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Growth promotion of peanut (*Arachis hypogaea* L.) and maize (*Zea mays* L.) plants by single and mixed cultures of efficient phosphate solubilizing bacteria that are tolerant to abiotic stress and pesticides

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ABSTRACT

The aims of this study were, to analyze *in vitro* phosphate solubilization activity of six native peanut bacteria and to determine the effect of single and mixed inoculation of these bacteria on peanut and maize plants. Ability to produce organic acids and cofactor PQQ, to solubilize $FePO_4$ and $AlPO_4$ and phosphatase activity were analyzed. Also, the ability to solubilize phosphate under abiotic stress and in the presence of pesticides of the selected bacteria was determined. The effect of single and mixed bacterial inocula was analyzed on seed germination, maize plant growth and in a crop rotation plant assay with peanut and maize. The six strains produced gluconic acid and five released cofactor PQQ into the medium. All bacteria showed ability to solubilize phosphate from $FePO_4$ and $AlPO_4$ and phosphatase activity. The ability of the bacteria to solubilize tricalcium phosphate under abiotic stress and in presence of pesticides indicated encouraging results. Bacterial inoculation on peanut and maize increased seed germination, plantís growth and P content.

Phosphate solubilizing bacteria used in this study showed efficient phosphate mineralizing and solubilization ability and would be potential P-biofertilizers for peanut and maize.

1. Introduction

Legumes are the third largest family of higher plants, wherein peanut (Arachis hypogaea L.) is one of the most important species. It is one of the choicest world agriculturally economic important crop (Krishna et al., 2015). Argentina is one of the world's leading peanut exporters together with USA and China. In the cultivating area of Argentina about 91% is produced in the province of Córdoba (Barberis et al., 2015). In this producing area, maize (*Zea mays* L.) is the main crop used in rotation with this legume reaching 50% of national production (Bongiovanni, 2008; Bolsa de cereales de Córdoba, 2015). Nevertheless, in the province of Cordoba intense agricultural practices have decreased the nutritional quality of the soils. Within macronutrients, low levels of available phosphorus (P) for plant nutrition (5–15 ppm) were reported in this area (Ascheri, 2007; Sainz Rosas et al., 2012). P deficiency is one of the most important chemical factors limiting crop production in many soils worldwide (Arcand and Schneider, 2006). For peanut and maize the optimal P content is 10 and 13–18 ppm, respectively (Cope et al., 1984; IPNI, 2009).

P exists in nature in a variety of organic and inorganic forms. Despite P compounds are abundant in agricultural soils, more than 80% of soluble P in soil becomes immobile and unavailable for plant uptake due to low solubility and fixation in soil (Gulati et al., 2008; Miller et al., 2010). Also, soluble forms of P fertilizers applied to the soil are easily precipitated (Haque and Dave, 2005). Sundara et al. (2002) reported that the recovery rate of P fertilizer by plants is only about 25%. The remaining 75% is accumulated in soil in an immobile form bound to Al or Fe in acid soils, or Ca and Mg in alkaline soils (Prochnow et al., 2006; Yang et al., 2010).

In the soil, rhizosphere, rhizoplane and phylosphere, a high number of bacterial species are able to exercise a beneficial effect, by different mechanisms, on plant growth and have been termed

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plant growth promoting bacteria (PGPB) (Glick, 1995; Bashan and de-Bashan, 2005). Within these, phosphate solubilizing bacteria (PSB) have been described to play an important role in plant nutrition through an increase in P uptake by plants (Rodríguez et al., 2006). These bacteria can solubilize and mineralize P from inorganic and organic insoluble sources, respectively, through different mechanisms which improve the mobilization and availability of P for plant nutrition (Fernández and Rodríguez, 2005; Rodríguez and Fraga, 1999). Mineral phosphate solubilization by bacteria is widely associated with the production of low-molecular-weight organic acids, mainly gluconic and 2-cetogluconic acids (Deubel et al., 2000). The best characterized mechanism of mineral phosphate solubilizing, in many Gram negative bacteria involves direct oxidation pathway of glucose via the membrane-bound quinoprotein glucose dehydrogenase (GDH) (Kim et al., 1997; Lin et al., 2006; Patel et al., 2008) to produce gluconic acid. This enzyme requires the cofactor redox pirroquinolin quinone (PQQ), whose biosynthesis involves a *pqq* operon consisting of at least five genes (*pqqA*, *B*, *C*, *D*, *E*) (Meulenberg et al., 1992; Kim et al., 1998, 2003; Choi et al., 2008). The role of PQQ has been described to be essential for the phosphate solubilizing phenotype in several bacteria (Kim et al., 2003; Han et al., 2008; Ludueña et al., 2016).

In modern agriculture, agrochemicals are frequently applied to the crop fields to increase the production (Ramanathan and Lalithakumari, 1999). As a result of the retention of certain pesticides in soil particles, the beneficial microorganisms are subject to biochemical changes, reducing its activity as biofertilizer and their plant growth promoting effect (Paoletti, 1999). Although some soil microorganisms use these compounds as C or energy source (Johnsen et al., 2001), they predominantly cause damage to the habitat and cause changes in the densities of bacteria and fungi in the soil, promote or suppress the growth and microbial activity and generate detectable changes in the population structure of the soil (Girvan et al., 2004; Kremer and Means, 2009; Barriuso et al., 2010; Anzuay et al., 2015a).

Considering P deficiency in agricultural soils of Córdoba, the use of PSB is an economically and a most friendly alternative than fertilizers to increase the content of this nutrient in the soil. In our laboratory 433 PSB isolated from peanut plants growing at field, have been screened for tricalcium phosphate solubilizing activity (Taurian et al., 2010). From this collection, selected bacteria were inoculated on peanut plants in a microcosm assay to analyze their potential beneficial effects (Anzuay et al., 2013, 2015b). Results obtained indicated that several PSB showed to promote this legume growth and enhance its P content. Considering that maize constitutes the main rotation crop used in peanut cultivation area of Córdoba, the long aim of this research is to propose a potential P-biofertilizer for both maize and peanut crop. Thus, in this study six PSB belonging to different genera were selected in order to analyze their ability to solubilize other P sources, to characterize the organic acids produced and ability to release PQQ and finally, to analyze the effect of abiotic stresses and pesticides on the phosphate solubilizing ability. Selected PSB were further tested in single and mixed inocula on maize growth, seedlings emergence and on the growth of peanut and maize plants in a simulated crop rotation system.

2. Material and methods

2.1. Bacterial growth and maintenance

Six phosphate-solubilizing bacteria isolated from peanut plants cultivated in central and southern region of Córdoba, Argentina (latitude, 32°–34°, longitude, 63°–65°) (Taurian et al., 2010; Anzuay et al., 2013) were used in this study. Bacteria were grown and maintained in TSA (trypticase soy agar) (Britania) or TY (Beringer, 1974) media. *Pseudomonas fluorescens* PMT1 used in commercial inoculant formulation ("RIZOFOS"® -RIZOBACTER) was used as reference strain and grown in LB (Luria-Bertani) media (Miller, 1972). Bacteria were maintained in 20% glycerol (v/v) at −80 °C. For *in vitro* phosphate solubilizing and mineralizing assays NBRIP-BPB medium was used (Glucose 10 g/l, $Ca_3(PO_4)_2$ 5 g/l, $MgCl_2.6H_20$ 5 g/l, $MgSO_4.7H_20$ 0,25 g/l, KCl 0,2 g/l, $(NH_4)_2SO_4$ 0,1 g/l, bromo phenol blue 0,0025 g/l, pH 7, Mehta and Nautiyal, 2001).

2.2. Production of organic acids by the PSB

Organic acids produced by PSB were detected in the supernatant of bacterial cell culture. Bacteria were grown in NBRIP medium devoid of BPB, at 28 °C and 150 rpm. Samples were obtained at the time in which maximum levels of soluble P were released by each bacterium (Anzuay et al., 2013) and centrifuged at 10,000 rpm for 12 min. The supernatant were passed through 0.22 μm nylon filter and 20 μl of filtrates were injected. Detection and quantification of organic acids was done on Waters 2996 PAD High Performance Liquid Chromatogram (HPLC). The organic acid separation was carried out on Hamilton PRP-X300 column (250 mm × 4.1 mm and 7 μm particle size). Mobile phase consisted of 0.04 N H_2SO_4 run at a flow rate of 2 ml/min. HPLC profiles of the culture filtrates were analyzed by comparison with the elution profiles of standards organic acids (gluconic, citric, lactic and succinic acid). The software used for HPLC analyses was the software Empower and the organic acids detected were identified by comparing their retention times and the peak areas of their chromatograms with those of standards.

2.3. PQQ cofactor quantification

To measure PQQ production, bacteria were grown in NBRIP medium as described previously. At the time in which maximum levels of soluble P were released by each bacterium one volume of cell culture was sampled and diluted with nine volumes of methanol. The precipitated materials were removed by using nylon filters (0.22 μm pore size), methanol was evaporated and the samples were lyophilized and resuspended in 10 ml of mobile phase. Reverse phase HPLC was performed using a Perkin Elmer 200 Series HPLC System equipped with autosampler and fluorescence detection. Fluorescence was monitored at e_x 360 and e_m 480 nm. A Phenomenex LUNA RP C-18 column (250 mm x 4.5 ID, 5 μm pore size) (Phenomenex Inc., Torrance, CA, USA) was used for analytical separation fitted with a C18 guard column using a gradient mode. RP-HPLC was performed as described previously by Stites et al. (2000) with modifications. The initial mobile phase for the HPLC protocol consisted of 30% methanol and 70% 0.06 M phosphoric acid. A linear gradient was applied from 5 to 30 min with a final concentration of 70% methanol and 30% 0.06 mol/l phosphoric acid. PQQ derivatization: PQQ was derivatized with acetone to form the acetone adduct (5-acetonyl-PQQ) to aid in identity and validation. PQQ (200 nmol, 0.2 ml) was derivatized in 0.1 M sodium carbonate (pH 9.2, 0.1 ml) with the addition of 16% acetone (v/v, 0.1 ml) at 37 °C for 30 min.

2.4. Quantification of soluble P released from Fe-P and Al-P sources

The quantity of soluble P released to the supernatant was determined in modified NBRIP-BPB medium following Fiske and Subbarow (1925) method. One hundred microlitres of an overnight inoculum (approximately 10^9 CFU/ml) in TY medium was transferred to 15 ml of NBRIP-BPB medium containing $FePO_4$ (1 g/l) or $AlPO_4$ (2 g/l) in replacement of $Ca_3(PO_4)_2$. To replace the calcium source $CaCl_2$ (1 g/l) was added. After 24, 48, 72 h and 7 days of growth, 1.5 ml of bacterial cultures were sampled and cen-

trifuged for 12 min at 10.000 rpm. The amount of P was quantified spectrophotometrically by measuring absorbance at 660 nm. At each incubation time, CFU/ml by drop plate method (Hoben and Somasegaran, 1982) in TY medium and supernatants' pH of each sample were determined.

2.5. Acid and alkaline phosphatase activity measurments

Phosphatase activities were analyzed in the supernatant of the bacteria according to Freitas et al. (1997) modified protocol. PSB were grown 24 h in 15 ml NBRIP liquid broth at 28 °C (150 rpm). Aliquots of 50 μl of each supernatant were added to 100 μl ml of 0.05 M *p*-nitrophenyl phosphate (pNPP) solution, 50 μl of $MgCl_2$ (0.02 M) and 300 μl universal buffer 0.1 M, pH 6.5 or pH 11 for acid or alkaline phosphatase activity, respectively. The samples were incubated at 37 °C for 1 h. The reaction was stopped by the addition of 500 μl of NaOH (2 N). The absorbance was measured using visible spectrophotometer at 410 nm (Tabatai and Bremmer, 1969). The acid or alkaline phosphatase activity corresponds to the amount of released *p*-nitrophenol (pNP) per min per ml (μg pNP/min/ml).

2.6. Evaluation of the effect of abiotic stresses and pesticides on bacterial growth and on the in vitro phosphate solubilizing activity of PSB

The determination of *in vitro* phosphate solubilizing ability of PSB under stress conditions of salt, pH and temperature was analyzed. In all cases NBRIP-BPB medium was used. For stress salt conditions bacteria were grown in plates containing the medium with the addition of 50 or 150 mM NaCl. Acidic and alkaline conditions were performed by adjusting pHís medium to 5 or 8, respectively. The effect of high temperatures on phosphate solubilizing ability was analyzed by incubating bacteria at 37 °C or 42 °C. In all cases bacteria were grown for 7 days in which ability to growth and to solubilize TCP were determined. The ability to solubilize TCP was measured qualitatively by observation of a halo of clearance around the bacterial colony.

Pesticides assayed were those commonly used on peanut and maize crops in the producing area of the province of Córdoba. Nitrocellulose paper disks were embedded with each pesticide at the doses employed on field: Insecticide Lamdacialotrine (25 ml/ha) for both plants; fungicides azoxystrobin + difeconazole (1 l/ha) and pyraclostrobin + epoxiconazole (0.75 l/ha) for peanut and maize, respectively; herbicides glyphosate (1.5 kg/ha), s-metaclor (1 l/ha) and imazethapyr (1 l/ha) for both plants and diclosulam (20 g/ha) and imazapic (85 g/ha) for peanut and atrazine (2 l/ha) for maize. The disks were deposited into Petri dishes containing TY medium or NBRIP-BPB previously seeded with each isolate (Fabra et al., 1998). The plates were incubated at 28 °C during 24 h and 7 days for TY and NBRIP-BPB media, respectively; time in which ability to growth and solubilize TCP was determined.

2.7. Bacterial coexistence in plate assays

Plate assays were performed following the technique described by Dey et al. (2004) with some modifications. Fresh culture of each microorganism was streaked on one of the two halves of plates containing TY or NBRIP-BPB medium. All possible combinations between bacteria analyzed were performed. Plates containing TY medium were incubated at 28 °C for 24 h and the plates supplemented whit NBRIP-BPB for 7 days.

2.8. Greenhouse evaluation of PSB in single and mixed inocula on peanut and maize seeds and plants

2.8.1. Seeds germination in plate assay

Seeds of peanut (var. Granoleico) and maize (Hybrid NK900 TD MAX) were surface disinfected according to Vincent (1970) and Pereira et al. (2011) and and, respectively. Seeds were inoculated by immersing them in a plastic Petri dish containing 3 ml of bacterial suspension (10^9 CFU/ml) under stirring at 150 rpm, 30 min and then transferred to glass Petri dishes (150 × 30 mm), containing semi solid (agar 11.5 g/l) Hoagland medium (Hoagland and Arnon, 1950) supplemented with tricalcium phosphate (2 g/l) as the sole source of P. Three seeds of maize or peanuts were deposited in each plate. For mixed inocula 1.5 ml and 1 ml of each culture were used, in the case of double inoculations and triple inoculations, respectively.

Single and mixed inoculations treatments were performed as follows: (1) *Pantoea* sp. J49; (2) *Bacillus* sp. L55; (3) *Serratia* sp. S119; (4) *Acinetobacter* sp. L176; (5) *Enterococcus* sp. L191; (6) *Serratia* sp. J260; (7) *Pantoea* sp. J49 + *Serratia* sp. S119; (8) *Acinetobacter* sp. L176 + *Serratia* sp. J260; (9) *Bacillus* sp. L55 + *Serratia* sp. J260; (10) *Enterococcus* sp. L191 + *P. fluorescens*; (11) *Bacillus* sp. L55 + *Enterococcus* sp. L191; (12) *Bacillus* sp. L55 + *Serratia* sp. J260 + *P. fluorescens*; (13) Uninoculated maize seeds; (14) Uninoculated peanut seeds; (15) Uninoculated maize seeds supplemented with 20 mM KH_2PO_4 (P fertilized maize seeds; C+); (16) Uninoculated peanut seeds supplemented with 20 mM KH_2PO_4 (P fertilized peanut seeds; C+); (17) maize seeds inoculated with commercial strain *P. fluorescens* PMT1 and (18) peanut seeds inoculated with commercial strain *P. fluorescens* PMT1.

Plates were half-covered to protect radicles from light, incubated under controlled environmental conditions (light intensity of 200 $\mu R\, m^{-2}\, s^{-1}$ 16 h day/8 h night cycle, at a constant temperature of 28 °C and a relative humidity of 50%). After 7 days of incubation, length (cm) and dry weight (g $plant^{-1}$) of radicles were determinated.

2.8.2. Maize growth in microcosm assay

Seeds of maize (Hybrid NK900 TD MAX) were surface disinfected as described previously and transferred to sterilized Petri dishes with one layer of Whatman N°1 filter paper and moist cotton for germination. Plates were incubated at 28 °C until the radicle reached 2 cm length. Maize seedlings were transferred to sterilized plastic pots (5.7 cm-diameter, 8.4 cm height) that contained a mixture of sterile vermiculite and sand (2:1) and 40 mg/kg $Ca_3(PO_4)_2$ (Rivas et al., 2007). The bacterial inoculation treatments and controls were the same as described in seed germination assay. Bacterial inocula were obtained by harvesting 3 ml cultures of each bacterial culture grown at 28 °C and 150 rpm in TY culture medium (10^9 CFU/ml). Each inoculum was deposited on the crown of the root. In the case of mixed inocula 1.5 or 1 ml of each culture were used, in the case of double and triple inoculations, respectively. Number of inoculated bacteria in plant growth substrate was analyzed in the first hour after bacterial inoculation by streaking serial dilutions of 1 g samples of substrate in phosphate buffered saline (PBS) on NBRIP-BPB plates supplemented with the appropriate antibiotics, depending on the resistance profile of each bacterium (Anzuay et al., 2015b).

Maize plants were grown under controlled environmental conditions, watered regularly with sterilized tap water and once a week, with the nutrient solution Hoagland, which was devoid of soluble P. Plants were harvested at 21 days post inoculation and the following growth parameters were determined: length (cm), dry weight (g/plant) and P content (mg/g) of aerial and root tissues. P content of both aerial and radical was determined using the method described by Jackson (1973) with modifications. P content and pH of plant growth substrate were determined at the end of

the experiment. pH was analyzed by potenciometry (1:2.5) and P available by incubating 1 g of each sample in 15 ml NBRIP-BPB liquid medium at 28 °C for 15 days followed by estimation of soluble P (Fiske and Subbarow, 1925).

Survival of inoculated bacteria in plant growth substrate was analyzed at harvest by determining CFU/g substrate, following the same procedure as described before, and by genomic fingerprinting. Approximately 5 colonies from NBRIP-BPB plates obtained from the quantification of number of bacteria, were selected to obtain bacterial DNA template. Total bacterial DNA was obtained by using the procedure described by Walsh et al. (1991). Repetitive genomic regions (rep-fingerprint) of bacterial genomes were amplified using primers ERIC and BOX (Versalovic et al., 1994). PCR amplifications were performed as described by Tonelli et al. (2010) in a Mastercycler gradient block (Eppendorf) and products were separated according to molecular size by horizontal electrophoresis on 2% (w/v) agarose gels stained with SYBR Green II (Molecular Probes). Genomic fingerprint patterns obtained were compared with those obtained from pure culture of inoculated bacteria.

2.8.3. Crop rotation assay peanut-maize-peanut in test pots

Growth promotion of peanut and maize plants by selected PSB were tested in a simulated crop rotation assay (peanut-maize-peanut). Plastic pots (30 cm-diameter, 35 cm height) that contained sieved unsterilized soil with low P content from the peanut cultivation area of Córdoba (organic matter: 1.40% (Walkley-Black method), pH: 6.82 (Potenciometry 1:2.5), N: 11.5 μg/g (phenolsulfonic acid), P: 8.5 μg/g (Kurtz and Bray I method)) were used. The soil was supplemented with 40 mg/kg $Ca_3(PO_4)_2$. Firstly, peanut seeds (var. Granoleico) were surface disinfected as described previously, germinated and transferred to the pots. The treatments were: plants inoculated with (1) *Pantoea* sp. J49; (2) *Bacillus* sp. L55; (3) *Serratia* sp. S119; (4) *Enterococcus* sp. L191; (5) *Serratia* sp. J260; (6) *Pantoea* sp. J49 + *Serratia* sp. S119; (7) *Bacillus* sp. L55 + *Enterococcus* sp. L191; (8) Uninoculated plants (negative control plants, C−); (9) uninoculated plants supplemented regularly with 20 mM KH_2PO_4 (plants fertilized with P; C+) and (10) plants inoculated with commercial strain *P. fluorescens* PMT1.

Bacterial inocula were obtained as described previously in the microcosm maize assay. Bacterial inoculation was performed one time at the beginning of the trial. Peanut plants were grown under controlled environmental conditions, watered regularly with sterilized tap water and, once a week, with Hoagland nutrient solution which was devoid of soluble P. Peanut plants were extracted at 45 days post inoculation (first harvest of peanut). Subsequently, disinfected maize seeds were sown in the same pots and at 21 days (66 days post inoculation), maize plants were extracted. Subsequently peanut disinfected seeds were sown and at 45 days (111 days post inoculation) peanuts plants were extracted (second harvest of peanut). In each plant harvest the following parameters were determined: length (cm), dry weight (g/plant) and P content (mg/g) of aerial and root tissues, as described above, as well as P content (μg/ml) and pH of soil.

2.9. Data analysis

The data were subjected to analysis of variance (ANOVA) and differences among treatments were detected by Tukey test ($P < 0.05$) for biochemical parameters and LSD test ($P < 0.05$) for plant assays. Pearson correlation coefficient between soluble P released by bacteria and pH was calculated. In all cases Infostat software was used.

Table 1
Organic acids and PQQ cofactor production, maximum amounts of P-liberated, time of growth, pH and colony forming units in NBRIP-BPB medium with $FePO_4$ and $AlPO_4$ and phosphatase activity by the six native peanut PSB and *P. fluorescens* PMT1.

Strain	Organic acids (μg/ml)				PQQ (nmol)	NBRIP–BPB medium with $FePO_4$				NBRIP–BPB medium with $AlPO_4$				Phosphatase activity (μg pNP/ml/h)	
	Gluconic	Citric	Lactic	Succinic		Solubilized phosphate (μg/ml)[a]	Time of growth (h)[b]	pH[c]	CFU/ml[d]	Solubilized phosphate (μg/ml)[a]	Time of growth (h)[b]	pH[c]	CFU/ml[d]	Acid	Alkaline
Serratia sp. S119	7960	ND	ND	ND	289.7	17.2 ± 1.1^{A}	24	3.12 ± 0.05^{AB}	9×10^{6}	141.6 ± 2.4^{D}	48	3.19 ± 0.09^{A}	6×10^{2}	50.7 ± 1.5^{B}	58.7 ± 2.2^{BC}
Pantoea sp. J49	3510	ND	ND	ND	282.0	15.0 ± 1.1^{A}	24	3.49 ± 0.04^{BC}	2×10^{8}	27.3 ± 2.1^{B}	168	3.39 ± 0.03^{AB}	8×10^{3}	43.9 ± 0.6^{A}	42.8 ± 1.2^{A}
Acinetobacter sp. L176	4010	ND	ND	ND	81.7	16.3 ± 1.2^{A}	24	3.48 ± 0.11^{BC}	8×10^{7}	12.5 ± 0.9^{A}	24	3.50 ± 0.08^{B}	2×10^{8}	44.0 ± 0.7^{A}	42.1 ± 1.1^{A}
Bacillus sp. L55	1240	ND	ND	ND	137.9	16.2 ± 0.5^{A}	24	3.89 ± 0.0^{C}	6×10^{6}	9.9 ± 0.5^{A}	24	4.15 ± 0.04^{C}	1×10^{6}	42.0 ± 0.7^{A}	42.1 ± 1.3^{A}
Enterococcus sp L191	1950	ND	ND	ND	ND	14.5 ± 0.7^{A}	24	4.67 ± 0.16^{D}	7×10^{6}	11.9 ± 1.2^{A}	24	3.95 ± 0.04^{C}	2×10^{7}	43.8 ± 0.8^{A}	41.2 ± 1.5^{A}
Serratia sp. J260	8920	ND	ND	ND	80.5	37.4 ± 2.0^{B}	48	2.84 ± 0.03^{A}	1×10^{6}	73.3 ± 2.2^{C}	48	3.15 ± 0.02^{A}	2×10^{3}	49.7 ± 0.8^{B}	52.7 ± 2.0^{B}
P. fluorescens	ND*	ND*	ND*	ND*	ND*	13.9 ± 0.3^{A}	48	3.31 ± 0.01^{B}	5×10^{7}	22.2 ± 1.1^{B}	48	3.51 ± 0.04^{B}	5×10^{6}	56.1 ± 1.4^{C}	65.9 ± 2.8^{C}

Data are means ± S.E., of 6 replicates, $p < 0.05$ according to Tukey test ($P < 0.05$). Different letters indicate differences among isolates.
ND*: no determinated.
ND: no detected.
[a] Maximum levels of soluble phosphorus released.
[b] Time of growth (h) in which maximum levels of soluble P were released.
[c] Supernatants pH at time of maximum levels of soluble P released by each bacterium.
[d] Colony-forming units at time of maximum levels of soluble P released by each bacterium.

3. Results

3.1. Native PSB from Cordoba soils produce gluconic acid and PQQ

All strains showed to produce gluconic acid and quantities detected ranged from 1240 to 8920 μg ml^{-1} (Table 1). Although other minor peaks did appear in the HPLC chromatographs, none of the retention times of these peaks corresponded to those of the other organic acids tested. With the exception of *Enterococcus* sp. L191, PQQ cofactor was detected in the supernatants of all analyzed bacteria and levels detected ranged from 80.5 to 289.7 nmol (Table 1). Results indicated that Gram negative bacteria secreted the highest level of gluconic acid while PQQ production was not directly correlated with these concentrations (r = 0.27).

3.2. Native peanut PSB solubilize strong insoluble P sources and show ability to mineralize insoluble organic P sources

Results obtained showed that all bacteria solubilized both $FePO_4$ and $AlPO_4$ accompained with a decrease of the pH of supernatants (Table 1). The concentration of soluble P released by the PSB in liquid medium NBRIP-BPB containing $FePO_4$ ranged from 14.5 to 37.4 μg/ml. The strain *Serratia* sp. J260 secreted the highest amounts of available P in the medium (37.4 μg/ml) and showed the lowest value of pH in the supernatant (2.84, respectively). The values of soluble P released from $AlPO_4$ ranged from 9.9 to 141.6 μg/ml and the strains *Serratia* sp. S119 and *Serratia* sp. J260 secreted the highest amounts of available P into the medium (141.6 and 73.3 μg/ml, respectively) and showed the lowest value of pH in their supernatants (3.19 and 3.15, respectively). In general, the values of soluble P released by the bacteria in NBRIP-BPB medium containing $AlPO_4$, were higher than those observed with $FePO_4$. Phosphate solubilization was associated with pH decrease of the media. Correlation analysis showed a low negative relationship between soluble P secreted in liquid medium NBRIP-BPB with $FePO_4$ and $AlPO_4$ and supernatants pH (r = −0.56 and −0.77, respectively). Viability of isolates was not affected along the assay and almost all bacteria, released maximum levels of soluble P within of 48 h of growth (Table 1).

All bacteria analyzed presented both acid and alkaline phosphatase activity (Table 1). The values of pNP produced ranged from 42.0 to 50.7 μg/ml/h and 41.2 to 58.7 μg/ml/h, for acid and alkaline phosphatases, respectively. The strains *Serratia* sp. S119 and *Serratia* sp. J260 showed the highest activities of both acid and alkaline phosphatases.

3.3. Effect of abiotic stresses and pesticides on growth and the in vitro phosphate solubilizing activity of the PSB

It was observed that three bacteria (*Serratia* sp. J260, *Pantoea* sp. J49 and *Acinetobacter* sp. L176) showed TCP solubilization ability in all conditions tested except for 42 °C incubation temperature in which they were not able to growth. *Bacillus* sp. L55 showed phosphate solubilizing ability only at 37 °C while *Enterococcus* sp. L191 and *Serratia* sp. S119 showed no TCP solubilization ability in none of the abiotic stress conditions tested (Table 2).

In order to analyze if the application of pesticides modifies the phosphate solubilizing capacity of bacteria analyzed, the ability to grow and solubilize TCP in the presence of pesticides regularly applied in peanut and maize crops were analyzed. In general, all bacteria showed ability to grow and solubilize TCP in the presence of most pesticides used in the concentrations applied in the field. All bacteria were able to solubilize TCP in presence of Imazapic and the fungicide Pyraclostrobin + Epoxiconazole. In addition, with the exception of *Pantoea* sp. J49, all bacteria solubilized TCP in NBRIP-BPB medium containing the insecticide Lambda-cyhalothrin. On the contrary, herbicide Glyphosate and the fungicide Azoxystrobin + Difenoconazole, affected negatively the ability to solubilize TCP of several isolates. When bacteria were exposed to the pool of pesticides (used for both peanut and maize) none of the PSB were able to solubilize TCP. Although pool assayed include all pesticides applied in soil for both crops, application is performed in different plantís growth times, so soil bacteria are probably not exposed to all compounds simultaneously. Strains *Serratia* sp. J260 and *Pantoea* sp. J49 were able to grow in TY medium against all pesticides analyzed (data not shown) but lost this ability when grown in NBRIP-BPB. This could be because TY is a rich medium while NBRIP-BPB is a minimal medium in which the concentration of nutrients is more limited, and therefore, the bacteria finds it difficult to change their metabolism to resist the effect caused by the application of pesticides.

3.4. Plant inoculation experiments

3.4.1. Phosphate solubilizing bacteria coexistence in plate assays

In order to select bacterial combinations for mixed PSB inoculation plant experiments, plate assays were performed with all selected bacteria. Coexistence assays indicated no negative effect within bacteria in both TY and NBRIP-BPB medium (data not shown).

3.4.2. Seed germination of peanut and maize is increased by inoculation with selected PSB

The results obtained indicated that the root dry biomass of bacterial inoculated maize seeds was significantly increased, compared to negative control plants, with two the single inoculations (*Serratia* sp. S119 and *Serratia* sp. J260) and three mixed inoculations (*Pantoea* sp. J49 + *Serratia* sp. S119, *Acinetobacter* sp. L176 + *Serratia* sp. J260 and *Enterococcus* sp. L191 + *P. fluorescens*) (Fig. 1B). In the case of peanut seeds, the simple inoculation (*Acinetobacter* sp. L176) and mixed inoculations (*Acinetobacter* sp. L176 + *Serratia* sp. J260 and *Bacillus* sp. L55 + *Serratia* sp. J260 + *P. fluorescens*) showed statistically significant increases, compared to C− in root dry weight (Fig. 1D). Root length of both peanut and maize seeds increased only in P-fertilized treatment (Fig. 1A and C). In general, it was observed that treatments with mixed bacterial inocula were more efficient than single inoculations.

3.4.3. Inoculations with native peanut PSB on Zea mays L. produce increase of plant growth

In the producing area of Argentina, maize is the main crop used in rotation with peanut and both crops are of great agricultural importance. Results obtained from this assay showed that the inoculation, both in single and mixed combinations, with the six selected PSB and the reference strain *P. fluorescens*, increased at least one of the plant growth parameters analyzed with respect to uninoculated maize plants (Fig. 2; Table 3). The inoculation with three treatments (*Pantoea* sp. J49, *Serratia* sp. J260, and *Pantoea* sp. J49 + *Serratia* sp. S119) significantly increased plant aerial length, compared with uninoculated plants. The root length was significantly increased with five bacterial treatments (*Pantoea* sp. J49, *Serratia* sp. J260, *Serratia* sp. S119, *Enterococcus* sp. L191 and *Acinetobacter* sp. L176) (Fig. 2). The results obtained indicated a significant increase in aerial dry weight of plants inoculated with three treatments (*Serratia* sp. S119, *Serratia* sp. J260 and *Pantoea* sp. J49 + *Serratia* sp. S119) compared with uninoculated plants. Moreover, root dry weight showed a significant increase when plants were inoculated with four treatments (*Serratia* sp. J260, *Pantoea* sp. J49 + *Serratia* sp. S119, *Enterococcus* sp. L191 + *P. fluorescens* and *Bacillus* sp. L55 + *Enterococcus* sp. L191) (Fig. 2).

In general it was possible to observe improved plant growth promoting effect of maize plants treated with single inocula. Inoc-

Table 2
Phosphate solubilizing ability under abiotic stress and in presence of pesticides by the six native peanut PSB and *P. fluorescens* PMT1 in NBRIP-BPB medium

		Strains						
		Serratia sp. S119	*Serratia* sp. J260	*Pantoea* sp. J49	*Acinetobacter* sp. L176	*Bacillus* sp. L55	*Enterococcus* sp. L191	*P. fluorescens*
Stress								
	NaCl (mM) 50	–	+	+	+	–	–	ND*
	150	–	+	+	+	–	–	ND*
	pH 5	–	+	+	+	–	–	ND*
	8	–	+	+	+	–	–	ND*
	Temp. (°C) 37	–	+	+	+	+	–	ND*
	42	–	–	–	–	–	–	ND*
Pesticides	Lambda Cyhalothrin	+	+	–	+	+	+	+
	S-metolachlor	–	+	+	–	–	+	+
	Glyphosate	–	–	+	+	–	–	–
	Imazethapyr	–	–	+	+	+	+	–
	Imazapic	+	+	+	+	+	+	+
	Diclosulam	+	–	+	+	+	+	–
	Atrazine	+	+	+	+	–	–	+
	Azoxystrobin + Difenoconazole	–	–	–	+	–	+	+
	Pyraclostrobin+ Epoxiconazole	+	+	+	+	+	+	+
	Pool (maize)[a]	–	–	–	–	–	–	–
	Pool (peanut)[b]	–	–	–	–	–	–	–
	Pool (all pesticides)[c]	–	–	–	–	–	–	–

ND*: no determinated
[a] Pool of pesticides (insecticides, fungicides and herbicides) commonly used on maize crops in the agricultural area of the province of Córdoba.
[b] Pool of pesticides (insecticides, fungicides and herbicides) commonly used on peanut crops in the agricultural area of the province of Córdoba.
[c] Pool of pesticides (insecticides, fungicides and herbicides) commonly used on maize and peanut crops in the agricultural area of the province of Córdoba.

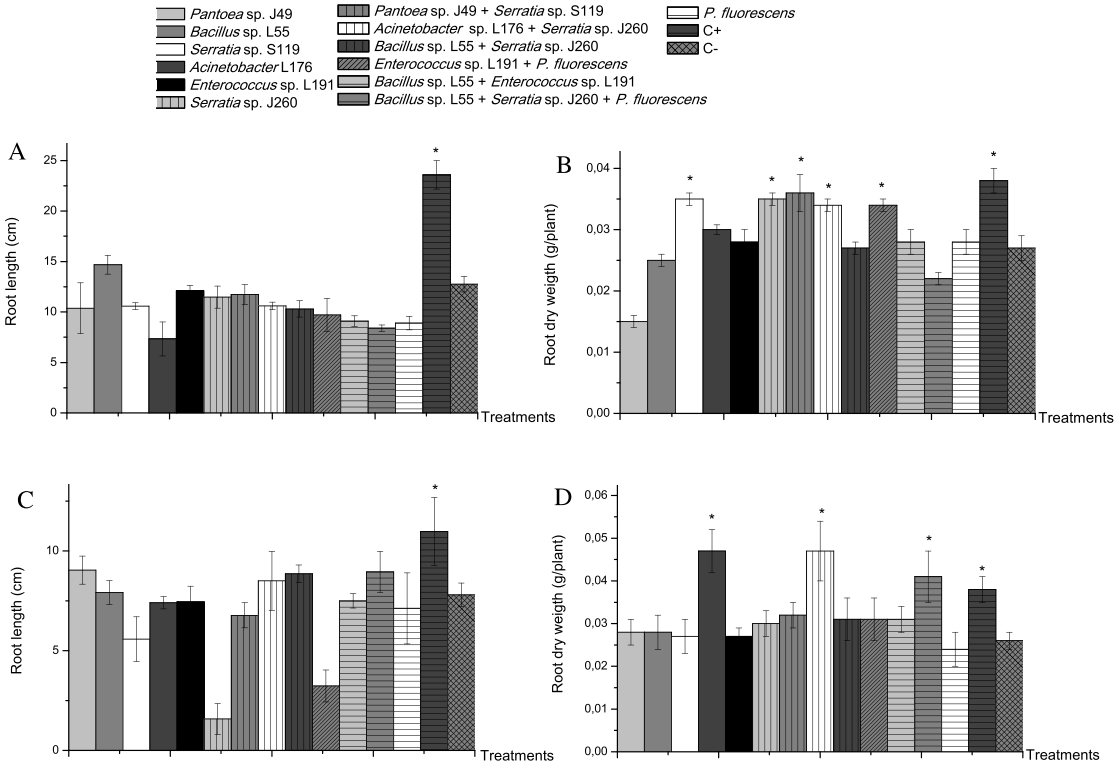

Fig. 1. Length (cm) and root dry weight (g/plant) of maize (A and B) and peanut (C and D) seeds inoculated with PSB and *P. fluorescens* PMT1. Data are means ± S.E., of 9 replicates, $p < 0.05$ according to LSD test ($P < 0.05$). *: indicates statistically significant difference compared with uninoculated plants.

ulation with *Serratia* sp. J260, *Serratia* sp. S119 or *Pantoea* sp. J49 increased almost all parameters analyzed with respect to uninoculated plants. On the other hand, the mixed inoculation with *Pantoea* sp. J49 + *Serratia* sp. S119 produced significant increases in most parameters analyzed on maize plants.

Values of maize P content indicated that all treatments produced increases greater than 40% in aerial and/or radical tissues with respect to uninoculated plants (Table 3). All mixed bacterial treatments and four single inoculations (*Pantoea* sp. J49, *Serratia* sp. S119, *Serratia* sp. J260 and *Enterococcus* sp. L191) increased P content of both aerial and root maize tissues. Moreover, it was observed that in most treatments, the increase in the P content was higher in aerial than in root tissues. Maize plants treated with the reference pseudomonas strain showed an increase in the biomass of

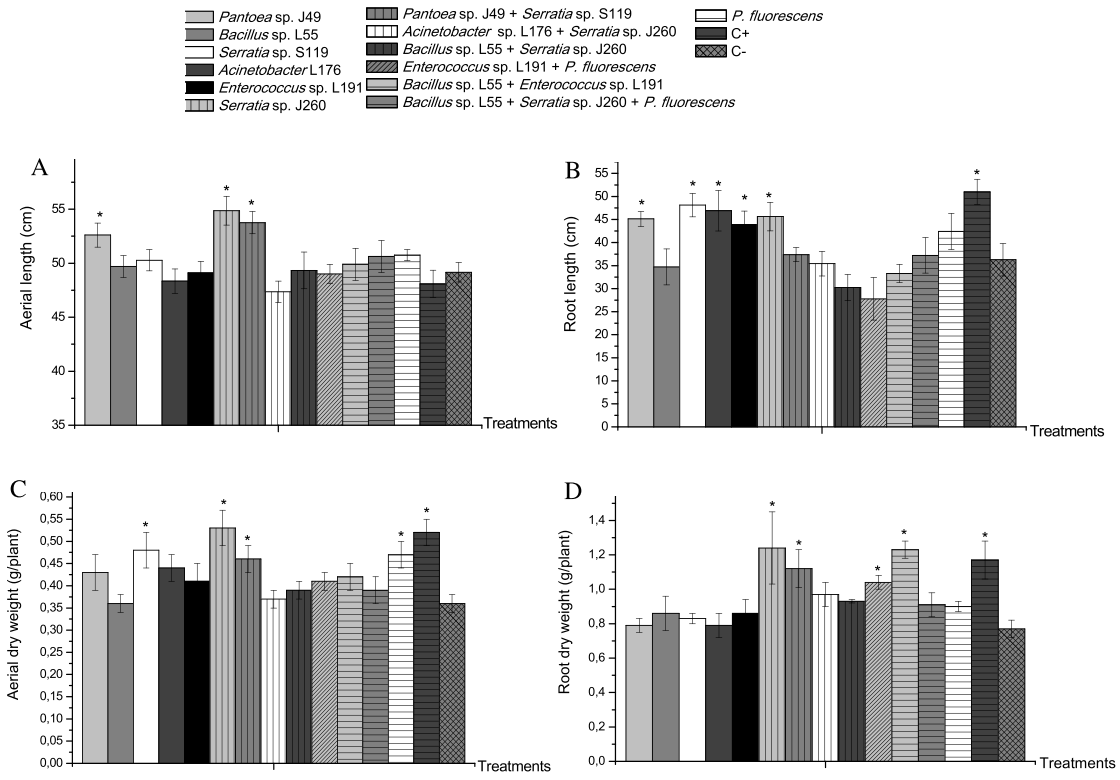

Fig. 2. Aerial and root length (cm) (A and B) and aerial and root dry weight (g plant^{-1}) (C and D) of maize plants inoculated with PSB and *P. fluorescens* PMT1. Data are means ± S.E., of 8 replicates, $p < 0.05$ according to LSD test ($P < 0.05$). *: indicates statistically significant difference compared with uninoculated plants.

Table 3
Aerial and root P content of maize plants inoculated with PSB and *P. fluorescens* PMT1, P content and pH of plant growth substrate at the end of the experiment and survival of PSB.

Treatments	P content (mg/g plant) aerial root		P content of plant growth substrate (μg/ml)	pH of plant growth substrate	CFU/g of plant growth substrate[a]	CFU/g of plant growth substrate[b]
Pantoea sp.J49	4.4 ± 0.2^{E}	2.5 ± 0.2^{DEFG}	284.9 ± 22.6^{CDEF}	7.4 ± 0.1	2×10^8	2×10^6
Bacillus sp.L55	2.5 ± 0.3^{AB}	2.4 ± 0.2^{CDEF}	323.3 ± 23.22^{DEFG}	7.5 ± 0.2	4×10^7	4×10^6
Serratia sp. S119	7.2 ± 0.2^{H}	2.9 ± 0.1^{G}	345.3 ± 20.3^{FG}	7.5 ± 0.1	2×10^8	3×10^6
Acinetobacter sp. L176	3.0 ± 0.4^{BC}	1.5 ± 0.1^{AB}	344.85 ± 29.9^{FG}	7.7 ± 0.2	2×10^8	4×10^5
Enterococcus sp.L191	3.0 ± 0.2^{BC}	2.2 ± 0.1^{CDE}	302.2 ± 30.4^{CDEF}	7.4 ± 0.1	8×10^7	4×10^6
Serratia sp. J260	4.1 ± 0.1^{DE}	3.5 ± 0.4^{H}	395.5 ± 41.6^{G}	7.6 ± 0.2	2×10^8	3×10^3
Pantoea sp.J49 + *Serratia* sp. S119	5.8 ± 0.5^{G}	2.0 ± 0.1^{BC}	145.9 ± 51.9^{A}	7.8 ± 0.1	2×10^8 8×10^6	4×10^7/ 5×10^5
Acinetobacter sp. L176+ *Serratia* sp. J260	3.4 ± 0.6^{BCD}	2.3 ± 0.1^{CDEF}	241.5 ± 27.0^{ABCDE}	7.5 ± 0.1	1×10^8 2×10^3	2×10^6 2×10^3
Bacillus sp.L55 + *Serratia* sp. J260	4.6 ± 0.3^{EF}	3.9 ± 0.1^{H}	224.9 ± 24.2^{ABC}	7.7 ± 0.1	2×10^7 7×10^3	3×10^6 3×10^3
Enterococcus sp.L191 + *P. fluorescens*	4.7 ± 0.4^{EF}	2.7 ± 0.1^{EFG}	208.3 ± 41.7^{AB}	7.7 ± 0.1	1×10^8 4×10^6	5×10^5 2×10^6
Bacillus sp.L55 + *Enterococcus* sp.L191	5.5 ± 0.4^{FG}	2.4 ± 0.1^{CDEF}	208.5 ± 29.7^{AB}	7.7 ± 0.1	7×10^6 5×10^6	4×10^6 2×10^6
Bacillus sp.L55 + *Serratia* sp. J260 + *P. fluorescens*	3.5 ± 0.1^{CD}	2.1 ± 0.2^{CD}	247.4 ± 39.6^{BCDE}	7.8 ± 0.1	3×10^6 5×10^4 1×10^7	$9 \times 10^5 1 \times 10^4 2 \times 10^6$
P. fluorescens	3.83 ± 0.68^{CDE}	2.82 ± 0.10^{FG}	227.9 ± 23.0^{ABC}	7.5 ± 0.1	1×10^8	3×10^6
C+	4.42 ± 0.08^{E}	1.55 ± 0.03^{AB}	333.6 ± 21.6^{EFG}	7.1 ± 0.2	ND*	ND*
C-	1.78 ± 0.21^{A}	1.46 ± 0.10^{A}	235.5 ± 15.1^{ABCD}	7.7 ± 0.1	ND*	ND*

Data are means ± S.E., of 8 replicates, $p < 0.05$ according to LSD test ($P < 0.05$). Different letters indicate differences among isolates. C+: uninoculated maize plants supplemented regularly with 20 mM KH_2PO_4 (plant fertilized with P); C-: Uninoculated maize plants. ND*: no determinated. Inoculum: 10^9 CFU/ml; [a]: CFU/g support, final concentration even inocula were deposited on the crown; 1 h post-inoculation; [b]: CFU/g support at the end of the experiment, 21 days post inoculation.

aerial tissues and in P content both in radical and aerial tissues, with respect to uninoculated plant. P content of plant growth substrate was determined at the end of the experiment and values obtained ranged from 145 to 400 ppm. A significant increase of P content was observed in pots with maize plants inoculated with *Acinetobacter* sp. L176, *Serratia* sp. S119 or *Serratia* sp. J260. Interestingly, these treatments also showed growth promotion of maize plants (Fig. 2).

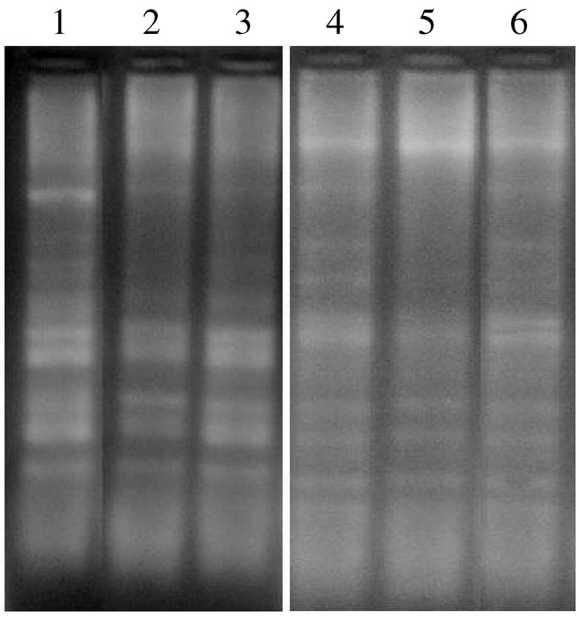

Fig. 3. ERIC-PCR profiles of DNA obtained from *Pantoea* sp. J49 of maize plant assay. Profiles of *Pantoea* sp. J49 inoculated (1), *Pantoea* sp. J49 recovered from simple inoculums *Pantoea* sp. J49 (2) and *Pantoea* sp. J49 recovered from mixed inoculums *Pantoea* sp. J49 + *Serratia* sp. S119. (3). Profiles of *Serratia* sp. S119 inoculated (4), *Serratia* sp. S119 recovered from simple inoculums *Serratia* sp. J49 (5) and *Serratia* sp. S119 recovered from mixed inoculums *Pantoea* sp. J49 + *Serratia* sp. S119. (6).

The values of plant growth substrate pH at the end of the experiment ranged from 7.1 to 7.8 (Table 3).

All inoculated bacteria were isolated from plant growth substrate at the end of the experiment. In maize rhizosphere the number of bacteria ranged from 10^3 to 10^6 CFU/g substrate (Table 3). The identical ERIC/BOX-PCR profiles obtained from DNA of bacteria isolated from rhizosphere samples and those from the inoculated strain confirmed the presence of the PSB at the end of the experiment (Fig. 3).

In general it was possible to observe on maize microcosm assay, that the treatments that highlighted were *Serratia* sp. J260, *Serratia* sp. S119, *Pantoea* sp. J49 and *Pantoea* sp. J49 + *Serratia* sp. S119.

3.4.4. Phosphate solubilizing bacteria promote growth of peanut and maize plants in a simulated crop rotation system

The results obtained indicated that both peanut and maize plants, inoculated with single and mixed inocula increased most plant growth parameter analyzed. In general, the plant growth parameters values obtained from both harvest of peanut were similar. Inoculation with PSB significantly increased aerial length of peanut plants of all treatments analyzed, compared with uninoculated peanut plants (Fig. 4A). The inoculation with five bacterial treatments (*Pantoea* sp. J49, *Bacillus* sp. L55, *Enterococcus* sp. L191, *Serratia* sp. J260 and *Bacillus* sp. L55 + *Enterococcus* sp. L191) on maize plants increased this parameter. Root length of peanut plants increased significantly with four bacterial treatments (*Pantoea* sp. J49, *Bacillus* sp. L55, *Serratia* sp. J260, *Bacillus* sp. L55 + *Enterococcus* sp. L191) and the treatments with *Bacillus* sp. L55, *Serratia* sp. J260 or *Bacillus* sp. L55 + *Enterococcus* sp. L191 enhanced this parameters on maize plants (Fig. 4B). The results showed a significant increase on aerial dry weight, compared with uninoculated plants, in both peanut and maize plants in five treatments (*Pantoea* sp. J49, *Bacillus* sp. L55, *Serratia* sp. J260, *Pantoea* sp. J49 + *Serratia* sp. S119 and *Bacillus* sp. L55 + *Enterococcus* sp. L191). On the other hand, the inoculation with *Serratia* sp. S119 increased aerial dry weight in peanut plants (Fig. 4C). The results indicated a significant increase in root dry weight in peanut plants inoculated with six treatments (*Pantoea*

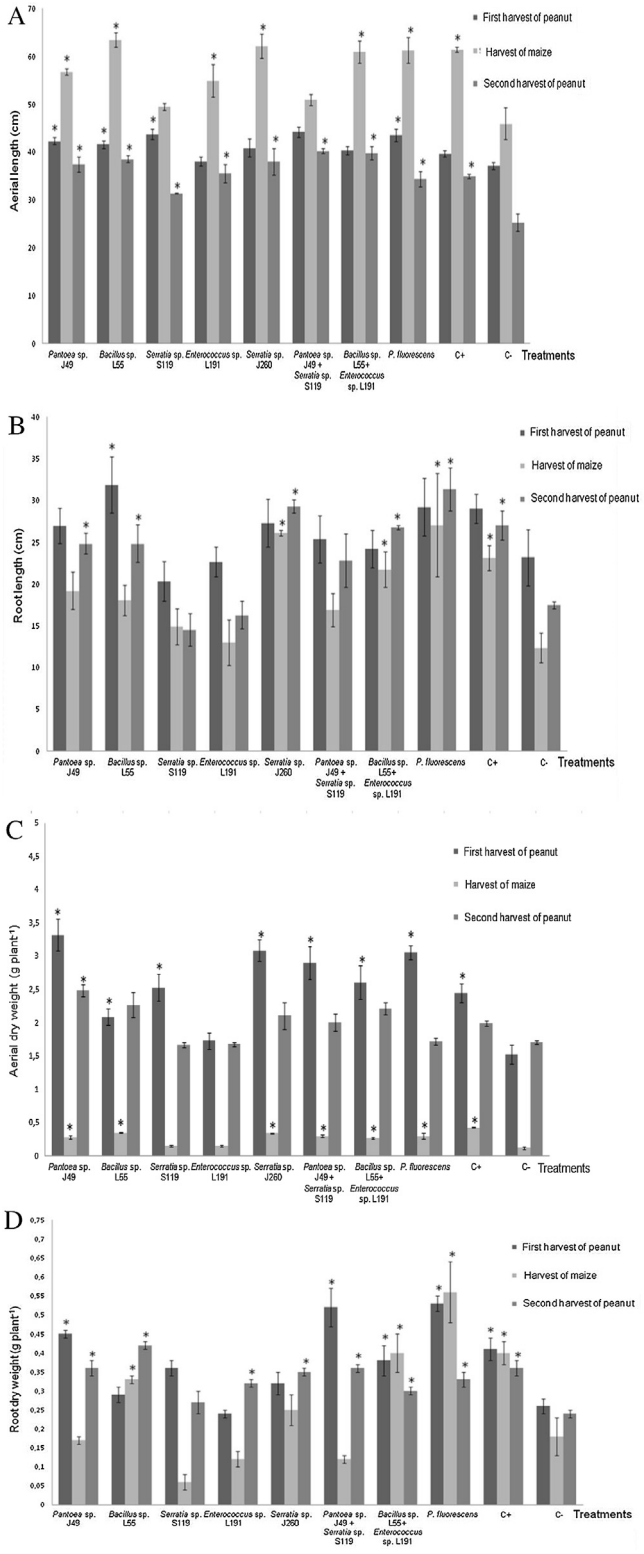

Fig. 4. Aerial and root length (cm) (A and B) and aerial and root dry weight (g plant^{-1}) (C and D) of simulating crop rotation on peanut-maize-peanut plants inoculated with peanut associated native isolates and *P. fluorescens* PMT1. Data are means ± S.E., of 8 replicates, $p < 0.05$ according to LSD test ($P < 0.05$). *: indicates statistically significant difference compared with uninoculated plants.

sp. J49, *Bacillus* sp. L55, *Enterococcus* sp. L191, *Serratia* sp. J260, *Pantoea* sp. J49 + *Serratia* sp. S119 and *Bacillus* sp. L55 + *Enterococcus* sp. L191), compared with uninoculated peanut plants. In maize plants, an increase of root dry weight with respect to uninoculated plants was observed in those plants inoculated with *Bacillus* sp. L55 or the mixed inoculum *Bacillus* sp. L55 + *Enterococcus* sp. L191 (Fig. 4D).

In this assay, the treatments *Pantoea* sp. J49, *Bacillus* sp. L55, *Serratia* sp. J260 and the mixed inocula *Pantoea* sp. J49 + *Serratia* sp. S119 and *Bacillus* sp. L55 + *Enterococcus* sp. L191 promoted growth on peanut and maize plants.

An increase in aerial or root P content in both peanut and maize plants was observed with the bacterial treatements *Serratia* sp. J260 and *Pantoea* sp. J49 + *Serratia* sp. S119 (Table 4). Aerial P content of peanut plants inoculated with *Pantoea* sp. J49 + *Serratia* sp. S119 increased compared with uninoculated peanut plants. Also, this treatment and the single inocula *Serratia* sp. J260 or *Enterococcus* sp. L191 showed an increase in the aerial P content of maize plants. Inoculation with *Pantoea* sp. J49 + *Serratia* sp. S119, *Bacillus* sp. L55 + *Enterococcus* sp. L191, *Pantoea* sp. J49 and *Serratia* sp. S119 increased root P content in both plants compared with negative control. In addition, the root P content of peanut plants increased with the bacterial treatments *Serratia* sp. J260, *Enterococcus* sp. L191 and *Bacillus* sp. L55.

The soil P content in both peanut and maize plants with treatments in each harvest and end of the experiment showed increment respect to measured soil P content at the beginning of the assay (8.5). The data obtained ranged from 8.5 to 26.5 ppm (Table 4). All treatments, except for *Bacillus* sp. L55 and *Bacillus* sp. L55 + *Enterococcus* sp. L191, showed a significant increase in soil P content in peanut plants compared with uninoculated peanut plants. On the other hand, the treatments *Pantoea* sp. J49, *Serratia* sp. J260 and the mixed inocula *Pantoea* sp. J49 + *Serratia* sp. S119 increased el soil P content in maize plants. In this assay, the treatments *Pantoea* sp. J49, *Serratia* sp. J260 and the mixed inocula *Pantoea* sp. J49 + *Serratia* sp. S119 increased soil P content in both peanut and maize plants. These treatments increased also aerial and/or root P content of both plants.

The soil pH at the end of the experiment showed similar values to those of the beginning of the assay (6.8) (Table 4) probably due the buffering capacity of soil particles that limits changes in pH values.

In general it was possible to observe from the results obtained from this simulated crop rotation assay, that the treatments *Serratia* sp. J260, *Serratia* sp. S119 and *Pantoea* sp. J49 and *Pantoea* sp. J49 + *Serratia* sp. S119 were the most efficient.

4. Discussion

The basic principle of the transformation of insoluble phosphates resides in the bacterial production of organic acids that dissolve phosphates making them available for the uptake by plants (Paredes-Mendoza and Espinosa-Victoria, 2010). Direct periplasmic oxidation of glucose to gluconic acid is considered as the metabolic basis of inorganic phosphate solubilization by many Gram-negative bacteria as a competitive strategy to transform the readily available carbon sources into less readily utilizable products by other microorganisms (Goldstein, 1995). The finding that only gluconic acid was detected in culture media of selected PSB agree with those obtained by Oteino et al. (2015) when analyzed pseudomonas strains in similar growth conditions. Also, Vyas and Gulati (2009) reported that the quantity of organic acids produced differed with the nature of phosphate substrates and suggested than in presence of tricalcium phosphate, higher gluconic acid production was detected. PQQ production was not directly correlated with concentrations of gluconic acid. This could probably be due to alternative

Table 4
Aerial and root P content of bacterial inoculated peanut and maize plants, P content and pH of soil in the simulated crop rotation assay in test pots.

Treatments	Peanut-first harvest			Maize			Peanut –second harvest		
	P content (mg/g plant) aerial root	P soil content (μg/ml)	pH soil	P content (mg/g plant) aerial root	P soil content (μg/ml)	pH soil	P content (mg/g plant) aerial root	P soil content (μg/ml)	pH soil
Pantoea sp.J49	1.8 ± 0.1^{AB}	3.3 ± 0.1^{E}	7.0 ± 0.5^{A}	6.1 ± 0.3^{A}	3.2 ± 0.4^{A}	4.9 ± 0.1^{E}	20.2 ± 1.7^{C}	6.4 ± 0.1^{B}	2.4 ± 0.3^{A}	2.6 ± 0.1^{BCD}	23.7 ± 1.4^{BC}	6.2 ± 0.5^{A}
Bacillus sp.L55	2.0 ± 0.1^{AB}	2.7 ± 0.3^{CD}	10.5 ± 2.5^{A}	6.5 ± 0.4^{A}	3.5 ± 0.3^{AB}	1.5 ± 0.3^{AB}	8.5 ± 0.4^{A}	5.9 ± 0.3^{AB}	2.3 ± 0.3^{A}	1.9 ± 0.2^{AB}	17.0 ± 3.0^{ABC}	6.0 ± 0.5^{A}
Serratia sp. S119	1.5 ± 0.2^{A}	2.7 ± 0.1^{CD}	11.0 ± 1.5^{A}	6.9 ± 0.2^{A}	3.2 ± 0.4^{A}	3.3 ± 0.4^{CD}	8.9 ± 1.9^{A}	6.1 ± 0.2^{AB}	3.0 ± 0.1^{ABC}	3.0 ± 0.5^{CDE}	24.0 ± 3.0^{BC}	5.9 ± 0.2^{A}
Enterococcus sp.L191	2.6 ± 0.2^{C}	2.8 ± 0.2^{CDE}	9.3 ± 3.0^{A}	6.7 ± 0.5^{A}	5.3 ± 0.3^{DE}	1.8 ± 0.1^{AB}	9.4 ± 2.5^{AB}	6.4 ± 0.3^{B}	1.9 ± 0.3^{A}	1.4 ± 0.2^{A}	26.5 ± 2.5^{C}	5.9 ± 0.5^{A}
Serratia sp. J260	2.6 ± 0.2^{C}	2.6 ± 0.1^{BC}	14.7 ± 3.1^{AB}	6.3 ± 0.5^{A}	6.5 ± 0.8^{E}	1.5 ± 0.1^{AB}	22.0 ± 1.7^{CD}	5.8 ± 0.2^{A}	3.6 ± 0.2^{BC}	3.7 ± 0.3^{E}	26.3 ± 2.4^{C}	6.1 ± 0.3^{A}
Pantoea sp.J49 + *Serratia* sp. S119	3.4 ± 0.3^{D}	3.1 ± 0.2^{DE}	25.8 ± 0.8^{D}	6.6 ± 0.4^{A}	4.9 ± 0.5^{CD}	4.2 ± 0.7^{DE}	20.3 ± 1.5^{CD}	5.8 ± 0.1^{A}	2.3 ± 0.4^{A}	2.2 ± 0.3^{ABC}	25.80 ± 0.80^{D}	6.1 ± 0.3^{A}
Bacillus sp.L55 + *Enterococcus* sp.L191	1.7 ± 0.1^{AB}	2.1 ± 0.1^{B}	9.7 ± 3.2^{A}	6.4 ± 0.3^{A}	3.2 ± 0.2^{A}	2.5 ± 0.5^{BC}	$9.9 \pm 0,3^{AB}$	6.1 ± 0.1^{AB}	1.9 ± 0.4^{A}	3.2 ± 0.5^{CDE}	13.1 ± 3.9^{A}	6.8 ± 0.3^{A}
P. fluorescens	2.2 ± 0.2^{BC}	1.1 ± 0.1^{A}	25.5 ± 2.5^{CD}	6.3 ± 0.4^{A}	3.6 ± 0.3^{ABC}	1.9 ± 0.1^{AB}	17.9 ± 4.1^{BC}	5.8 ± 0.2^{A}	3.6 ± 0.5^{C}	2.3 ± 0.1^{ABC}	24.0 ± 2.0^{BC}	5.9 ± 0.1^{A}
C+	4.1 ± 0.2^{E}	4.7 ± 0.1^{F}	22.2 ± 4.1^{BCD}	6.1 ± 0.3^{A}	4.7 ± 0.5^{BCD}	3.7 ± 0.9^{CDE}	28.8 ± 5.2^{D}	6.0 ± 0.1^{AB}	2.4 ± 0.3^{A}	3.5 ± 0.3^{DE}	26.0 ± 2.0^{C}	6.0 ± 0.1^{A}
C–	2.4 ± 0.2^{BC}	1.6 ± 0.1^{A}	15.5 ± 0.5^{ABC}	6.1 ± 0.4^{A}	2.9 ± 0.1^{A}	1.3 ± 0.4^{A}	9.8 ± 1.8^{AB}	5.9 ± 0.1^{AB}	2.6 ± 0.4^{AB}	2.3 ± 0.3^{ABC}	13.1 ± 3.9^{A}	6.0 ± 0.1^{A}

Data are means ± S.E., of 8 replicates, $p < 0.05$ according to LSD test ($P < 0.05$). Different letters indicate differences among isolates. C+: uninoculated maize plants supplemented regularly with 20 mM KH_2PO_4 (plant fertilized with P); C–: Uninoculated maize plants.

pathways, independent of PQQ, for the biosynthesis of gluconic acid in some bacteria (García Ortega and Ponce Rivas, 2003). Another explanation to low levels of PQQ detected in efficient PSB is that this cofactor is required in low quantities.

The PSB analyzed in this study have been previously characterized in their *in vitro* tricalcium phosphate solubilizing ability (Anzuay et al., 2013). Although this P source was selected considering that calcium phosphates are predominant in the agricultural soils of Córdoba, it was of interest to analyze the ability of selected strains to solubilize more insoluble inorganic P sources. Bashan et al. (2013) reported that soils vary greatly by pH and several chemical factors and therefore, study of only one metal-P source (Fe-P, Al-P or Ca-P) is not suitable as an universal selection factor for phosphate solubilizing bacteria. Our results indicate that the values of soluble P released by the bacteria in NBRIP-BPB medium containing $AlPO_4$ were higher than those observed with $FePO_4$. Similar results were found by several authors when analyzing phosphate solubilizing ability (Gadagi and Sa, 2002; Fankem et al., 2008; Collavino et al., 2010) since aluminum phosphate is considerably more soluble than the corresponding ferric salt (Ishio et al., 1986; Fankem et al., 2008). The supernatantís pH values detected when the bacteria grew with $FePO_4$ or $AlPO_4$, was lower than those detected in calcium phosphate (Anzuay et al., 2013). While the solubility of calcium phosphate increases exponentially with decreasing pH (Merbach et al., 2009), the behavior of $AlPO_4$ and $FePO_4$ in culture medium is different. The solubility of ferric phosphate decreases with lower pH down to 4.5–3.5, and aluminum phosphate has the lowest solubility within pH 5.5–4.5. Hence, acidification of the medium per se cannot account for phosphate mobilization in bacterial cultures in the cases of the insoluble phosphates sources (Fankem et al., 2008; Merbach et al., 2009). In addition to phosphate solubilization ability all selected PSB shoed phosphatase activity enhancing its potential to be used as P-biofertilizers.

Considering that the use of PSB as P-biofertilizers is an alternative to chemical fertilizers to increase P in the soil, it is necessary that the selected strains conserve this beneficial phenotype under adverse environmental conditions. The phosphate solubilizing capacity is severely influenced by environmental factors especially under stress conditions (Pal, 1998). It was interesting to observe that the inorganic phosphate solubilization property of the analyzed bacteria was conserved in presence of pesticides regularly applied in maize and peanuts crops and under abiotic stress conditions.

Although there are several previous reports tested with the application of PSB in peanut or maize plants with single inoculations (Mudalagiriyappa et al., 1997; Dey et al., 2004; Hameeda et al., 2008; Vyas and Gulati, 2009; Ibañez et al., 2014; Taurian et al., 2010, 2013; Anzuay et al., 2015b) and co-inoculations between PSB and other beneficial microorganisms in crops of peanuts, sorghum and barley (Glick, 1995; Bashan and Holguin, 1997; Taurian et al., 2013), there are few studies of co-inoculations between PSB. The presence in the PSB collection of bacteria belonging to differentes genera encouraged us to test mixed inocula. Iniatially, the effects of PSB inoculations in the early growth stages of peanut and maize plants was analyzed in a germination assay. From this experiment it was noteworthy that almost all treatments increased root dry biomass being this a promising result since the acquisition of soil nutrients is carried out by the roots.

Plants assays indicated that mixed inocula showed variable results depending on the plant and the growth substrate used. In addition, these treatments showed no synergistic effect with respect to single inocula. Results obtained in peanut and maize plants in a simulated crop rotation assay confirmed the previously described ability of the strains to promote peanut growth (Anzuay et al., 2015b). In addition, Ibañez et al. (2014) in a simulated crop rotation assay with maize found that other peanut associated bacteria from Cordoba soils, isolated from root nodules, promoted both plants growth when they were co-inoculated with *Bradyrhizobium* sp. SEMIA 6144. Treatments analyzed showed better plant growth promotion on peanut with respect to maize. This could be explained because, as mentioned above, the PSB were isolated from peanut plants. Dey et al. (2004) found that PSB significantly increased peanut crop because increased levels of P available soil. The increase in plantsí P content observed in plant inoculation assays suggests that the PSB exert their beneficial effect either increasing the P content of the plant or leaving this nutrient available in the plant growth substrate.

It is noteworthy that those PSB that showed greatest efficiency on plant promoting growth are Gram negative bacteria. This is in accordance to previous results that show encouraging beneficial results of Gram negative PSB in both single and mixed inoculations on plants of agricultural interest increasing plant growth and P content of plantís growth substrate (Goldstein, 1995; Chen et al., 2006; Rosas et al., 2006; Kuldeep et al., 2010; López-Ortega et al., 2013).

5. Conclusion

In conclusion, the six selected PSB used in this study showed efficient phosphate mineralizing and solubilization ability. Treatments with *Serratia* sp. J260 and *Pantoea* sp. J49 gave promising results showing not only a beneficial effect on the growth of both peanut and maize plants but also an increase of P content values in plant and/or plant growth substrate. Hence, these PSB strains would be potential P-biofertilizers for peanut and maize in the producing area of Argentina.

Conflict of interest

The authors declare they have no conflict of interest in this work.

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