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SHORT COMMUNICATION

Phosphate-solubilization activity of bacterial strains in soil and their effect on soybean growth under greenhouse conditions

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Abstract The efficiency of 13 phosphate-solubilizing bacteria (PSB; four *Burkholderia* sp., five *Enterobacter* sp., and four *Bradyrhizobium* sp.) was assessed in a soil plate assay by evaluating soil phosphorus (P) availability. A commercial argentine strain, *Pseudomonas fluorescens*, was used for comparing solubilizing activity. *Burkholderia* sp. PER2F, *Enterobacter* sp. PER3G, and *Bradyrhizobium* sp. PER2H strains solubilized the largest quantities of P in the soil plate assay after 60 days as compared with the other strains, including the commercial one. The effect of PSB inoculation on growth and nutrient uptake of soybean plants was also studied under greenhouse conditions. Plants inoculated with *Burkholderia* sp. PER2F had the highest aerial height and showed an appropriate N/P ratio. However, none of the PSB increased P uptake by plants. This suggests that PSB inoculation does not necessarily improve P nutrition in soybean, nor was there any relationship between P availability in the soil plate assay and P content in the soybean shoot in the greenhouse. We concluded that the selection of efficient PSB strains as possible inoculation tools for P-deficient soils should focus on the integral interpretation of soil assays, greenhouse experiments, and field trials.

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Introduction

Phosphorus (P) is one of the major plant nutrients, the lack of which limits plant growth. Most agricultural soils contain large reserves of total P, commonly in the range of 200 to 5,000 mg P kg^{-1} with an average of 600 mg P kg^{-1}, and a part of P accumulates depends on regular application of chemical fertilizers or sludge from wastewater treatment (Gyaneshwar et al. 2002; De-Bashan and Bashan 2004). Both P fixation and precipitation occur in soil because of the large reactivity of phosphate ions with numerous soil constituents (Rodríguez and Fraga 1999). Interest has been focused on the inoculation of phosphate-solubilizing microorganisms (PSM) into the soil so as to increase the availability of native fixed P and to reduce the use of fertilizers (Illmer and Schinner 1992).

Many phosphate-solubilizing bacteria (PSB) belonging to the *Pseudomonas*, *Bacillus*, *Rhizobium*, *Agrobacterium*, *Burkholderia*, *Achromobacter*, *Micrococcus*, *Aerobacter*, *Enterobacter*, *Flavobacterium*, and *Erwinia* genera have been isolated from soils (Rodríguez and Fraga 1999). These bacteria can grow in media containing calcium–phosphate complexes as the sole source of P, solubilize a large proportion of P, assimilate it, and release it in higher amounts. Phosphate solubilization occurs by organic acids synthesized and released by microorganisms; this release also decreases pH (Rodríguez et al. 2006; Puente et al. 2004). This reaction, shown as a halo or clear zone on the plate, is used to assess the P solubilizing activity of these bacteria. However, the reliability of this technique is

questionable when it is the only one used detecting potential PSB (Nautiyal 1999; Rodríguez and Fraga 1999).

Therefore, the objective of the present work was to assess the efficiency of different PSB strains in solubilizing P by using the soil plate assay and by monitoring soybean plants growing under greenhouse conditions.

Materials and methods

PSB strains

Thirteen soil bacteria with the ability to solubilize tricalcium phosphate in solid and liquid medium were isolated from five soils from a soybean region. The fields were located at the Pergamino department (33°52′59.6″ South, 60°35′00.3″ West) in the northern region of Buenos Aires, Argentina. Most of the strains (PER3A, PER1B, PER3C, MAN1D, PER2F, PER3G, MAN1K, MAN1L, and MAN1LL) were maintained on nutrient agar. *Bradyrhizobia* (Per2H, Per2I, Per2J, and Per2E) were maintained on yeast extract mannitol agar (Vincent 1970) and stored at 4°C. A commercial argentine bacterium, *Pseudomonas fluorescens*, was obtained from a private company, Rizobacter Argentina, S.A., and was used for the purpose of comparing solubilization activity.

To determine the taxonomic identity of the organisms, a gene region of 1,300 bp encoding 16S rDNA was amplified by polymerase chain reaction using universal 16S rDNA primers Po and P6 (Picard et al. 2000) and was sequenced. These determinations were made by LIB-Q (Universidad Nacional de Quilmes, Argentina).

Inocula preparation

Each strain was grown in 125-ml Erlenmeyer flasks containing 30 ml of nutrient broth at 28°C for 72 h. *Bradyrhizobium* sp. was cultured in Bergensen medium (Bergensen 1961) at the same temperature for 168 h. The suspensions were grown up to obtain an optical density at 620 nm wavelength of 0.6 (ca. 10^5 to 10^6 CFU ml^{-1}) and 0.9 (ca. 10^8 to 10^9 CFU ml^{-1}) using a Uv/Vis spectrophotometer (Metrolab; Buenos Aires, Argentina).

Soil type

About 70 kg of soil was sampled from the 0- to 10-cm soil layer at the experimental field of the Universidad Nacional del Sur. The soil was classified as sandy loam, termic typic Ustipsament, and had the following characteristics: silt plus clay comprised 15% of the total mineral fraction; organic C content, 9.69 g kg^{-1}; total N, 1.78 g kg^{-1}; and extractable P, 5 mg kg^{-1}. These determinations were made by LANAIS N-15 CONICET-UNS (Universidad Nacional del Sur, Argentina).

Phosphorus solubilization in soil plate assay

Fresh soil (40 g) was placed in petri dishes, and it was inoculated with 2 ml of each bacterial suspension (OD_{620nm} 0.6) at the beginning of incubation and with 1 ml after 7 days. All tests were replicated three times. Two treatments were added: uninoculated soil and uninoculated soil with the addition of 1 ml of 0.1 M $KH_2PO_4{\cdot}2H_2O$. The addition of an available source of P to one treatment was to compare a chemical input with respect to an inoculated strain. Petri dishes were incubated at 28°C in a moist chamber, and the soil was moistened with sterile distilled water. After 15 days, the soluble P was analyzed (Illmer and Schinner 1992).

According to the results, a second assay was performed with an inoculation of 4 ml of bacterial suspension (OD_{620nm} 0.9) of seven isolates from each genera. A second inoculum (1 ml) was added once a week for the first 30 days. Petri dishes were incubated at 28°C in a moist chamber for 90 days. Soluble P was analyzed at 30, 60, and 90 days.

Mobilization of phosphorus to soybean plants

According to the results, isolates PER2F, PER3G, PER2H, and the commercial bacterium (*Pseudomonas*) were selected for the greenhouse assay. Twenty-five soybean seeds (*Glycine max* L. Merrill, cv. Nidera 4423) were sterilized with sodium hypochlorite (25 g l^{-1}) followed by six rinses in sterile distilled water. The seeds were placed in a sterile glass and mixed with 4 ml of the inoculant preparation (OD_{620nm} 0.9). Five inoculated seeds were sown in each pot (180×160 mm), which had been previously filled with 2 kg of soil and five replicates (pots) per each bacterial inoculum were made. There were two controls as follows: uninoculated soil/seeds and uninoculated soil/seeds with the addition of 10 ml of 0.1 M $K_2HPO_4{\cdot}2H_2O$ during the V1 and V3 vegetative growth phases. In addition, these controls were replicated five times.

Plants were grown in the greenhouse for a 100-day period under natural temperature (average day, 21±3°C and average night, 11±1°C) and light conditions (140 μEm^{-2} s^{-1}). One milliliter of the inocula preparation was added per plant by a micropipette at 30, 60, and 90 days. N amendment was carried out 5 days after planting by adding 0.1 M of NH_4NO_3 (except for *Bradyrhizobium* sp. treatment). Plants were watered when required with sterile distilled water. At harvest, height, dry weight, P, and N content of the aerial part of soybean plants were determined.

Analytical methods

Soil phosphate-solubilization estimations were carried out by the molybdenum blue method (Murphy and Riley 1962). The total N and P content in soybean plants was determined

using an auto analyzer equipment (Biichi Distillation Unit, Switzerland) after digestion in nitric and perchloric acid (Johnson and Ulrich 1959).

Statistical analysis

The assays were carried out in a completely randomized design, and the data were analyzed statistically with the analysis of variance. When a significant F value was detected, least significant difference (Fisher LSD) was used (Steel and Torrie 1997).

Results

The isolates described as phosphate solubilizers were identified as strains of *Burkholderia* sp. PER3A, PER3C, MAN1D, and PER2F; *Enterobacter* sp. PER3G, MAN1L, MAN1LL, PER1B, and MAN1K; and *Bradyrhizobium* sp. PER2H, PER2I, PER2J, and PER2E according to 16S rDNA sequencing. It is well known that many bacterial species from these genera are able to exert a beneficial effect upon plant growth as extensively studied.

In the first experiment, soluble P was measured in the soil plate assay after 15 days of incubation. A maximum available P of 60 mg P Kg^{-1} soil was recorded in the soil with soluble phosphate. Inoculation with isolates *Burkholderia* sp. PER3C, MAN1D, and PER2F and *Enterobacter* sp. PER3G, MAN1K, and MAN1L increased significantly ($P \leq 0.05$) available P in the soil (up to 8.25- to 9-mg P Kg^{-1} soil) with respect to the uninoculated soil (control; 6.4-mg P Kg^{-1} soil). However, none of the four isolates of *Bradyrhizobium* or the commercial strain tested was able to solubilize statistically more inorganic P than that present in the control.

A second soil plate assay lasting 90 days was performed with two controls (uninoculated and incubated soil and uninoculated and not incubated soil) and testing of *Burkholderia* and *Enterobacter* genera and 1 *Bradyrhizobium* isolates. The results of phosphate solubilization by these strains after 90 days are presented in Table 1. After 30 days of incubation, the PER2F, PER3G, MAN1K, and MAN1D isolates showed significantly higher values of phosphate solubilization (over 10-mg P Kg^{-1} soil) than the controls and the rest of the studied strains. After 60 days, all the strains, with the exception of MAN1L, solubilized from 11.6 to 15.4 mg P Kg^{-1} of soil, values statistically higher than those of both controls. Soil soluble P continuously increased during the 60-day incubation period with PER3G, PER3C, and PER2H strains. After 90 days, solubilization of soil P by all the studied strains was statistically higher than the value of the controls. However, MAN1D, PER2F, PER2H, and PER3G strains solubilized less quantities of P at 90 days than in the 30- and 60-day incubation periods.

Table 1 Evaluation of the P solubilization (mg Kg^{-1} soil) by seven PSB strains after 30, 60, and 90 days in a soil plate assay at 28°C in a moist chamber

	Incubation period (days)		
	30	60	90
Control 1*	5[a]±0.02[b] a[c]	5±0.02 a	5±0.02 a
Control 2**	8.1±0.22 b	6.6±0.22 a	7.1±0.06 b
Enterobacter			
PER3G	10.5±0.27 cd	14.5±0.27 bc	10.7±0.05 c
MAN1K	11.9±0.13 d	12.1±0.97 bc	11.7±1.15 c
MAN1L	9.5±0.18 bc	8±0.37 a	11.9±0.30 c
Burkholderia			
MAN1D	14.2±0.14 e	14.2±0.12 bc	10.8±0.08 c
PER2F	14.5±0.12 e	15.4±0.33 c	10.7±0.10 c
PER3C	8.06±0.27 b	11.6±0.13 b	11.7±0.08 c
Bradyrhizobium			
PER2H	9.33±0.10 bc	14.6±0.20 bc	11.3±0.17 c

*Control 1: uninoculated and not incubated soil
**Control 2: uninoculated and incubated soil
[a] Available P (mg Kg^{-1} soil)
[b] Data are average values of three replicates±SE
[c] Means with different letters in the same column differ significantly at $P \leq 0.05$ according to the Fisher LSD

The results of P mobilization in pots with soybean plants are presented in Table 2. Under greenhouse conditions, plants inoculated with *Burkholderia* sp. PER2F had the highest aerial height, which were 40 and 60% greater than that of uninoculated soil/seed and that of uninoculated soil/seed treated with soluble P, respectively. Although visual appearance of better root density and integral crop development (number of nodes in the main stem, leaf area, and soybean vegetative aerial development) were observed in plants inoculated with *Burkholderia* sp. PER2F, no statistically significant differences were found in aerial dry weights between treatments. Furthermore, there were no differences in shoot N content, but plants inoculated with *Burkholderia* sp. PER2F exhibited more favorable contents. Only in plants fertilized with soluble P was the P content higher than that of plants grown in uninoculated soil and soil with inoculated seeds. Plants inoculated with *Burkholderia* sp. PER2F showed an N/P ratio of 11.3, whereas the plants of other treatments including both controls exhibited a ratio lower than 8.8.

Discussion

According to the results, PSB strains could be differentiated in two groups with different mineral phosphate-solubilizing activity (Fig. 1): the group A included both *Enterobacter* and *Burkholderia* sp. strains, whereas group B included *Bradyrhizobium* sp., *Burkholderia* sp., and *Enterobacter* sp.

Table 2 Plant growth estimated by aerial height and N and P contents of soybean (*Glycine max* L. Merrill) shoots

Inoculation treatment	Aerial height (cm)	Aerial dry weight (g)	Shoot P content (mg g^{-1})	Shoot N content (mg g^{-1})
Control 1*	38,06±0,24[a] a[b]	7,58±0,34 a	0,84±0,09 a	7.42±0,03 a
Control 2**	33.41±1.33 a	7.07±0.24 a	1.88±0.04 b	7.94±0.30 a
Pseudomonas fluorescens	42.68±1.46 ab	6.94±0.19 a	1.02±0.01 a	8.92±0.43 a
Enterobacter sp. Per3G	42.69±1.35 ab	8.42±0.29 a	0.95±0.01 a	7.95±0.18 a
Burkholderia sp. Per2F	53.47±3.61 b	7.51±0.37 a	0.89±0.03 a	10.1±0.47 a
Bradyrhizobium sp. Per2H	34.10±0.85 a	7.25±0.29 a	1.13±0.02 a	7.41±0.17 a

Seeds were inoculated with *Burkholderia* sp. PER2F, *Enterobacter* sp. PER3G, *Pseudomonas fluorescens* (a commercial bacterium), or *Bradyrhizobium* sp. PER2H and grown under greenhouse conditions.
*Control 1: uninoculated soil/seeds
**Control 2: uninoculated soil/seeds with the addition of 10 ml of 0.1 M $K_2HPO_4 \cdot 2H_2O$
[a] Data are average values of five replicates±SE
[b] Means with different letters in the same column differ significantly at $P=0.05$ according to the Fisher LSD

The bacteria of group B solubilized greater amounts of P than the bacteria of group A, and solubilization levels decreased at the end of the experiment. It is well known that P dynamics can be affected by microorganisms in different ways as follows: (1) an increase in PSM biomass as result of rhizodeposition can increase solubilization in the rhizosphere (Gyaneshwar et al. 1998); (2) the release of P once microbial cells die can occur; microbial biomass can immobilize from 1 to 10% of the total soil P (Richardson 2001); and (3) mineralization of organic P to inorganic P can occur through phosphatase activity (Oehl et al. 2001).

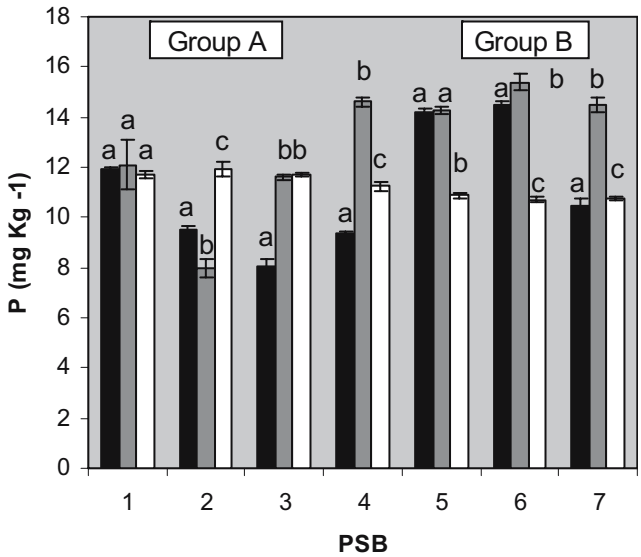

Fig. 1 Amounts of soluble P (mg Kg $soil^{-1}$) in the soil plate assay inoculated with seven phosphate-solubilizing bacterial strains (*PSB*) after 30 (*darkbars*), 60 (*greybars*), and 90 (*whitebars*) days of incubation at 28°C in a moist chamber. The *bar* represents the standard error ($n=3$). *Bars* labeled with the same letter were not significantly different (Fisher LSD $P \leq 0.05$). The PSB strains were differentiated in two groups with a different mineral phosphate-solubilizing activity. *Group A 1 Enterobacter* sp. MAN1K, *2 Enterobacter* sp. MAN1L, and *3 Burkholderia* sp. PER3C; and *Group B 4 Bradyrhizobium* sp. PER2H, *5 Burkholderia* sp. MAN1D, *6 Burkholderia* sp. PER2F, and *7 Enterobacter* sp. Per3G

Our results are similar to those reported for growth chamber and greenhouse experiments. Peix et al. (2001) observed an increase in growth and yield parameters of barley and chickpea inoculated with *Mesorhizobium mediterraneum*, a PSB strain; however, the P content of plants was higher when soil was treated with soluble phosphate than when seeds were inoculated with PSB. De Freitas et al. (1997) observed that *Bacillus* and *Xanthomonas*, both PSB, significantly increased the height and biomass of canola but not the P content of the plants with respect to uninoculated plants. Therefore, PSB can stimulate plant growth through other mechanisms than P uptake.

Plants inoculated with *Burkholderia* sp. PER2F showed an N/P ratio of 10–11 (Fageira et al. 1997). It is important to underline that in soybean leaves, the ratio can increase during the flowering stage (Blevins 1989).

Rodríguez and Fraga (1999) reported that the plate halo screening method produced contradictory results. However, this method can be regarded as generally reliable for isolation and preliminary characterization of PSM. Gyaneshwar et al. (1998) found that some PSM showing phosphate-solubilizing ability under laboratory conditions were not able to release P when inoculated into alkaline vertisols even when supplemented with other nutrients. Probably, this was because of the high buffering capacity of the alkaline soils coupled with the inability of bacteria to secrete high concentrations of organic acids (Gyaneshwar et al. 2002). It has been estimated that the amount of P released in the medium without a buffer ranged from 3 to 24% of the total P; whereas with a buffer, the values ranged from 0.07 to 4.82% (Fernández et al. unpublished data). Therefore, it would be better to test PSM under conditions resembling those occurring in soil.

Generally, the availability of P in soil increases with PSB inoculation. Illmer and Schinner (1992) observed an increase in available P in soils inoculated with *Pseudomona*

and *Penicillium* isolates, and this increase was almost as high as that of the respective soil amended with 0.007 M of $KH_2PO_4 \cdot 2H_2O$. Kucey et al. (1989) measured increased levels of available P in soils that had been amended with farmyard manure, rock phosphate, and PSM isolates of *Bacillus*, *Streptomyces*, *Penicillium*, and *Aspergillus* sp. As in this work, they used a simple soil plate assay more suitable for screening PSM strains than the method based on the production of halo or clear zone on the plate.

In this work, no relationship was found between P availability estimated by the soil plate assay and the P content of soybean plants grown in greenhouse and inoculated with PSB. The ability of an inoculated strain to supply P to plants may be affected by the following: (1) degradation of the compounds released by PSB to solubilize P and (2) by precipitation of the solubilized P before it can reach the root surface (Barea et al. 2005).

In conclusion, the efficiency of 13 PSB in solubilizing phosphate has been studied by the soil plate assay and by the monitoring of the growth of inoculated soybean plants under greenhouse conditions. Strains of *Burkholderia* sp. PER2F, *Enterobacter* sp. PER3G, and *Bradyrhizobium* sp. PER2H solubilized higher quantities of P in the soil plate assay after 60 days than the other strains. Under greenhouse conditions, soybean plants inoculated with *Burkholderia* sp. PER2F had the highest aerial height and the most suitable N/P ratio. However, none of the PSB strains increased the P content of plants. This suggests that PSB inoculation does not necessarily improve P uptake in soybean plants. The selection of an efficient PSB as possible inoculants for P-deficient soils should be based on the combination of soil assays and greenhouse and field experiments.

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