosfatasa. La capacidad de los organismos para solubilizar Pi se relacionó indirectamente con la cantidad de Pi soluble presente en los medios. La capacidad de los microorganismos solubilizadores de Pi está principalmente asociada con el efecto de los exudados presentes en el rizoplaneo, sin embargo, esto no ocurrió en el caso de las bacterias evaluadas en el presente trabajo. Además de promover el crecimiento de las plantas, estas bacterias demostraron ser antagónicas de patógenos de plantas como *Fusarium graminearum* y *F. solani*.

**Key words:** PGPB (Plant Growth Promoting Bacteria), solubilization of Pi, siderophores, AIA, antagonistic

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## INTRODUCTION

Plant growth-promoting bacteria (PGPB) are rhizospheric bacteria that enhance plant growth by a wide array of mechanisms like Pi solubilization, siderophore production, biological nitrogen fixation, production of 1-Aminocyclopropane-1-carboxylate deaminase, and phytohormone production (Bhattacharyya & Jha, 2012). In addition, some of these organisms exhibit antifungal activity, produce volatile organic compounds, induce systemic resistance, promote beneficial plant-microbe symbioses, interfere with pathogen toxin production, etc. (Beneduzi et al., 2012; Effmert et al., 2012; Husen, 2013). The interest in the application of PGPB in agriculture has been increased as they might offer a way to replace the use of chemical fertilizers, pesticides and other supplements (Prévost & Antoun, 2007; Babalola, 2010; Ahemad & Kibret, 2014). Recently, the concept of PGPB has been unconfined and now it refers to bacterial strains that can carry out at least two of three criteria aggressive colonization, plant growth stimulation and biocontrol (Beneduzi et al., 2012; Bhattacharyya & Jha, 2012).

Phosphorous (P) is among nutrients, together with nitrogen, that most frequently limits crops yield, due to its essential role on plant growth and development (Hameeda et al., 2008; Khan et al., 2009; Ghosh et al., 2015). It may represent up to 0,2 % of plant dry weight (Aadarsh et al., 2011; Sharma et al., 2013). Even in soils with high levels of P, like those fertilized with 4-5 kg/ha, P may not be available for plant growth; since it precipitates it is frequently and therefore is available at low concentrations such as 5-11 ppm (Alvarez, 2017) Pi solubilization might be the result of the release by microorganisms of metabolites, such as organic acids, whose hydroxyl and carboxyl groups chelates the cations bound to Pi, which becomes soluble (Shahid et al., 2012). Other biological mechanisms involved in Pi solubilization include extrusion of cytoplasmic by proton pumps (Ahmed & Shahab, 2011).

Several bacterial genus, including *Pseudomonas*, *Bacillus*, *Enterobacter*, *Rhizobium*, *Mesorhizobium*, *Klebsiella*, *Acinetobacter*, *Achromobacter*, *Micrococcus*, and *Erwinia* have the ability to solubilize Pi and, as a result, are known as Phosphate Solubilizing Microorganisms (PSM) or PGPB (Sperber, 1958; Goldstein & Liu, 1987; Illmer & Schinner, 1992; Naik et al., 2008; Prasanna et al., 2011; Ahemad & Kibret, 2014). Many of these genus solubilize Pi by releasing organics acids, which have a liming effect on rock P (Ahmed & Shahab, 2011; Sharma et al., 2013). Furthermore, some bacteria were found to produce  $\alpha$ -hydroxyacids, which were reported as the most reactive organic acids. However, in some instances no relation was found between acid production (media pH) and Pi solubilization (Sperber, 1958; Kucey, 1983). While Illmer and Schinner (1992) suggested that something else other than acidification is responsible for Pi solubilization, Goldstein & Liu (1987) suggested that the process is regulated by the available P, though many steps in the biological solubilization of Pi remain unknown (Ahmed & Shahab, 2011).

In many cases, PGPB species also promote growth by means of indol acetic acid (IAA) and/or siderophores synthesis, which are among the most frequent

metabolites released by Pi solubilizing microorganisms (Vassilev et al., 2006). Auxins, like IAA, are involved in host-parasite relationships such as plant-pathogen interactions (Vinale et al., 2008; Badri et al., 2009). Indol acetic acid might be involved in the inhibition of spore germination and mycelium growth of different pathogenic fungi (Vassilev et al., 2006; Parra Gonzalez et al., 2009; Effmert et al., 2012). On the other hand, rhizobacteria can impact on growth of various phytopathogens in a variety of ways, including competition for nutrients and space, limiting available Fe supply through siderophores production, synthesis of lytic enzymes, and/or antibiosis (Effmert et al., 2012; Bhattacharyya & Jha, 2012; Ahemad & Kibret, 2014). Among PGPB, the broad-spectrum antagonism activity of *P. fluorescent* against several phytopathogens has been widely reported.

Furthermore, bacteria that produce siderophores promote plant growth indirectly by sequestering iron from the rhizosphere, especially in neutral and alkaline soils, and, in this way, they also reduce the availability for pathogens (Alexander & Zuberer, 1991). Under conditions of iron limitation, siderophores act also as solubilizing agents of this metal (Ahemad & Kibret, 2014). They might also form stable complexes with other heavy metals, such as Al, Cd, Cu, Ga, In, Pb and Zn (Rajkumar et al., 2010). Binding of metal to siderophores increases the soluble metal concentration (Rajkumar et al., 2010). Hence, bacterial siderophores help to alleviate the stresses imposed on plants in soil with high levels of heavy metals.

The mechanisms of plant growth promotion do not function independently, such as Pi solubilization, dinitrogen fixation and antifungal activity, IAA and siderophore biosynthesis, etc., might together promote plant growth and increase yield (Beneduzi et al., 2012; Bhattacharyya & Jha, 2012; Ahemad & Kibret, 2014). Currently, in Argentina farmers are particularly interested in using microorganisms as PGPB (biological fertilizer) and/or organisms for biocontrol that might be developed by means of the isolation and identification of potentially useful microorganisms. We describe here the isolation of different species of PGPB that solubilize Pi and synthesize IAA. We characterized the isolates regarding their ability to solubilize Pi in different culture media and evaluated their ability to inhibit fungal growth.

## MATERIALS AND METHODS

Soil microorganisms were isolated by plating a dilution of a protocalcic argiudol soil from Tres Arroyos, Province of Buenos Aires, Argentina on selective media (Sperber, 1958). One gram of soil, adhered to soybean roots, was released by shaking them; detached soil particles were supplemented with 100 mL of sterile distilled water and were stirred for 1 h. A serial dilution was made by transferring 1 mL of the soil suspension to a tube containing 9 mL of sterile water. The last two dilutions,  $10^{-5}$  and  $10^{-6}$ , were plated on media (see below) with insoluble  $\text{Ca}_3(\text{PO}_4)_2$ . They were incubated at 30 °C for 5 days. Those colonies surrounded with a translucent halo were picked and subcultured three times on the same media.

### Identification.

The organisms's identification was performed by means of two complementary methods, 16S rDNA sequence and the biochemical profile in Gram (-) Biolog microtiter plates (Gamo & Shoji, 1999). Additional biochemical reactions (Bergey's manual of Determinative Bacteriology) were performed, as required, to confirm the identity of the isolates.

Genomic DNA was isolated as described by Meinhardt et al. (1994). Then, the 16S rDNA was amplified by means of PCRs with two universal primers p27F and p800RT (Saldaña et al., 2003). The DNA amplicons were resolved in 1 % agarose gels. Gels were documented in a Syngene Image Analyzer. The 800 bp fragment amplified were sequenced using 16S rDNA universal primers with an ABI PRISM® BIGDYE™ Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, USA) in an automatic sequencer Sanger et al. (1977) and sequences were analyzed by means of the Basic Local Alignment search tool. Sequences were annotated at the NCBI database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) under the respective access number. Sequences were aligned by means of the multiple sequence alignment program Clustal W. A UPGMA phylogenetic tree was constructed with the Molecular Evolutionary Genetics Analysis version 6 (MEGA6) program.

### P-Solubilization.

Isolates were cultured on plates with two source of insoluble Pi:  $\text{Ca}_3(\text{PO}_4)_2$  4.6 mM or hydroxyapatite that was generated by adding to a 50 °C medium 0.5 mL of a 57.8 mM  $\text{K}_2\text{HPO}_4$  and 1 mL of 90 mM  $\text{CaCl}_2$  to a 50 °C medium as described by Sperberg (1958). The size of the transparent halos surrounding bacterial colonies in solid media were used to estimate the amount of Pi solubilized. By adding bromothymol to the media, we determined the variations in pH. Liquid cultures were carried on Erlenmeyer flasks filled with 25 mL of media containing 55.5 mM glucose, 4.6 mM  $\text{Ca}_3(\text{PO}_4)_2$ , 4.05 mM  $\text{MgSO}_4$ , and 0.9 mM NaCl, pH 7.2 (Sperberg, 1958), were grown in 125 mL. Cultures were incubated in a Labeline shaker at 200 rpm. Soluble Pi was determined by the ascorbic acid and ammonium molybdate reaction described by Chen et al. (1956) and Bencini et al. (1983). Before each test, glassware was immersed for 1 h in 0.3 N HCl and then rinsed with distilled water. Bacterial growth in liquid cultures was estimated by reading the absorbance at 625 nm in a Shimadzu UV-160A spectrophotometer.

Phosphate solubilizing microorganisms usually grow in the rhizosphere or rhizoplane of roots. To examine if plant exudates act as a signal of the presence of a higher demand of soluble Pi, we studied their effect on the ability of bacterial Pi solubilization, both on solid and liquid media. Seed and root exudates were collected from surface sterilized seeds of wheat, tomato and soybean that were soaked for 5 m in 50 % ethanol followed by 5 m in 50 % Clorox and then were rinsed with sterile water. Seeds were suspended in 125 mL flasks with 20 mL of sterile water and softly shaken for 72 h at 30 °C. The supernatant was sterilized by filtering twice through a 0.22 µm Millipore membrane. Exudates ability to induce bacterial Pi solubilization was tested by adding 1 µL of sterile supernatant per mL of culture. Sterile distilled water was used as a control.

### Phosphatase activity.

To estimate phosphatase activity, 1 mL of the culture was centrifuged at 10000 rpm for 10 m (Kumar et al., 2014). Aliquots of each sample (150 µL) were mixed with 0.48 mL of universal buffer 0.1 M, pH 6.5 and 120 µL of 0.05 M p-nitro phenyl phosphate (pNPP) solution, followed by incubation at 37 °C. The enzymatic activity was evaluated by absorbance measurement at 405 nm (Tabatabai & Bremmer, 1969) and expressed in terms of units (U). P-nitrophenol (pNP) was used as the standard. The amount of enzyme required to release 1 µg pNPP/mL from the culture filtrate under the assay conditions was considered one unit of phosphatase activity. After 72 h of growth the pH of the media was determined.

### Synthesis of IAA.

Indole acetic acid production was tested by growing bacteria on Tris-YMRT medium pH 6.8 (mannitol, 10 g;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.15 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 g; Tris-HCl, 1.21 g; yeast extract, 0.2 g; casamine acid, 1.0 g per liter). Tryptophan was added at a final concentration of 0.3 mM after sterilization by filtering through a 0.22 µm membrane. Indole acetic acid was determined as described by Ferreira and Hungria (2002).

### Siderophores Production.

Siderophores production was tested qualitatively using chrome azurol S (CAS) agar as described by Alexander and Zuberer (1991). The CAS agar was made by mixing three solutions that were prepared and sterilized separately. The 10 mL Fe-CAS indicator solution (solution 1) contained 1 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  dissolved in 10 mM HCl, 50 mL of an aqueous solution of CAS (1.21 mg/mL), and 40 mL of an aqueous solution of hexadecyl-trimethylammonium bromide (HDTMA) (1.82 mg/mL). Solution 2 (buffer solution) was prepared by dissolving 30.24 g of PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid]) in 750 mL of salt solution, distilled water was added to bring the volume to 800 mL. Once the pH was adjusted to 6.8 with 50 % KOH and 15 g of agar were added, the solution was autoclaved. Solution 3 contained 2 g glucose, 2 g mannitol and trace elements in 70 mL distilled water. When the solution 3 was mixed with the solution 1, the color changed to a dark green. Siderophores-production was determined by the appearance of an orange halo around colonies after an incubation period of 24 h in CAS agar plates. Three replicates of bacteria were cultured on this media.

### Antifungal activity.

The antagonistic capacity against *F. graminearum* and *F. solani* of 6 of the isolates obtained (GP2, GP3, GP6, GP7, GP8, and GP9) and of a strain of *P. fluorescens*, was evaluated in two experiments using CAS agar and nutritive agar as culture media. To do so, the plates were divided in four quadrants and inoculated each one with a 5 mm agar cylinder of a culture of *F. graminearum* or *F. solani*. Then, we inoculated bacteria by cross slants that divided the plates on quadrants. The antifungal activity and siderophores production were evaluated as described by Filippi et al. (1984). The plates were incubated at 26 °C and the diameter of fungal colonies was measured after 2 and 7 days of incubation. Controls consisted in colonies of the fungi

that grew in the absence of bacteria. Each treatment was replicated three times.

### PGPB on cultivated wheat

The plant growth promotion of the isolated bacteria was examined on wheat plants cultivated in the greenhouse. Seeds were germinated on water agar plates and transferred aseptically to 10 L growth pots filled with autoclaved soil after 4 days of seedling emergence. Before the transplant, pots were watered with 200 mL of 1/2 strength Hoagland's nutrient solution. Bacterial cultures of two isolates (GP2 and GP9) were grown in 50 mL falcon tubes filled with 25 mL nutritive broth and incubated at 200 rpm for 16 h. At the time of seedling transplant, 1 mL inoculum ( $\sim 10^8$  bacterial cells  $\text{ml}^{-1}$  suspended in 0.85 % saline solution; OD = 0,45) was applied at the base of each seedling. The uninoculated pots were supplied with same amount of sterile saline solution. Two seedlings were maintained per growth pot and placed in growth chamber at 20–22 °C with a day length of 12 h and relative humidity was set at 70 %. The experiment was set up in randomized complete design (CRD) using 10 replicate pots per treatment. The uninoculated pots were watered with full strength Hoagland solution (supplemented with N, P) while inoculated pots received full strength Hoagland, supplemented with 1 g inorganic tricalcium phosphate as sole P-source. Plants were harvested 90 days after transplant and data was recorded for leaf dry weight, and tillers and head numbers.

### Statistical design and analysis

All experiments were conducted in triplicate and data were subjected to analysis of variance (ANOVA). Mean

values were compared using one-way ANOVA Tukey's test and significant differences were detected at the  $P=0.05$  level.

## RESULTS

Bacteria isolated from soil were identified on the basis of their 16S rDNA sequence. A BLAST search revealed that these bacteria were all enterobacteria. Most of the isolates belonged to the genus *Enterobacter* or *Pseudomonas*: GP6, GP7 and GP8 were identified as *Enterobacter* spp., GP2 and GP3 as *P. cichorii* and *P. corrugata*, respectively. Isolate GP9, on the other hand, was identified as *Kluyvera cryocrescens*. The ribosomal sequences of the isolates were annotated at the gene bank NCBI (access numbers: GP2–KC463904; GP3–KC463905; GP6–KC463906; GP7–KC463907; GP8–463908 and GP9–KC463909).

Organisms were organized into three clusters in a UPGMA phylogenetic tree (Figure 1). The first group was supported by a bootstrap value of 92 % and included four strains (GP6, GP8, GP7 and GP9) that clustered with four reference sequences of *Enterobacter* sp., *K. ascorbata* and *K. cryocrescens*. The second cluster was supported by bootstrap values of 100 % and included a representative of *B. subtilis* and the *P. fluorescens* strain. Isolates GP2 and GP3 were clustered in a separate group supported by a bootstrap value of 100 %, together with the reference sequences of *P. cichorii* and *P. corrugata*. These isolates probably have to be studied further either to confirm the identity or to determine, on which bases they were not clustered to other isolates of the same species, which might be

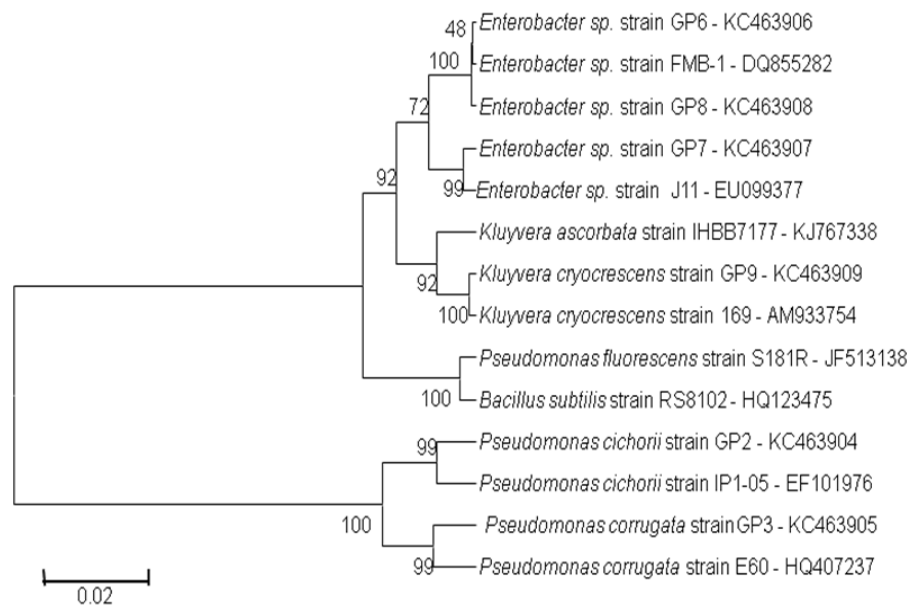


Figure 1. Dendrogram showing the identity and relationship among bacteria isolated from soil. Numbers on the branches represents bootstrap values obtained from 1000 replications. The bar indicates 0.02 substitutions per site. Species names are followed by the National Center for Biotechnology Information (NCBI) Gen-Bank database accession numbers.

reflected in phenotypic differences. In any case, the organisms belong to a monophyletic cluster that included closely related organisms with high level of similarity at the 16S rDNA sequence level, which are clustered with species such as *B. subtilis*, *P. fluorescens*, *P. corrugate*, *P. cichorii*, *K. ascorbata*, *K. cryocrescens* and *Enterobacter sp.* Identities of the isolates were confirmed using the microliter plates developed by Biolog Inc, that contain 95 different nutrient sources. Furthermore, we confirmed that the isolated bacteria were genetically distinct by fingerprinting the genome (de Bruijn, 1992) with ERIC (Enterobacter Repetitive Sequences) and REP (Repetitive Element Palindromic) primes (data not show).

Six Gram (-) bacterial strains isolated from soil (GP2, GP3, GP6, GP7, GP8, and GP9) and the control strain *P. fluorescens*, proved to be able to solubilize tricalcium phosphate  $\text{Ca}_3(\text{PO}_4)_2$  or hydroxyapatite [ $3\text{Ca}_3(\text{PO}_4)_2 \text{Ca}(\text{OH})_2$ ] both on solid and liquid media. These phenotypes remained stable after subculturing them at least three times.

All isolates produced a halo on solid media amended

with insoluble Pi. The halo formed on the medium with  $\text{Ca}_3(\text{PO}_4)_2$  was twice as big as that formed on the medium supplemented with hydroxyapatite [ $3\text{Ca}_3(\text{PO}_4)_2 \text{Ca}(\text{OH})_2$ ]. By adding bromothymol to the media, we found that within the halos the pH was acid. Furthermore, all the isolates presented low levels of phosphatase activity.

When bacteria were grown in liquid media, we found that growth resulted in a 3 unit drop in the pH of the media, which decreased from 7.3 to 4.5 after 24 h of cultivation. Isolates differed in their ability to solubilize Pi in liquid culture, GP2, GP6, GP8 and GP9 solubilized twice as much Pi as GP3 and GP7 (Figure 2). This suggests that Pi solubilization is an intrinsic characteristic of the organisms that vary within isolates, and may be related to the organic acids released. When the insoluble Pi source was hydroxyapatite, the amount of Pi solubilized was lower than with  $\text{Ca}_3(\text{PO}_4)_2$ . However, the intrinsic solubilization ability of the different bacteria remained the same (Figure 2).

By culturing the PSM on different media we found that the amount of Pi solubilized was dependent on the amount and type of carbon (C) source (Table 1).

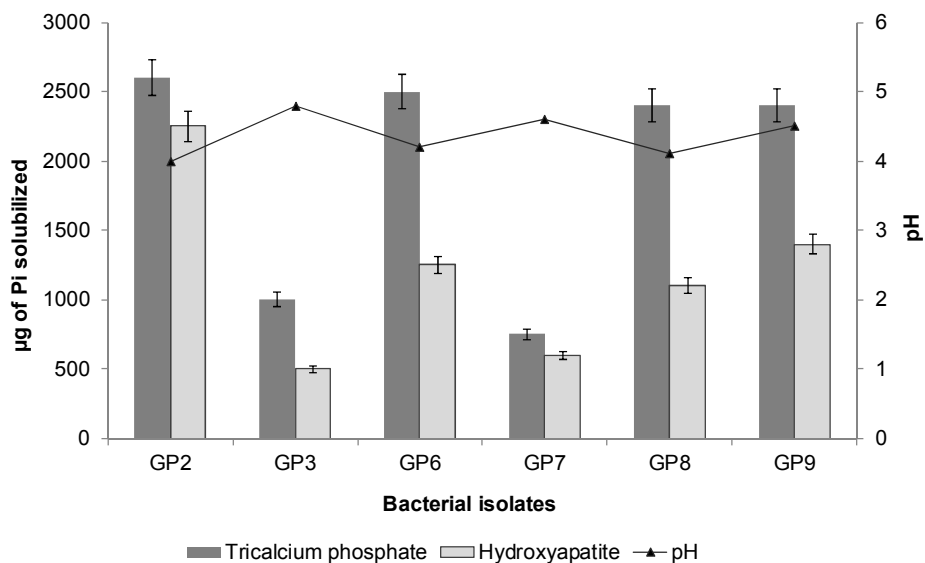


Figure 2. Bacterial solubilization of tricalcium phosphate and hydroxyapatite. Pi solubilization is expressed as  $\mu\text{g}$  of Pi solubilized by  $10^8$  cells on 7 days. Bars indicate LSD at the 5% level. pH values at the end of the solubilization period on tricalcium phosphate were measured. Three independent experiments with three replicates per experiment were done.

Table 1. Pi solubilization by *Kluyvera cryocrescens* (GP9). Cells cultured in media supplied with different amounts of nutrients. Pi solubilization is expressed as  $\mu\text{g}$  of Pi solubilized by  $10^8$  cells in 7 days. The experiments had three replicates per treatment and were performed twice. Different letters indicate statistically significant differences at the 5% level.

Culture Media	$\mu\text{g}$ Pi solubilized
A: 55.5 mM glucose, 4.6 mM $\text{Ca}_3(\text{PO}_4)_2$ , yeast extract 0.5 g/L, pH 7.2	2049.0 <sup>a</sup>
B: 111 mM glucose, 55.5 mM $\text{Ca}_3(\text{PO}_4)_2$ , yeast extract 2 g/L, pH 7.0	969.4 <sup>c</sup>
C: 55.5 mM mannitol, 4.6 mM $\text{Ca}_3(\text{PO}_4)_2$ , yeast extract 0.5 g/L, pH 7.2	1117.2 <sup>b</sup>
D: 55.5 mM glucose, 4.6 mM hydroxyapatite, yeast extract 0.5 g/L, pH 7.2	776.2 <sup>cd</sup>

Based on these results, we decided to study the effect of the C source on the PSM phenotype of GP9. When glucose was the C source, the rate of Pi solubilization was higher than with mannitol, sucrose, arabinose, lactose, maltose, citrate, fructose, glycerol, sorbose and xylose (Figure 3). On the other hand, the release of soluble phosphates does not correlate directly with the acidity.

The ability of GP9 to solubilize Pi was negatively correlated with the concentration of soluble Pi in the media (Figure 4).

Wheat, tomato and soybean exudates enhanced bacterial growth and induced larger halos of P-

solubilization in solid media. However, the specific activity of Pi solubilization remained the same. In liquid media, the amount of soluble Pi released by bacteria followed the same pattern described on solid media (Table 2).

The isolates collected from the soils can be considered PGPB based upon their Pi solubilizing ability, but these bacteria might also enhance plant growth by additional mechanisms such as the synthesis of siderophores and/or auxins. All the isolated bacteria formed an orange halo around the colonies in CAS media, which confirms that they produce siderophores. Furthermore, with the exception of GP3, all isolates synthesized between 40 and 260 microgram of IAA per unit OD.

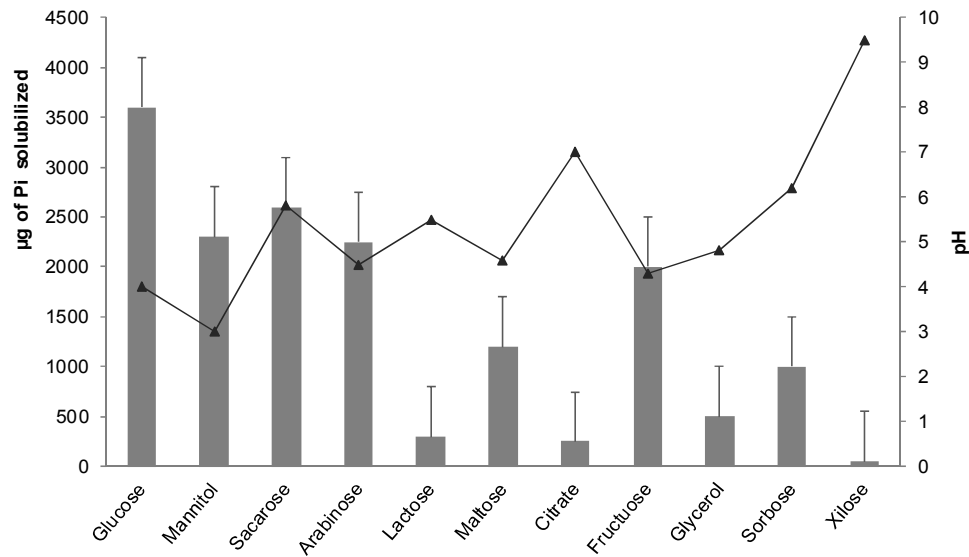


Figure 3. Carbon source effect on the ability of *Kluyvera cryocrescens* (GP9) to solubilize Pi. Sugars were added to a minimal medium (concentration 10 g/L), sterilized twice through a 0.22 µm millipore filter, and the pH was adjusted to 7.3. Bars represent the LSD at the 5 % level. Pi solubilization is expressed as µg of Pi solubilized by 10<sup>8</sup> cells on 7 days.

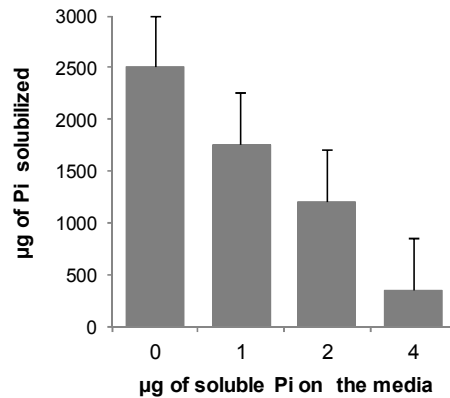


Figure 4. Pi solubilization ability of *Kluyvera cryocrescens* (GP9) in the presence of increasing amounts of soluble Pi. Bars represent the LSD at the 5 % level. Pi solubilization is expressed as µg of Pi solubilized by 10<sup>8</sup> cells on 7 days. Five independent experiments were performed with three replicates per experiments.

Table 2. Effect of seeds exudates on growth rate and Pi solubilization of PMS bacteria in liquid culture. The assay was repeated three times and three replicates were included in each experiment. *Kluyvera* spp. cells were cultivated in media with insoluble P. The control consisted of sterile distilled water. Pi solubilization is expressed as µg of Pi solubilized by 10<sup>8</sup> cells on 7 days. Different letters indicate statistically significant differences at the 5 % level.

Exudates source	Bacterial cell number OD <sub>625nm</sub>	µg of Pi solubilized
Wheat	1.28 <sup>a</sup>	1486.8 <sup>a</sup>
Tomato	0.90 <sup>ab</sup>	1664.4 <sup>a</sup>
Soybean	1.23 <sup>a</sup>	1569.1 <sup>a</sup>
Control	0.70 <sup>b</sup>	1653.1 <sup>a</sup>

Isolates GP7 and GP9 produced the highest levels of IAA (Figure 5), while GP2 and GP9 secreted of the highest amounts of siderophores.

Regarding the antagonistic effect of bacteria or bacterial metabolites on fungal growth, dual culture experiments showed that bacterial isolates GP2 and GP9 significantly inhibited *F. graminearum* growth after 48 h. After 7 days *F. graminearum* was inhibited by all of the isolates. On the other hand, except for GP3, which hardly grew on CAS media, all isolates inhibited growth of *F. solani* after 48 h of incubation. In addition, isolates GP2 and GP9 maintained the inhibition of *F. solani* growth during an incubation period of 7 days.

The biocontrol ability of two PGPB strains, selected on the basis of the results obtained in preliminary experiments, were evaluated in their ability to promote growth of wheat plants when they were inoculated at seeding. The results are presented on figure 6. Isolates GP2 and GP9 increased plant dry weight in 11 and 8 %, respectively. Furthermore, both strains increased the number of tillers per plant in a 26.25 and 20.25 % when compared with uninoculated plants, respectively.

## DISCUSSION

Plant growth promoting bacteria might induce changes in root architecture as well as promote plant growth and development by means of a wide array of mechanisms. These may act alone or concomitantly to synergistically enhance growth, which might result in changes in the bacterial competitive ability to colonize roots, enhance plant growth and biocontrol plant pathogens (Bhattacharyya & Jha, 2012). The ability of PGPBs to solubilize mineral Pi, therefore, has been of interest to agricultural microbiologists since it could enhance the availability of P for effective plant growth and represent a possible mechanism of plant growth promotion under field conditions (Kumar et al., 2014).

Several bacterial genera have been reported as PSM (Pi solubilizing Microorganism), including: *Escherichia freundii* (Sperber, 1958), *E. coli* (Goldstein & Liu, 1987), *Erwinia herbicola* (Goldstein & Liu, 1987), *Rhizobium* (Prasanna et al., 2011), *Enterobacter* (Shahid et al., 2012), *Arthrobacter*, *Agrobacterium* and others (Bhattacharyya & Jha, 2012). In the present work, most of the bacteria isolated were representatives of enterobacteria, with the exception of one isolates which was identified as *K. cryocrescens*. Because this isolate showed a high capacity to solubilize P, we concentrated

most of our studies on this organism. Solubilization of Pi might be a characteristic feature of bacteria from this genus as *K. ascorbata* isolates have also been described as PGPB (Son et al., 2014).

Phosphate solubilization ability showed by the isolated bacteria was a stable character since in no case it was lost after being subcultured. The isolates described in the current report might solubilize Pi by secreting organics acids. This is supported by the decrease of pH observed in the culture media of Pi solubilizing isolates. Gluconic, citric and/or malic acids could be responsible for the low pH of the media after bacterial growth. Scervino et al. (2011) and Shahid et al. (2012) found that in closely associated bacteria the Pi solubilization occurred due to the synthesis and release of gluconic, citric as well as malic acid. The production of citric acid by *K. cryocrescens* isolate GP9 could be responsible of its solubilization capacity, as the media containing citrate prevented Pi solubilization, possibly because the acid in the media prevented the synthesis of the molecule by the bacteria.

In accordance with previous reports, we found that carbon source of the media affects the rate of solubilization of Pi. Illmer and Schinner (1992) as well as Scervino et al. (2011) observed that the carbon source provided in the media can alter Pi solubilization, hypothesizing that glucose could be a more amenable substrate for the bacterial metabolism, producing and releasing more organic acids. Although a positive trend was observed between acidification and solubilization of Pi in media with different carbon sources, no direct relationship between these parameters was found, something that was also described by Gulati et al. (2008).

We found a straight negative correlation between the presence of soluble Pi on the media and the ability of bacteria to solubilize calcium phosphate. This agrees with previous findings by Kucey (1983), Golstein & Liu (1987) and Illmer & Schinner (1992). Goldstein & Liu (1987) suggested that Pi solubilization is process tightly regulated by the environmental conditions. Repression of Pi solubilization under certain ecological situations might be a natural strategy of bacteria to manage energy within cell metabolism.

Phosphate solubilizing microorganisms are usually found growing in the rhizosphere or the rhizoplane of plants. Katznelson et al. (1962) demonstrated that the plant type exerted an important effect on the number of PSM. This is probably due to exudates released to the rhizosphere by the plants.

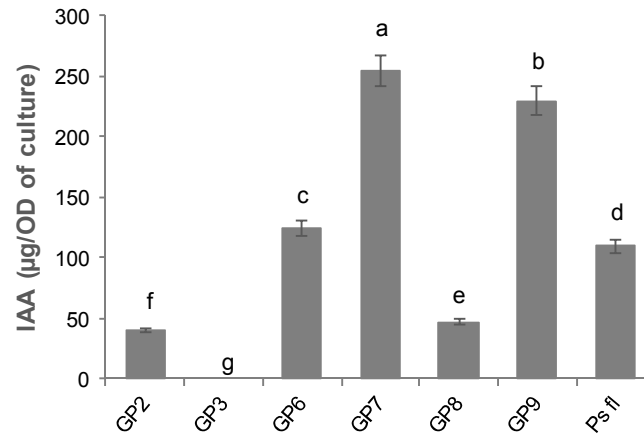


Figure 5. Ability of the bacterial isolates to synthesize IAA. The production of the auxin is expressed as  $\mu\text{g}$  of IAA per unit of crop OD and values are the average of three replicates. Different letters indicate statistically significant differences at the 5 % level.

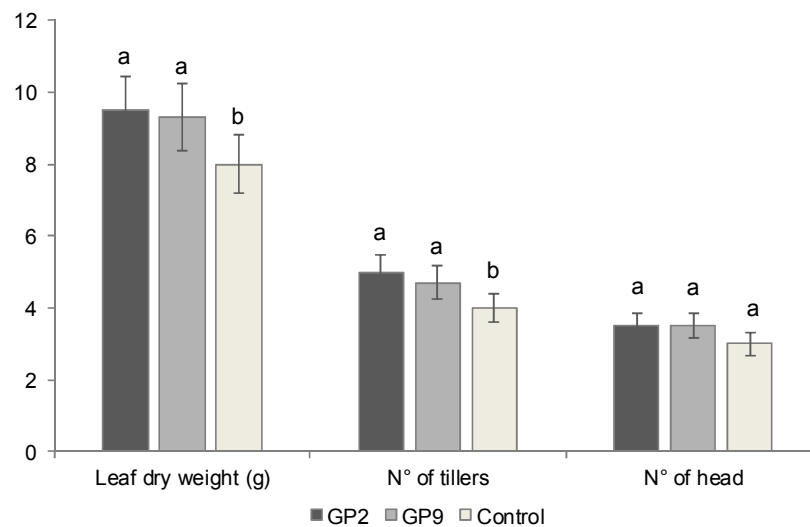


Figure 6. Effect of inoculation with GP2 and GP9 in leaf dry weight, number of tillers and number of heads of wheat plants. Different letters indicate statistically significant differences at the 5 % level.

However, in the present work, the exposure of the bacteria to the exudates of the seeds had no effect on their Pi solubilization capacity.

Rhizobacteria can suppress the growth of plant pathogens in a variety of ways, including competition for nutrients and space, increasing availability of Fe by siderophores synthesis, and/or the synthesis of lytic enzymes and antibiosis (He & Yang, 2007). Among PGPBs, *P. fluorescens* is probably the bacteria with the broadest antagonistic spectrum against plant pathogens. In our experiments, the ability of *P. cichorii* (GP2) and *K. cryocrescens* (GP9) to inhibit fungal growth was directly correlated with the ability to produce siderophores. Furthermore, these bacteria proved the ability to promote wheat growth.

Inoculation of plants with PGPB is becoming a common tool in agriculture to enhance crop yields (Glick, 2012;

Reed & Glick, 2013; Bashan et al., 2013). Some bacterial strains isolated from the rhizosphere of roots showed potential as PGPB (Wisniewski-Dyé et al., 2013) and could be used to promote growth. Under both natural agro-ecological niches and controlled soil environments, significant increases in yields of different crops have been observed following PGPB applications. In a context of increasing reluctance to consume foods produced by genetically modified plants, PGPB may prove advantageous to promote plant growth. The large scale application of PGPB may decrease the global dependence on agricultural chemicals. Furthermore, it is a technology readily accessible to farmers in both developed and developing countries (Gamalero et al., 2009).

In the present work, both *Pseudomonas* and enterobacteria, synthesized metabolites that make them



potential PGPB and/or biocontrol agents. Furthermore, this is the first report of *K. cryocrescens* isolates with potential PGPB ability and the capacity to produce siderophores. We also confirmed the ability to solubilize phosphorus of this species as previously reported (Vazquez et al., 2000; Sharma et al., 2013). As a result, isolates GP2 and GP9 are potential candidates to develop inoculants of PGPB bacteria considering that they not only promote growth, but are antagonistic of at least some plant pathogenic organisms.

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