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Beneficial effects of native phosphate solubilizing bacteria on peanut (Arachis hypogaea L) growth and phosphorus acquisition

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Abstract This study analyses the effect of inoculation of native phosphate solubilizing bacteria on peanut (*Arachis hypogaea* L.) growth and phosphorus acquisition. Peanut plants were inoculated individually with 18 native phosphate solubilizing bacteria in microcosm studies using a low content P soil from the producing area. Survival of inoculated bacteria in soil at the end of the experiment was determined by streaking serial dilutions of dry soil samples and subsequent repfingering analysis of the colonies. The ability of peanut plants to increase P levels without bacteria was determined in hydroponic culture. The results obtained indicated that inoculation of native phosphate solubilizing bacteria on peanut seedlings led to an increase in at least one of the plant growth parameters analyzed. The beneficial effect of bacteria inoculation was mainly observed in aerial organs of peanut plants. Inoculation of *Serratia* sp. J260, *Enterobacter* sp. J33, *Acinetobacter* sp. L176, *Enterococcus* sp. L185, *Enterococcus* sp. L191 and *Bacillus* sp. L55 on peanut plants led to an increase in plant or soil P content. Plant assay in hydroponic conditions indicated that peanut plants growing with tricalcium phosphate were able to release soluble P into the growth medium reaching values similar to those of plants growing with available P. The beneficial effects of the bacteria analyzed in this study and their ability to survive encourage us to consider them for the production of a potential P-bioinoculant for peanut crops.

Keywords Peanut · Phosphate solubilizing bacteria · Plant growth promotion · P plant increase · Survival of bacteria in soil

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1 Introduction

Peanut (*Arachis hypogaea* L.) is a widespread leguminous plant of great agricultural and economic significance. Argentina is one of the major peanut producers in the world along with China and USA, and has become the world's main exporter. More than 90 % of its production is concentrated in the province of Córdoba (Bolsa de Cereales de Córdoba 2013). Peanut growing soils of Córdoba have decreased phosphorus availability due to the intense agriculture (Sainz Rozas et al. 2012).

After nitrogen, P is the second most important macronutrient in plants as it plays an important role in energy transfer, cell division, photosynthesis, biological oxidation, metabolism, and reproduction (Sashidhar and Podile 2010). P is a limiting nutritional factor affecting the process of biological nitrogen fixation in peanut-rhizobiasymbiosis (Freire 1984). In plants, the concentration of P varies with age and organ. In peanut, only 10 % of the P incorporated into the plant is absorbed in the vegetative phase, while 39 and 51 % are incorporated in the reproduction and maturation stages, respectively (Giabastani 1996). In most agricultural soils, only a very low percentage of soil-P is available for plants due to its low solubility and lack of mobility which limits plant growth (Gulati et al. 2008). Less than 5 % of soil P is taken up by plants in the form of HPO_4^{2-} and $H_2PO_4^-$ (Rendig and Taylor 1989). In addition, nearly 75 % of the inorganic phosphates applied as fertilizers are also immobilized in soil (Podile and Kishore 2006). P forms insoluble metal ion complexes with iron and aluminum in acidic soils (Whitelaw 2000), or with calcium carbonate in alkaline soils (Gyaneshwar et al. 1998).

Soil microorganisms are very important for the biogeochemical cycles of nutrients and for soil health and quality (Jeffries et al. 2003). Plant growth promoting bacteria (PGPB) may exert their beneficial effects in two ways: directly by enhancing nutrient availability due to enhanced N-uptake and possibly nitrogen fixation, phosphate solubilization or modulating plant hormone levels, and indirectly by protecting plants against phytopathogenic organisms (Glick 1995). Phosphate solubilizing microorganisms mobilize insoluble phosphates in the soil and increase plant growth under conditions of poor P availability (Tripura et al. 2007). The relationship between phosphate solubilizing bacteria (PSB) and plants is synergistic in nature, as bacteria provide soluble phosphate and plants supply root borne carbon compounds (mainly sugars) that can be metabolized for bacterial growth (Pérez et al. 2007). Phosphate solubilizing bacteria have been found to belong to different genera such as *Pseudomonas, Bacillus, Rhizobium, Agrobacterium, Burkholderia, Acromobacter, Micrococcus, Aerobacter, Flavobacterium, Erwinia*, *Pantoea, Acinetobacter, Enterococcus* and *Enterobacter* (Rodríguez and Fraga 1999; Pérez et al. 2007; Ogut et al. 2010; Walpola and Yoon 2013; Kaur and Sudhakara Reddy 2014).

The solubilization of inorganic P occurs mainly by the action of low molecular weight organic acids such as gluconic and citric acid, synthesized by different soil bacteria (Goldstein 1995; Rodríguez and Fraga 1999; Wan and Wong 2004). Several studies with different phosphate solubilizing microorganisms have reported that the application of both simple and mixed inocula to different crops produce a significant yield increase (Richardson et al. 2001; Trivedi and Sa 2008; Vyas and Gulati 2009; Mohammadi et al. 2011). It is also known that some plant species are able to utilize non-labile P effectively in either inorganic or organic forms (Shibata and Yano 2003). For example, lupine roots excrete large quantities of citrate which mobilizes calcium-bound phosphate (Dinkelaker et al. 1989), and pigeonpea excretes piscidic acid able to chelate iron bound to phosphate (Ae et al. 1990). In the particular case of peanut, it has been shown that its roots exude several organic acids but their release does not fully correlate with the solubilization of non-labile P (Otani and Ae 1996). However, encouraging results were found when the rhizosphere of peanut plants was inoculated with phosphate solubilizing bacteria (Mudalagiriyappa et al. 1997; Dey et al. 2004; Taurian et al. 2010, 2013).

The P content in the peanut soils of Córdoba is low and the aim of our research was to assess the potential use of P- solubilizing bacteria (PSB) as a biofertilizer for peanut crops. Native PSB were isolated from peanut plants growing in native fields and analyzed for their in vitro plant growth promotion abilities (Taurian et al. 2010), especially in their phosphate solubilization capacity (Anzuay et al. 2013). The present study investigated the effect of inoculation of native phosphate solubilizing bacteria on the growth of peanut plants in soils containing P with low levels of available P.

2 Materials and methods

2.1 Bacterial growth and maintenance

Eighteen native phosphate solubilizing bacteria isolated from stems, roots and root nodules of peanut plants cultivated in central and southern region of Córdoba, Argentina (latitude, 32° to 34°, longitude, 63° to 65°) (Taurian et al. 2010) were used in this study. The bacteria belong to the genera *Serratia, Enterobacter, Pantoea*, *Acinetobacter, Bacillus* and *Enterococcus* (Table 1). *Pseudomonas fluorescens* PMT1 used in a commercial P-inoculant formulation ("RIZOFOS"® -RIZOBACTER) was employed as reference strain in plant based microcosm studies. Strains *Azospirillum brasilense* Cd and *Bradyrhizobium* sp. SEMIA 6144 (IPAGRO, Brasil) were used in in vitro nitrogen fixation assays as positive and negative controls, respectively. The bacteria were grown in TSA (trypticase soy agar) (Britania), YEMA (yeast extract mannitol agar) (Vincent 1970), LB (Luria-Bertani) (Miller 1972) or TY (tryptone yeast) (Beringer 1974) media. The bacteria were maintained in TY/YEM media supplemented with 20 % glycerol (*v/v*) at −80 °C.

2.2 Plant based microcosm studies

Seeds of *Arachis hypogaea* L cv. Tegua were surface disinfected in 96 % ethanol for 30 s, rinsed in sterile water, dipped in 15 % H_2O_2 for 10–15 min, and washed 5 times in sterile water. Then, they were germinated at 28 °C in sterilized Petri dishes with one layer of Whatman N°1 filter paper and moist cotton, until the radicle had reached a length of ca. Two centimeters (Taurian et al. 2002).

Peanut seedlings were transferred to sterilized plastic pots (30 cm-diameter, 35 cm height) that contained approximately 4.5 kg of sieved unsterilized soil with low phosphorus content from the peanut cultivation area of Córdoba (organic matter: 1.48 % (Walkley-Black method), pH: 6.7 (Potenciometry 1:2.5), nitrogen: 13.9 μg/g (Cadmium Reduction), phosphorus: 6.6 μg/g (Bray and Kurtz I method). The soil was supplemented with 0.2 % (*w/v*) $Ca_3(PO_4)_2$ to obtain a concentration of 40 mg/kg (Rivas et al. 2007). The following treatments were set up: (a) Uninoculated peanut plants (control); (b) uninoculated peanut plants supplemented regularly with 20 mM KH_2PO_4 (phosphorus fertilized peanut plants); (c) peanut plants inoculated with commercial strain *P. fluorescens* PMT1; (d) peanut plants inoculated with each of the 18 phosphate solubilizing isolates evaluated in this study. Bacterial inocula were obtained by harvesting 3–4 ml cultures of each bacteria grown at 28 °C and 120 rpm to stationary phase in TY

Table 1 Colony forming units per gram soil of phosphate solubilizing bacteria and their effect of inoculation on aerial and root length, stem, leaves, total aerial and root dry weight, aerial and root ratio, pod number, root nodule number and dry weight of peanut plants at 120 post inoculation

Treatments	CFU/g soil^{-a} (10^4)	Aerial length (cm)	Root length (cm)	Dry weight (g/plant)				AW/RW ratio	Pod number	Nodules	
				Stem	Leaves	Total aerial (stem + leaves)	Root			Number	Weight (mg)
Serratia sp. J21	180	54.9±2.6*[a]	14.7±1.0	0.92±0.15	1.11±0.17	1.98±0.43	0.20±0.02	9.5±1.4	3.2±0.4	1.3±0.2	6.0±1.0
Serratia sp. J145	2	38.0±3.2*	20.7±1.5*	1.65±0.13*	1.26±0.14*	2.88±0.19*	0.30±0.04	10.9±2.2	2.7±1.0	2.7±0.5	15.0±2.0
Serratia sp. J260	210	40.4±1.4*	19.2±1.2*	1.94±0.33*	1.47±0.16*	3.38±0.51*	0.51±0.05*[a]	7.0±0.6	1.5±0.3	10.5±0.5*	70.0±6.0*
Serratia sp. S93	60	46.8±3.1*	12.7±0.7	1.15±0.15	1.26±0.13*	2.28±0.30	0.26±0.05	10.8±2.2	2.5±0.6	8.0±0.9*	51.0±10.0*
Serratia sp. S119	18	32.1±1.9	19.7±1.7*	0.78±0.04	0.69±0.08	1.44±0.11	0.26±0.03	6.8±1.4	2.2±0.4	2.2±0.5	12.0±3.0
*Pantoea sp.*J49	60	46.8±1.8*	23.4±1.8*[a]	1.64±0.10*	0.88±0.05	2.44±0.14*	0.42±0.02*[a]	7.2±1.4	2.6±0.4	5.2±1.0*	33.0±7.0
Pantoea sp. J157	52	31.4±2.1	18.3±0.9*	0.97±0.14	0.82±0.10	2.00±0.23	0.27±0.02	7.0±0.4	3.0±0.4	1.4±0.2	15.0±6.0
Enterobacter sp. J33	12	36.6±1.6	17.6±1.4*	1.65±0.06*	1.36±0.19*	3.06±0.25*	0.41±0.05*[a]	7.9±1.1	2.0±0.6	7.2±0.2*	47.0±7.0*
Enterobacter sp. S57	110	47.5±2.1*	15.5±0.8	1.44±0.12	1.03±0.11	2.20±0.30	0.25±0.02	8.4±0.3	2.8±0.4	12.0±2.1*	166.0±78.0*
Acinetobacter sp. L176	3	41.2±0.4*	17.6±0.6*	1.69±0.16*	1.42±0.27*	2.66±0.46*	0.25±0.02	9.2±2.4	1.7±0.3	10.7±1.7*	68.0±17.0*
Bacillus sp. J9	430	54.6±4.0*[a]	17.5±0.9*	2.24±0.32*[a]	1.92±0.21*[a]	4.66±0.63*[a]	0.23±0.03	23.0±3.9*	4.0±0.6	1.5±0.3	7.0±3.0
Bacillus sp. J225	20	39.8±3.7*	18.7±1.8*	1.22±0.13	1.03±0.14	2.10±0.27	0.28±0.03	8.1±1.2	2.0±0.6	2.6±0.7	14.0±4.0
Bacillus sp. J255	40	34.5±1.3	14.1±0.8	1.32±0.14	1.06±0.13	2.40±0.16	0.39±0.05*	5.8±0.7	1.2±0.2	3.7±0.7	32.0±9.0
*Bacillus sp.*L54	160	33.7±2.6	16.0±1.0	1.16±0.26	0.82±0.11	1.98±0.32	0.32±0.04*	6.6±0.8	1.7±0.2	1.8±0.4	10.0±2.0
*Bacillus sp.*L55	220	41.1±1.7*	13.7±1.0	1.97±0.38*	1.48±0,24*	3.84±0.58*	0.43±0.07*[a]	9.6±1.8	1.2±0.2	1.7±0.2	9.0±4.0
Enterococcus sp. L177	60	36.1±1.3	15.6±0.3	1.46±0.37	1.37±0,36*	2.83±0.74*	0.30±0.04	10.4±3.8	1.5±0.3	1.2±0.2	8.0±2.0
*Enterococcus sp.*L185	39	48.8±4.4*	16.6±0.8	1.88±0.13*	1.56±0,10*	3.42±0.15*	0.23±0.01	14.2±0.7*	4.5±0.6*	2.0±0.3	14.0±3.0
*Enterococcus sp.*L191	700	37.0±2.3*	16.2±0.8	1.34±0.26	1.55±0,36*	3.34±0.45*	0.21±0.03	17.4±1.6*	3.0±0.3	5.2±0.7*	30.0±6.0
Pseudomonas fluorescens PMT1	2	29.6±2.8	16.2±1.5	0.99±0.17	1.03±0,08	2.06±0.23	0.23±0.02	9.3±1.4	2.0±0.6	4.0±0.6	23.0±5.0
Uninoculated peanut plants	130	25.2±1.8	10.7±1.4	0.68±0.08	0.47±0,10	1.11±0.12	0.14±0.01	7.8±0.8	2.2±0.6	1.7±0.2	9.0±2.0
P- fertilized peanut plants**	7	37.7±2.6*	15.3±1.0	1.15±0.13	1.33±0,09*	2.46±0.17*	0.22±0.03	10.2±1.9	2.8±0.6	6.7±0.9*	50.0±12.0*

Data are means±S.E., of 5–7 replicates, $p<0.05$ according to Tuckey test. * and [a] indicates statistically significant difference compared with non inoculated peanut plants and P fertilized plants, respectivelly

**Peanut plants fertilized with KH_2PO_4 (20 mM)

culture medium (10^9 CFU/ml). Inocula were deposited on the crown of the root, reaching a final concentration of 10^7 CFU/g soil. Equal volumes of sterile medium were deposited on uninoculated plants. The experiment was done with 5–7 replicates for each treatment.

Plants were grown under controlled environmental conditions (light intensity of 200 μR.m^{-2} s^{-1} 16 h day/8 h night cycle, at a constant temperature of 28 °C and a relative humidity of 50 %), watered regularly with sterilized tap water and, twice a month, with the nutrient solution described by Hoagland and Arnon (1950), which was devoid of soluble phosphate in this study.

Peanut plants were harvested at R3-R4 vegetative growth stages (Boote 1982) (120 days post inoculation). The following parameters were determined in the plants: aerial and root length, shoot and root dry weight, shoot-root dry weight ratio, pod number, number and dry weight of root nodules and aerial P and N content. Soil pH and P content were also determined at the end of the experiment, as described previously.

2.3 Survival of phosphate solubilizing bacteria in soil: counting and genomic fingerprint analysis

Survival of the bacterial inoculum in soil was determined at the end of the experiment by streaking serial dilutions of dry soil samples (1 g) on NBRIP-BPB plates (glucose 10 g/l, $Ca_3(PO_4)_2$ 5 g/l, $MgCl_2{\cdot}6H_20$ 5 g/l, $MgSO_4{\cdot}7H_20$ 0.25 g/l, KCl 0.2 g/l, $(NH_4)_2SO_4$ 0.1 g/l, Bromophenol blue 2.5 mg/l, pH 7.0, National Botanical Research Institute's phosphate growth medium, Mehta and Nautiyal 2001) supplemented with the appropriate antibiotics. After incubation of plates at 28 °C for 1–7 days, CFU per gram dry weight of rhizospheric soil was determined. Previously, bacteria were tested for their antibiotic resistance in liquid and agar TY media. The antibiotics tested were (final concentration): Streptomycin (30 μg/ml), Chloramphenicol (30 μg/ml), Neomycin (200 μg/ml), Kanamycin (50 μg/ml), Ampicillin (100 μg/ml), Nalidixic acid (1000 μg/ml), Rifampicin (200 μg/ml), Spectinomycin (200 μg/ml), Tetracycline (20 μg/ml), Gentamicin (10 μg/ml)). In order to confirm survival of inoculated bacteria in soil, repetitive genomic regions (rep-fingerprint) of bacterial genomes were amplified using primers ERIC and BOX (Versalovic et al. 1994). Approximately 5–8 colonies from NBRIP-BPB plates of the serial dilutions of soil (10^{-3}–10^{-4}) were selected to obtain bacterial DNA templates. Bacterial DNA was obtained by using the procedure described by Walsh et al. (1991). 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 2.5 % (*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.

2.4 Nitrogen fixation ability of phosphate solubilizing bacteria

Nitrogen fixation property of bacteria was estimated by analyzing their ability to grow in semi solid nitrogen free media NFB (Döbereiner et al. 1976) and JNF (Jensen's nitrogen-free) (Jensen 1942).

2.5 Peanut plants ability to increase P levels under hydroponic conditions

Disinfected and germinate peanut seeds were transferred to plant tubes containing 40 ml Hoagland liquid medium (Hoagland and Arnon 1950) supplemented with either 50 mM KH_2PO_4 or with 0.25 mM (2 g/l) $Ca_3(PO_4)_2$. Peanut plants were grown under controlled environmental conditions (light intensity of 200 μR m^{-2} s^{-1}, 16 h day/8 h night cycle, at a constant temperature of 28 °C and a relative humidity of 50 %), and were harvested after 2 weeks of growth. At harvest, aerial plant length was analyzed and, simultaneously, a sample of 1.5 ml of plant growth medium was obtained to analyze levels of soluble P and pH in it.

2.6 Data analysis

The data were subjected to analysis of variance (ANOVA) and differences among treatments were detected by a Tukey test ($P<0.05$). Analyses were performed on normal score transformed data. Pearson correlation coefficients between parameters analyzed were calculated. The statistical analysis was performed using Infostat software.

3 Results

3.1 Effect of inoculation with phosphate solubilizing bacteria on peanut plants growth

Inoculation of native PSB on peanut seedlings produced an increase in at least one of the plant growth parameters analyzed. Treatment of plants with nine (*Serratia* sp J145, *Serratia* sp J260, *Pantoea* sp J49, *Enterobacter* sp J33, *Acinetobacter* sp L176, *Bacillus* sp J9, *Bacillus* sp J225, *Enterococcus sp.*L185 and *Enterococcus sp.*L191) of the bacterial strains resulted in a significant increase of 50 % or more of the parameters measured, as compared with uninoculated plants. With five of these bacterial strains (*Serratia* sp. J21, *Pantoea* sp. J49, *Enterobacter* sp. J33, *Bacillus* sp. J9 and *Bacillus* sp. L55), some of the parameters showed a significant increase compared with P-fertilized plants (Table 1).

Plant aerial length significantly increased as a result of inoculation with 12 of the bacterial strains (*Serratia* sp J21, *Serratia* sp. J145, *Serratia* sp. J260, *Serratia* sp. S93, *Pantoea* sp. J49, *Enterobacter* sp. S57, *Acinetobacter* sp L176, *Bacillus* sp. J9, *Bacillus* sp. J225, *Bacillus* sp. L55, *Enterococcus* sp. L185 and *Enterococcus* sp. L191), while root length was significantly increased as a result of inoculation with nine (*Serratia* sp. J145, *Serratia* sp. J260, *Serratia* sp. S119, *Pantoea* sp. J49, *Enterobacter* sp. J157, *Enterobacter* sp. J33, *Acinetobacter* sp. L176, *Bacillus* sp. J9 and *Bacillus* sp. J225) of the strains. Bacterial isolates *Serratia* sp. J145, *Serratia* sp. J260, *Pantoea* sp. J49, *Acinetobacter* sp. L176, *Bacillus* sp. J9 or *Bacillus* sp. J225 promoted both aerial and root length. In addition, *Serratia* sp. J21 or *Bacillus* sp. J9 promoted aerial length while *Pantoea* sp. J49 enhanced root length to levels higher than those of plants fertilized with P.

The results showed a significant increase in aerial dry weight of plants inoculated with 11 (*Serratia* sp. J145, *Serratia* sp. J260, *Serratia* sp. S93, *Pantoea* sp. J49, *Enterobacter* sp. J33, *Acinetobacter* sp. L176, *Bacillus* sp. J9, *Bacillus* sp. L55, *Enterococcus* sp. L177, *Enterococcus* sp. L185 or *Enterococcus* sp. L191) of the strains and of those fertilized with P, as compared with control plants. Moreover, plants inoculated with *Bacillus* sp. J9 reached aerial dry weight values even higher than those found in P fertilized plants. In order to identify which of the two aerial organs was more sensitive to bacterial inoculation, dry biomass of plant aerial organs was determined separately. In plants inoculated with *Serratia* sp. J145, *Serratia* sp. J260, *Enterobacter* sp. J33, *Acinetobacter* sp. L176, *Bacillus* sp. J9, *Bacillus* sp. L55 or *Enterococcus* sp. L185 both stem and leaf biomass increased over control plants. However, inoculation with *Pantoea* sp. J49 only led to an increase in stem dry weight, and inoculation with *Serratia* sp. S93, *Enterococcus* sp. L177 and *Enterococcus* sp. L191 only led to an increase in leaf dry weight, as compared with negative control plants. After inoculation with *Bacillus* sp. J255 and *Bacillus* sp. L54, the dry weight of roots showed a significant increase compared to the negative controls. When plants were inoculated with *Serratia* sp. J260, *Pantoea* sp. J49, *Enterobacter* sp. J33 or *Bacillus* sp. L55, root dry weight reached values higher than those observed in P fertilized plants.

Root nodules were observed in all plants at the end of the assay. In those plants inoculated with *Serratia* sp. J260, *Serratia* sp. S93, *Pantoea* sp. J49, *Enterobacter* sp. J33, *Enterobacter* sp. S57, *Acinetobacter* sp. L176, or *Enterococcus* sp. L191 and those fertilized with P the number of nodules was higher than that observed in uninoculated plants. In addition, six of these treatments, including P-fertilized plants, showed also a significant increase in nodule dry weight per plant.

A similar aerial/root biomass ratio (AW/RW) was observed in bacteria-treated plants and control plants, except for those inoculated with *Bacillus* sp. J9, *Enterococcus* sp. L185 or *Enterococcus* sp. L191. Also, similar numbers of pods were observed in all plants, except in those inoculated with *Enterococcus* sp. L185, in which pod number was significantly higher than in control plants.

Correlation analysis between leaf and stem dry biomass and aerial plant length showed r values above 0.5 (r=0.51–0.56, p<0.02), while root length showed less than 0.4 correlation with root dry weight (r=0.39, p<0.1). As expected, both stem and leaf dry weight was directly correlated with total aerial dry weight (r=0.91 and 0.93, respectively, p<0.001). On the other hand, the AW/RW ratio indicated a direct relationship with total aerial dry biomass and pod number, which are two important crop yield parameters. Finally, the number of root nodules was highly correlated with the dry weight of nodules (r=0.89, p<0.001).

3.2 Effect of phosphate solubilizing bacteria inoculation on P content in peanut plants and soil

Aerial P content was measured in plants that showed a significant increase in 50 % or more of the parameters analysed. The results showed a significant increase in P aerial content of some of the treatments after inoculation with the bacteria *Serratia* sp. J260, *Enterobacter* sp. J33, *Acinetobacter* sp. L176, *Enterococcus* sp. L185 or *Enterococcus* sp. L191 compared with uninoculated or P-fertilized plants (Table 2). In all cases P increase was greater than 200 %, compared with both negative and positive control (P-fertilized plants). The highest increase (>500 %) was observed in plants inoculated with *Serratia* sp. J260, compared to P-fertilized plants. When the stem and leaf P content was analyzed separately, it was found that inoculation with the strains *Serratia* sp. J260, *Acinetobacter* sp. L176, *Enterococcus* sp. L185, *Enterococcus* sp. L191 increased P of both organs, while inoculation with *Enterobacter* sp. J33 increased only leaf P content compared with P-fertilized plants.

Soil P content was determined at the end of the experiment. Values obtained ranged from 9 to 26 μg/g (Table 2), which were higher than those determined before bacterial inoculation (6.6 μg/g). A significant increase in soil P content (>65 %) compared to negative control was observed in pots with plants inoculated with *Bacillus* sp. L55 or fertilized with P. The comparison of soil pH values measured at the beginning (pH 6.7) and at the end of the experiment (pH 6.93–7.23) did not show significant differences (Table 2).

Correlation analysis showed a negative relationship between soil and plant P content (r=−0.34, p<0,0001), while positive and high r values were observed between stem and leaf P content and total aerial P content (r=0.96 and 0.89, respectively, p<0.0001).

Table 2 Aerial P and N content of peanut plants inoculated with phosphate solubilizing bacteria and P content and pH of soil at the end of the experiment

Treatments	P content in shoot (mg/plant)	P content in leaves (mg/plant)	Total aerial P content (mg/plant)	Total aerial N content (mg/plant)	P soil content (μg/g soil)	Soil pH
Serratia sp. J260	5.72±0.34*[a]	5.33±1.11*[a]	11.05±1.45*[a]	112.30±15.76*[a]	21.35±1.9	7.21±0.14
Pantoea sp. J49	2.31±0.48	2.04±0.06	4.35±0.41	38.14±3.37	10.68±1.31	7.21±0.04
Enterobacter sp. J33	2.42±0.10	3.81±0.29*[a]	6.22±0.19*	77.39±10.23	10.23±0.78	7.23±0.03
Acinetobacter sp. L176	3.64±0.77*[a]	5.95±0.41*[a]	8.82±1.11*[a]	83.73±3.34*	14.6±1.04	7.23±0.08
Bacillus sp. J9	1.72±0.19	3.03±0.42	4.35±0.41	156.85±19.20*[a]	15.08±2.18	6.98±0.08
Bacillus sp. L55	1.51±0.05	2.69±0.38	4.21±0.43	91.99±5.78*	25.12±1.46*	7.00±0.12
Enterococcus sp. L185	4.01±0.15*[a]	3.84±0.33*[a]	7.85±0.19*[a]	119.61±9.69*[a]	14.51±0.68	6.93±0.07
Enterococcus sp. L191	3.06±0.55*[a]	5.39±0.14*[a]	8.99±0.69*[a]	116.33±6.17*[a]	9.28±0.28	7.17±0.16
Uninoculated plants	0.64±0.03	0.93±0.14	1.57±0.17	26.19±6.96	15.15±1.75	7.17±0.03
P- fertilized plants**	0.77±0.18	1.30±0.19	2.07±0.37	48.19±1.42	25.35±1.8*	7.13±0.03

Data are means±S.E., of 5–7 replicates, $p<0.05$ according to Tuckey test. * and [a] indicates statistically significant difference compared with non inoculated peanut plants and P fertilized plants, respectivelly. ** Peanut plants fertilized with KH_2PO_4 (20 mM)

3.3 Survival of phosphate solubilizing bacteria in soil

Results obtained indicated that inoculated bacteria survived in the peanut rhizosphere in a range of 10^4–10^6 CFU/g soil (Table 1), values that correspond to 0.1–10 % to initial inoculums dose (10^7 CFU/g soil). The identical ERIC/BOX-PCR profiles obtained from DNA of bacteria isolated from rhizospheric soil samples and those from the inoculated strain confirmed the presence of the PSB at the end of the experiment (Fig. 1 i.e.).

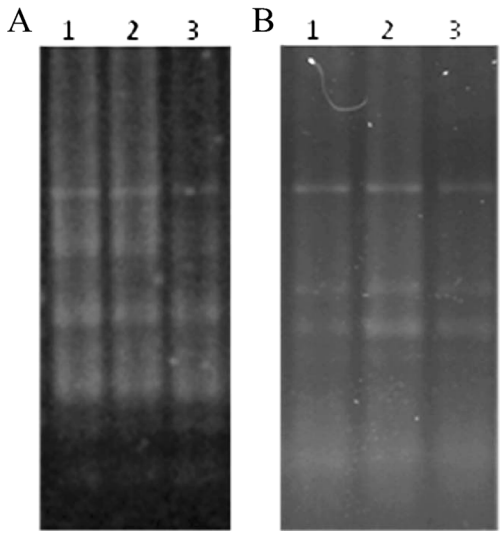

Fig. 1 ERIC-PCR profiles of DNA obtained from colonies recovered from survival assay of *Serratia* sp J21 (**a**) and *Serratia* sp J260 (**b**). Lanes 1: DNA from inoculated *Serratia* sp J21 and *Serratia* sp J260 culture; lanes 2 and 3 *Serratia* sp J21 and *Serratia* sp J260 recovered from soil

3.4 Nitrogen fixation ability of phosphate solubilizing bacteria

In vitro nitrogen fixation of phosphate solubilizing bacteria was determined. With the exception of *Enteroccous* sp. L177 and *Bacillus* sp. J255, all bacteria were able to grow in both nitrogen free media used, indicating nitrogen fixing ability. Reference strain *Azospirillum brasilense* Cd showed nitrogen fixation ability while *Bradyrhizobium* sp. SEMIA 6144 was not able to grow in either medium. A significant increase was observed in nitrogen content of aerial parts of those plants inoculated with six (*Serratia* sp. J260, *Acinetobacter* sp. L176, *Bacillus* sp. J9, *Bacillus* sp. L55, *Enterococcus* sp. L185 or *Enterococcus* sp. L191) of the eight bacterial strains that enhanced 50 % or more of the plant growth parameters analysed compared to uninoculated treatments (Table 2).

3.5 Peanut plants ability to increase P levels under hydroponic conditions

Peanut plants growing in the presence of an unavailable P source (tricalcium phosphate) significantly acidified the plant growth medium compared to plants growing with available P (Table 3). Levels of soluble P and aerial plant length estimated for both treatments did not differ.

4 Discussion

Exploitation of PSB through biofertilization is a potential strategy for making use of fixed P in the soil and natural reserves of phosphate rocks. To bring us closer to natural soil ecosystems, we analyzed the bacteria promoting effect on peanut plants growing on low P-content soil samples obtained

Table 3 Soluble P content and pH of plant growth medium and aerial length of peanut plants growing in Hoagland medium containing either soluble P or tricalcium phosphate

Peanut seedlings growing in the presence of:	Soluble P (μg/ml)	pH of medium	Aerial length (cm)
Available P source (KH_2PO_4, 20 mM)	14.00±2.96 [a]	5.77±0.1 [a]	12.38±0.39 [a]
Unavailable P source (PTC*)	19.16±3.92 [a]	4.97±0.11 [b]	11.38±0.81 [a]

Data are means±S.E., of 5–7 replicates, $p<0.05$ according to Tuckey test. Different letters indicate statistically significant difference between treatments, * PTC: Tricalcium phosphate

from the central and southern regions of Córdoba, Argentina. The beneficial effects of the bacteria was mainly observed, in plant based microcosm studies, on the aerial parts of peanut plants. Nearly 50 % of the bacterial strains used for the inoculation of peanut plants increased the number of root nodules compared to uninoculated plants. Dey et al. (2004) reported that the increase in the number of nodules in plants inoculated with PGPB could be attributed to the enhancement of root growth and root length. This enhancement provides more sites for nodulation by rhizobial strains in the soil. However, in the present study no correlation was found between the number of nodules and root length of peanut plants.

Although the majority of the bacterial strains were shown to exert their beneficial trait either by enhancing plant P content and/or by making this nutrient more available in soil, some did not enhance the plant P content under the experimental conditions used. Thus, the observed promotion of peanut growth must be due to more than an effect of bacterial activity on P solubilization. The eight bacterial strains that increased 50 % or more of plant growth parameters could grow in nitrogen free media. Moreover, an increase of aerial N content was observed in plants treated by almost all of these bacterial strains. This suggests that nitrogen fixation ability could be involved in the peanut plant growth promotion observed in this study. Alternatively, the increased nitrogen contents of the plants could also be attributed to increased N-uptake or to increased symbiotic nitrogen fixation by the nodules. In those treatments in which no significant increase in nodule number was observed it is suggested that nitrogen fixation ability of inoculated bacteria could have contributed to the increased N contents of plant aerial tissues. On the other hand, in those treatments in which a higher nodule number was observed, the presence of the bacterial inoculum could have promoted the N_2-fixing symbiosis which would explain the increased N content. Further studies are required to confirm this. Reyes et al. (2008) reported that plant inoculation with PSB can increase plant P contents and/or promote plant growth. In other studies, inoculation with PSB was shown to increase plant growth parameters without increasing plant P content (De Freitas et al. 1997; Peix et al. 2001; Fernández et al. 2007). Inoculation of different plant species with the same bacterial strain can produce different results. For instance, inoculation with PSB on lettuce did not lead to an increase in plant P content, but inoculation with these PSB on maize led to increased P levels (Chabot et al. 1996).

Serratia sp. J260, *Acinetobacter* sp. L176, *Enterobacter* sp. J33, *Bacillus* sp L55, *Enterococcus* sp. L185 and *Enterococcus* sp. L191 were found to be the most promising strains in the case of peanut. They promoted most of the plant growth parameters measured and also increased plant or soil P content. In previous studies the first three strains were shown to solubilize high and moderate levels of P and to release gluconic acid into the growth medium (Anzuay et al. 2013). Production of gluconic acid and other organic acids, probably synthesized by these bacteria, could be the main mechanism for phosphate solubilization. *Bacillus* sp. L55 was, however, found to release low levels of soluble P and gluconic acid in vitro (Anzuay et al. 2013) while *Serratia* sp. J260, *Enterobacter* sp. J33 and *Bacillus* sp. L55 were reported to produce siderophores (Taurian et al. 2010). The two other promising strains, *Enterococcus* sp. L191 and *Enterococcus* sp. L185, were known to produce low to moderate levels of soluble P and to secrete low or moderate quantity of gluconic acid (Anzuay et al. 2013). An interesting trait of these two bacteria is that they are endophytic, as they have been isolated from inside peanut root tissues (Taurian et al. 2010). It has been reported that endophytic bacteria belonging to genera such us *Bacillus, Enterococcus, Paenibacillus* and *Methylobacterium* can exert their beneficial trait directly inside the plant (Ferreira et al. 2008). Endophytism is a desirable trait for PGPB because it ensures bacterial survival (Reinhold-Hurek and Hurek 1998) and as a result they have the potential to improve plant growth (Hardoim et al. 2008). Survival of the bacteria in soil is also a desirable bioinoculant trait. It is interesting to note that the strains that caused a significant increase in plant or soil P content were found to exhibit the highest CFU per gram of soil.

The content of available P increased in almost all control and inoculated soil samples to values above 10 μg/g, which is the critical concentration for optimal peanut plant growth (Cope 1984). This enhancement in available soil P can be attributed either to the P-solubilizing activity of inoculated bacteria or to that of native microorganisms or to plant root exudates. Nevertheless, uninoculated peanut plants growing under hydroponic conditions with tricalcium phosphate did not significantly increase the P levels of the growth medium compared with plants growing with available P while a significant acidification was observed in the growth medium of these plants. Our results confirm those of Otani and Ae (1996) who found that organic acid release by peanut roots was not related to phosphate solubilization. Thus, it is possible to speculate that the enhancement in soil P observed in this study is due to the P-solubilizing activity of the bacterial inocula and to

that of native microorganisms. The fact that soil pH values remained almost constant suggests that even when PSB secrete organic acids into rhizosphere, the buffering capacity of soil particles limit changes in pH values. In conclusion, it seems clear that phosphate solubilizing microorganisms contribute greatly to P availabillty in soil and hence benefit peanut crops. (Delete last sentence as it's a repetition of the abstract)

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