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ORIGINAL ARTICLE

Characterization of phosphate-solubilizing bacteria exhibiting the potential for growth promotion and phosphorus nutrition improvement in maize (*Zea mays* L.) in calcareous soils of Sinaloa, Mexico

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Abstract Greenhouse bioassays were used to examine the ability of selected strains of the rhizobacteria *Sinorhizobium meliloti*, *Bacillus flexus* and *B. megaterium* to solubilize phosphorus (P) and to affect growth promotion and phosphorus nutrition in maize. These bacterial strains were found to decrease the pH and solubilize some forms of insoluble P, such as tricalcium phosphate and hydroxyapatite, as well as to exhibit acid and alkaline phosphatase enzymatic activities in culture medium, properties that are possibly involved in P solubilization. Inoculation of the strains separately and as a consortium of the three bacteria (*S. meliloti*, *B. flexus* and *B. megaterium*) in P-deficient soil (4.33 w/v P) fertilized without P improved plant height, shoot and root dry weight, as well as P nutrition in the maize plants. Use of the *B. flexus* and *B. megaterium* strains separately and in a consortium positively affected several growth parameters and P nutrition in plants supplemented with insoluble P. No effect was observed when pots in which the seedlings were growing were supplied with soluble fertilizer. A second assay using a P-deficient soil (6.64 w/v P) showed that inoculation with the consortium of *B. flexus* and *B. megaterium* significantly increased growth and total P content in maize plants. A dose–response P fertilization experiment using sterile P-deficient soil led us to conclude that inoculation to soil of the mixture of *B. flexus* and *B. megaterium* may improve P nutrition and growth to a level previously attained by the addition of soluble P-fertilizer at 40 w/v P. A non-sterile experiment showed a beneficial response with *B. megaterium* but not with *B. flexus*. We propose utilizing these bacteria in P-deficient alkaline soils in future field trials in order to evaluate their potential as biofertilizers.

Keywords Phosphate-solubilizing bacteria · Growth promotion · Phosphorus nutrition · Maize

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Introduction

Maize (*Zea mays* L.), an integral part of the Mexican culture and diet since antiquity, is the most important cereal crop in Mexico. Over half of the country's cultivated surface is dedicated to maize cultivation, with Sinaloa state leading the country in maize production (SIAP-SAGARPA 2016). A negative consequence of this intense cultivation practice is that high yields in maize production have required intensive phosphorus (P) fertilization (Tilman et al. 2002;Harvey et al. 2009). This has in turn contributed to the nutrient enrichment of water bodies, causing eutrophication and toxic algal blooms (Smith and Schindler 2009). Intensive P fertilization regimes also affect microbial diversity, leading to the loss of soil fertility and, consequently, a decrease in crop yield. Finally, estimates indicate that high-quality P sources are becoming increasingly limited (Gilbert 2009), while low-quality rock P is widely available.

Plants can only utilize 20–30% of the P in phosphate fertilizers applied to agricultural soils due to the reactivity of P with other cations (such as calcium in calcareous soils), which causes the rapid mineralization and insolubilization of P in the soil (Bashan et al. 2013). According to the soil taxonomy of the Food and Agriculture Organization of the United Nations

(FAO) classification, the soil types in Northern Sinaloa soil are vertisol, feozem and cambisol, and they are characterized by an alkaline pH ranging from 7.5 to 8.4 (Ramírez Soto et al. 2010). This results in the mineralization of P in insoluble compounds such as dicalcium or tricalcium phosphate (TCP) and hydroxyapatite (Bashan et al. 2013; Shen et al. 2011).

These problems linked to the use of P fertilizers have promoted the search for environmentally friendly alternative strategies that can improve crop production in low-P or P-deficient soils. Applying microbial inoculants or biofertilizers that have P-solubilizing activities to agricultural soils is considered to be an environmentally friendly alternative that can avoid/decrease the use of conventional P fertilizers (Sharma et al. 2013; Zaidi et al. 2009). However, a P source is still needed for these bacteria to function, and this source can be low-grade rock phosphate.

One such alternative approach for sustainable agriculture could use phosphate-solubilizing bacteria (PSB) to satisfy the P requirements for plant growth (Lavakush et al. 2014; Richardson et al. 2011). The mechanisms used by PSB to convert P-insoluble forms into available P-soluble forms involve acidification, chelation, oxidation-reduction reactions and secretion of strong organic acids (Bashan et al. 2013; Chen et al. 2006; Young et al. 2013), as well as the synthesis of several phosphatase enzymes (Richardson 2001). The application of PSB to the soil can improve the availability of soil P, the absorption of nutrients (N and K) and root development (Lopez-Arredondo et al. 2014). PSB application may also enhance plant growth through other mechanisms, such as symbiotic nitrogen fixation, aminocyclopropane-1-carboxylate deaminase activity, growth control of phytopathogenic microorganisms and the production of ammonia, siderophores and phytohormones (Bashan and de-Bashan 2010; Pereira and Castro 2014; Vessey 2003).

Sinorhizobium meliloti, *Bacillus flexus* and *B. megaterium* are native maize rhizospheric bacterial isolates from northern Sinaloa, Mexico. In a recent study these *Bacillus* strains were observed to exhibit antagonistic activity against *Fusarium verticillioides* (*Fv*) in vitro (Figueroa-López et al. 2016). In an earlier study, the application of *B. megaterium* B5 in field trials demonstrated that this strain is capable of reducing the incidence (by 75.1%) and severity (by 30%) of maize ear rot, in addition to increasing grain yield (Lizárraga-Sánchez et al. 2015). Nevertheless, these strains must still be tested in greenhouse and field trials to demonstrate their ability to perform as P solubilizers. The aim of this study was to characterize the phosphate solubilization of these strains in greenhouse bioassays and to evaluate their potential as biofertilizers for use in the promotion of growth and improvement of P nutrition in maize plants growing in P-deficient soil. Our hypothesis was that when bacterial strains possessing the ability to solubilize phosphate are tested under greenhouse conditions they should improve P nutrition in and growth of maize plants.

Materials and methods

Microorganisms

Bacillus flexus, *B. megaterium* and *Sinorhizobium meliloti* strains were used. Bacterial isolates were obtained from maize rhizospheric soils of northern Sinaloa, Mexico. Strains were identified based on their 16S rDNA gene sequence, as reported in Figueroa-López et al. (2016). The *Bacillus* strains were obtained from microorganisms maintained in a preliminary collection (CIIDIR-003; CIIDIR-Sinaloa, Mexico) and were stored at −70 °C in Luria Bertani (LB) broth with glycerol (15%, v/v). The *Sinorhizobium* strain was obtained from a collection that was specifically made to select for bacteria that can solubilize TCP [Ca_3 $(PO_4)_2$] on solid medium (Fierro-Coronado, unpublished results) as a first rough indicator of potential P solubilization. In this work, the *S. meliloti* strain was molecularly identified based on 16S rDNA gene sequencing (GenBank accession number KU230303). Strains were stored at −70 °C in peptone–yeast (PY) medium [5 g L^{-1} peptone and 3 g L^{-1} yeast extract with 10 ml L^{-1} $CaCl_2$ (0.7 M sterile solution added after sterilizing the culture medium)] with glycerol (15%, v/v). The three strains (*S. meliloti*, *B. flexus* and *B. megaterium*) were tested for their ability to solubilize TCP using the qualitative P-solubilization plate method as reported by Figueroa-López et al. (2016). After testing the ability of the strains to solubilize TCP solubilization in solid medium, their ability to solubilize hydroxyapatite was tested in liquid culture, as described in section P solubilization in liquid culture.

Phosphate solubilization in solid medium

Solid Pikovskaya medium (per liter: 10 g glucose; 0.5 g yeast extract; 5 g TCP; 0.5 g $MgCl_2 \cdot 6H_2O$; 0.2 g KCl; 0.2 g $NaCl_2$; 0.1 g $MgSO_4 \cdot 7H_2O$; 15 g bacteriological agar) was used to qualitatively assess the ability of bacterial isolates to solubilize phosphate. The final pH was adjusted to 7.00 ± 0.02. The strains were inoculated onto the solid agar plates and the plates incubated at 30 °C for 10 days. The experiment was performed twice using three plates each time. Phosphate solubilization was assessed by measuring the clear zone (area of P solubilization) surrounding each bacterial colony. The phosphate solubilization index was calculated using the formula: (colony diameter + halo zone diameter)/colony diameter.

P solubilization in liquid culture

Quantitative analysis of P solubilization was performed using 250-mL Erlenmeyer flasks containing 50 mL of Pikovskaya medium. Final bacterial cell suspension concentrations were adjusted to a cell density of approximately

3.0×10^7 CFU mL^{-1}. Three flasks containing Pikovskaya medium were inoculated with each presumptive isolate (100 μL inoculum with 3.0×10^7 CFU mL^{-1}) and incubated at 30 °C on a rotary shaker (200 rpm); pH and soluble P were measured at different time intervals (0, 1, 2, 3, 5, 7 and 10 days). Experiments were conducted twice per each isolate. The bacterial strains were assessed separately with two different sources of insoluble phosphate: TCP and hydroxyapatite [$Ca_5(PO_4)_3(OH)$]. After the predefined incubation period, the cultures were harvested by centrifugation at 2300 *g* for 15 min. Sterile, non-inoculated medium served as the control. The amount of soluble inorganic phosphate (Pi) remaining in the culture supernatant was measured using the phosphomolybdate blue colorimetric method (Murphy and Riley 1962). The amount of released Pi was calculated based on a standard KH_2PO_4 curve (Sigma-Aldrich; St. Louis, MO). Sample pH was measured in the bacterial supernatant using a pH meter (model 223; Hanna Instruments; Woonsocket, RI).

Phosphatase activity assays

Acid and alkaline phosphatase activities of the bacterial isolates were determined using a modified assay by Juma and Tabatabai (1988). Briefly, 100 μL of culture supernatant obtained by centrifugation of the bacterial cultures (2300 *g*, 15 min) was incubated at 37 °C with 100 μL of 25 mM *p*-nitrophenyl phosphate and 400 μL of modified universal buffer pH 5 (Öhlinger et al. 1996). After 1 h, the reaction was terminated by adding 100 μL $CaCl_2$ (0.5 M) and 400 μL $CaCO_3$ (0.5 M). After incubation, the absorbance was read at 410 nm, and the amount of product obtained in the reactions was determined by extrapolation using a *p*-nitrophenol (*p*-NP) calibration curve (μmol *p*-NP mL^{-1}). Each bacterial isolate was assayed enzymatically in two independent experiments with three biological replicates (three flasks) after overnight incubation (16 h) at 30 °C and 200 rpm.

Greenhouse pot assays

The soil used in the pot experiments was collected from a maize field in northern Sinaloa. Soil was milled, sieved (<2 mm) and mixed with vermiculite to a 1:3 ratio (v/v) and sterilized three times by Tyndallization (121 °C for 60 min on 3 consecutive days with subsequent drying at room temperature). After sterilization, the soil physico-chemical properties were measured as: pH 7.8, conductivity 0.19 mmhos cm^{-1}, organic matter 0.95% and (in w/v) NO_3 250, P Olsen 4.33, K 547, Ca 7876, Mg 1507, Na 161, Fe 10.36, Cu 5.02, Zn 2.30 and Mn 4.25. The greenhouse assay was based on a completely randomized design with three phosphate treatments: (1) control without P fertilization; (2) fertilized with soluble P (NPK 12/61/00); (3) fertilized with insoluble phosphate (TCP). This design also included five types of inoculation: (1) control without bacteria and (2) inoculated treatments with *S. meliloti*, *B. flexus*, *B. megaterium*, and a consortium of *S. meliloti*, *B. flexus* and *B. megaterium*, respectively. Commercial white maize hybrid seeds (DeKalb DK-2038; Guasave, Mexico) were disinfected using a hydrothermal treatment. Seeds were immersed in a Tween-20 solution (five drops of Tween 20 per 100 mL of sterile distilled water) and sonicated for 5 min. The Tween solution was then decanted and the seeds immersed in a 0.75% sodium hypochlorite solution and placed in a thermo-bath at 52 °C for 20 min. Finally, the seeds were washed three times in sterile distilled water and allowed to air-dry in a laminar flow hood (Leyva-Madrigal et al. 2015). For seed inoculation, *Bacillus* strains were grown in LB medium, whereas *S. meliloti* was grown in PY broth. Cells in the exponential phase of growth were harvested by centrifugation at 1000 *g* for 5 min. Bacterial inoculum was prepared by re-suspending pelleted cells in either LB or PY broth for *B. flexus* and *B. megaterium* strains and *S. meliloti*, respectively. The rhizobacteria inoculum density was adjusted to 2.0×10^8 CFU mL^{-1}. Seeds were pre-germinated for 5 days in petri dishes with water-agar, and seeds showing contamination even after disinfection were discarded (< 3%). Germinated seeds were immersed for 1 h in each of the bacterial suspensions (*S. meliloti*, *B. flexus*, *B. megaterium*) or a mix of the three strains, adjusted to a final concentration of 2.0×10^8 CFU mL^{-1}). The seeds were then planted in pots containing 1 kg soil/pot. Bacterial suspensions (5 mL/pot = 1×10^9 CFU) were also inoculated onto the surface of the soil and on top of the maize seeds at the time of planting. Two seeds were planted per pot; later, at the time of seedling emergence, one of the seedlings was removed so that there was one seedling per pot. This (1 seedling/pot) was considered to be an experimental unit, and five replicates per treatment were set up in a completely randomized design for a total of 75 plants. This experiment was repeated twice independently. Five days after maize seedling emergence, a second bacterial inoculation was performed, in which 5 mL of bacterial inoculum (1×10^9 CFU) was added per pot at the same concentration as used previously. Pots were placed in a controlled greenhouse (photoperiod 12 h, temperature range 28–30 °C, relative humidity range 60–75%). Plants were watered twice per week with 150 mL distilled water in order to reach substrate field water capacity. Depending on the P treatment scheme, different P fertilization regimes containing various fertilizers were applied to the pot substrates. Only N was applied in the treatment that did not receive any P fertilization, in the form of 0.33 g of urea (NPK 46/00/00). In the treatment for insoluble phosphate fertilization, P was added in the form of TCP (0.215 g TCP kg^{-1} of substrate), and N was supplemented in the form of urea, as described in the previous treatment.

In the treatment with soluble phosphate fertilization, 0.164 g of an NPK fertilizer (12/61/00) and 0.29 g of urea were added. Equivalence between both nutrients was maintained in all treatments: N and P were supplemented to reach 330 w/v of N and 160 w/v of P (soluble or insoluble).

Greenhouse pot assay using different P fertilization doses

Following the evaluation of the greenhouse assay described in the previous section, both *Bacillus* strains were selected based on their potential to promote growth and to increase P content in maize tissues; in contrast, *S. meliloti* was not selected for further use. Therefore, for the greenhouse pot assay described here, we designed a dose–response experiment that contained a control without bacteria, and treatments were inoculated with *B. flexus* and *B. megaterium* both separately and as a mixture. Fertilization doses were established at 0, 40, 80 and 120 w/v P. Substrate analysis for this experiment indicated the following physico-chemical properties: pH 7.8, conductivity 0.16 mmhos cm^{-1}, organic matter 0.98% and (in w/v) NO_3 25, P Olsen 6.64, K 581, Ca 9018, Mg 998, Na 168, Fe 11.4, Cu 4.60, Zn 2.00 and Mn 5.5. A completely randomized design with four treatments and four plants per treatment was utilized. The amounts used to fertilize each pot in each fertilization treatment were as follows (per kg of substrate): treatment (1) 0 g P, 0.33 g urea; treatment (2) 0.040 g P, 0.032 g urea; treatment (3) 0.080 g P, 0.031 g urea; treatment (4) 0.120 g P and 0.30 g urea. N concentration was established at 330 w/v, as in the first pot assay. NPK (12/61/00) was used as the P fertilizer, and urea (NPK 00/46/00) was used as the N fertilizer. A sterile soil/vermiculite mix (1:3 ratio, v/v) was used for this last experiment. At the end of the experiment, the available P remaining in the substrate of all treatments was determined according to the method described by Olsen et al. (1954). In a separate trial, the effect of bacterial inoculation was evaluated in non-sterile substrate fertilized without P (0 g P, 0.33 g urea). All other assay conditions were as described in preceding Materials and Methods subsections. Both experiments, the one performed under sterile conditions and the one performed under non-sterile substrate conditions, were repeated twice independently.

Plant sampling and analysis

Plants were harvested after 30 days and their roots thoroughly washed in tap water and deionized water. Shoot and root biomass and shoot height were then recorded. The dry biomass of shoots and roots was determined after they were oven dried at 70 °C for 72 h. Oven-dried tissues were finely ground, and tissue (0.5 g) was digested according to Kirkpatrick and Bishop (1971). The digested samples were used to determine the total P concentration in shoots by the phospho-vanadomolybdate colorimetric method (Ryan et al. 2007).

Statistical analysis

Data from greenhouse pot assays were subjected to two-way analysis of variance using SAS 9.0 (SAS Institute, Cary, NC). Greenhouse pot assays displaying different rates of P fertilization data were analyzed using a generalized linear model. Fisher's least significant difference test was used for the post hoc comparison of means. Correlations were performed between different variables, and Pearson's correlation coefficients were determined using the Statgraphics Centurion XVI.I statistical package (Statpoint Technologies, Inc., Warrenton, VA).

Results

Qualitative and quantitative assessment of phosphate solubilization

The bacterial strains tested differed in their ability to solubilize TCP, based on the formation of clear halos around colonies growing on Pikovskaya agar medium. Specifically, *S. meliloti* presented a solubilization index of 3.05. In contrast, the *Bacillus* strains did not produce any clear zone surrounding the colonies on this medium, even though these bacteria have previously been shown to dissolve TCP on Pikovskaya agar (Figueroa-López et al. 2016).

To verify that these strains had not lost their ability to solubilize TCP, we performed an experiment in liquid Pikovskaya medium. The amount of soluble Pi and changes in pH were monitored for 10 days in Pikovskaya broth (Fig. 1). Bacterial inoculation significantly increased TCP solubilization and caused a significant drop in pH by all strains. The non-inoculated control remained at the initial pH, and no P solubilization was recorded. *Sinorhizobium meliloti* had solubilized up to 592.85 μg P mL^{-1} medium at day 10 and decreased the pH to 4.48. The maximum P solubilization shown by *Bacillus* strains was 207.24 μg mL^{-1}, with a final pH of 4.85. This result indicates that the *Bacillus* strains had not lose their ability to solubilize phosphate. Moreover, the results show a clear negative correlation between the soluble P concentration and pH (*S. meliloti*, $r = -0.876$; *B. flexus*, $r = -0.812$; *B. megaterium*, $r = -0.760$; $P < 0.05$).

Both *S. meliloti* and *B. megaterium* were able to solubilize hydroxyapatite, which as a P source is more insoluble than TCP (Fig. 1). Furthermore, these strains demonstrated a negative correlation between hydroxyapatite solubilization and pH reduction in the culture medium (*S. meliloti*, $r = -0.774$, $P < 0.05$; *B. megaterium*, $r = -0.766$, $P < 0.05$). The *B. flexus* strain was unable to solubilize hydroxyapatite in liquid medium, even when a decrease in pH occurred.

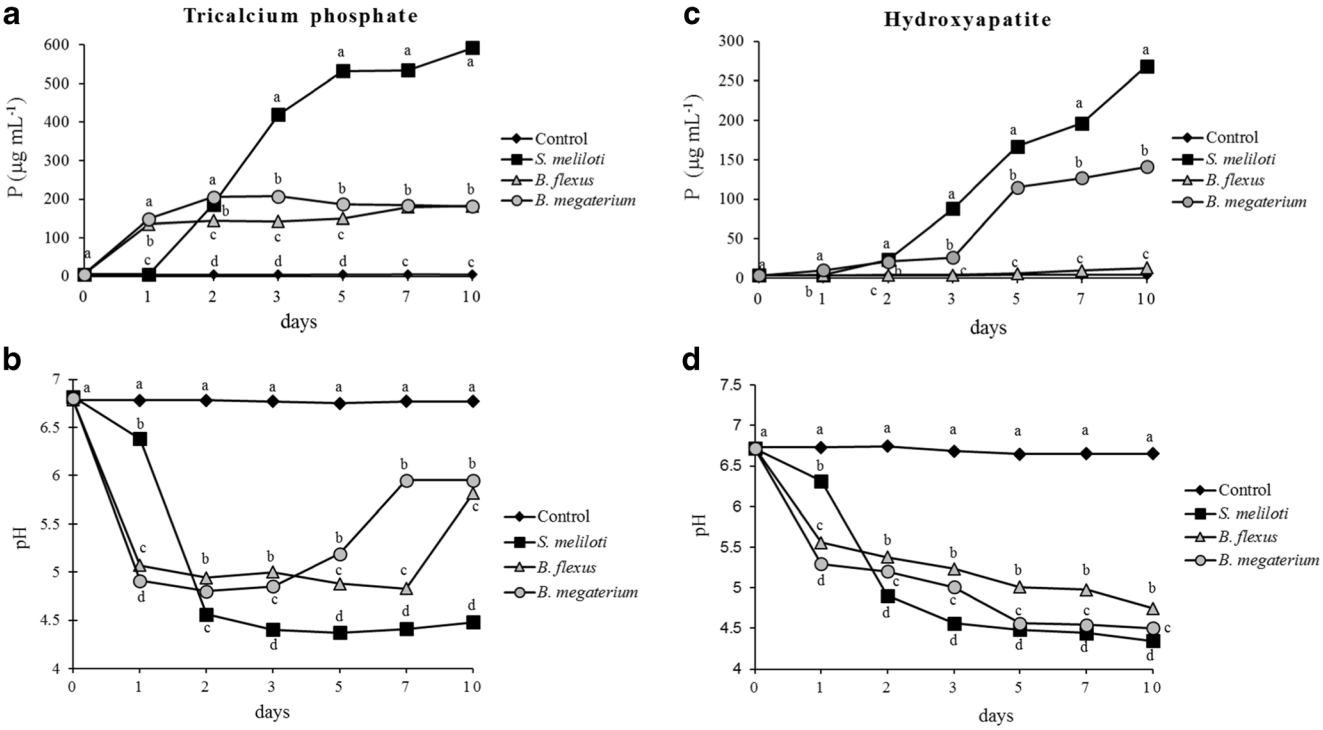

Fig. 1 Solubilization of different types of insoluble phosphate forms by the rhizobacteria strains *Sinorhizobium meliloti*, *Bacillus flexus* and *B. megaterium*. **a**, **b** Changes in solubilized tricalcium phosphate [TCP; $Ca_3 (PO_4)_2$] levels in liquid culture of the tested strains (**a**) and the accompanying changes in the pH of the TCP-containing medium (**b**). **c**, **d** Changes in solubilized hydroxyapatite levels in liquid culture of the tested strains (**c**) and the accompanying changes in the pH of the hydroxyapatite-containing medium (**d**). Different letters above the bars indicate significant differences at $P < 0.05$ between treatments according to the Tukey least significant difference test

Phosphatase activity

Alkaline and acid phosphatase activities for the two *Bacillus* strains were detected in the culture medium (Fig. 2). Alkaline phosphatase activity was lower than that of acid phosphatase in *B. flexus* and *B. megaterium* and required more time to reach its peak activity (144 vs. 48 h, respectively). At 48 h, acid phosphatase activity was 2.4- to 3.0-fold higher than that of alkaline phosphatase in all strains. *Sinorhizobium meliloti* showed significantly lower both phosphatase activities than the *Bacillus* strains.

Influence of rhizobacteria inoculation on *Z. mays* growth and P nutrition in greenhouse assays

In the P-deficient substrate (without no P fertilizer), all bacterial treatments significantly promoted plant height and root biomass, whereas treatments that included *B. flexus* increased biomass and total P shoot content relative to the control (Fig. 3). Plant height and total P shoot content increased significantly upon fertilization with insoluble P, relative to the control, when plants were inoculated with *B. flexus*, *B. megaterium* and the bacterial consortium (*S. meliloti*, *B. flexus* and *B. megaterium*); inoculation with *B. megaterium B5* or the consortium also enhanced production of shoot biomass (Fig. 3). No significant differences were observed in any of the analyzed parameters when maize plants were fertilized with soluble P [Electronic Supplementary Material (ESM) Table S1]. This experiment was repeated and the results found were as follows: in substrate without P fertilization, the presence of *B. flexus* and *B. megaterium* promoted growth and increased total P content in the plant tissue (ESM Table S2). Bacterial inoculation with strains *B. flexus* and *B. megaterium* of a substrate fertilized with insoluble P had a beneficial effect on plant height, shoot dry biomass and shoot total P content of the parameters evaluated with respect to the inoculated control (ESM Table S3). The data shown in ESM Table S4 confirmed that when maize plants growing in a P-deficient substrate were fertilized with soluble P, the addition of these bacterial strains had no significant effect.

Evaluation of inoculation by *Bacillus* strains and the bacterial mixture on maize plants in pot assays using different fertilization doses

Based on the results obtained in the previous greenhouse assay, we selected the *Bacillus* strains for further characterization of their potential to promote maize growth and improve P nutrition.

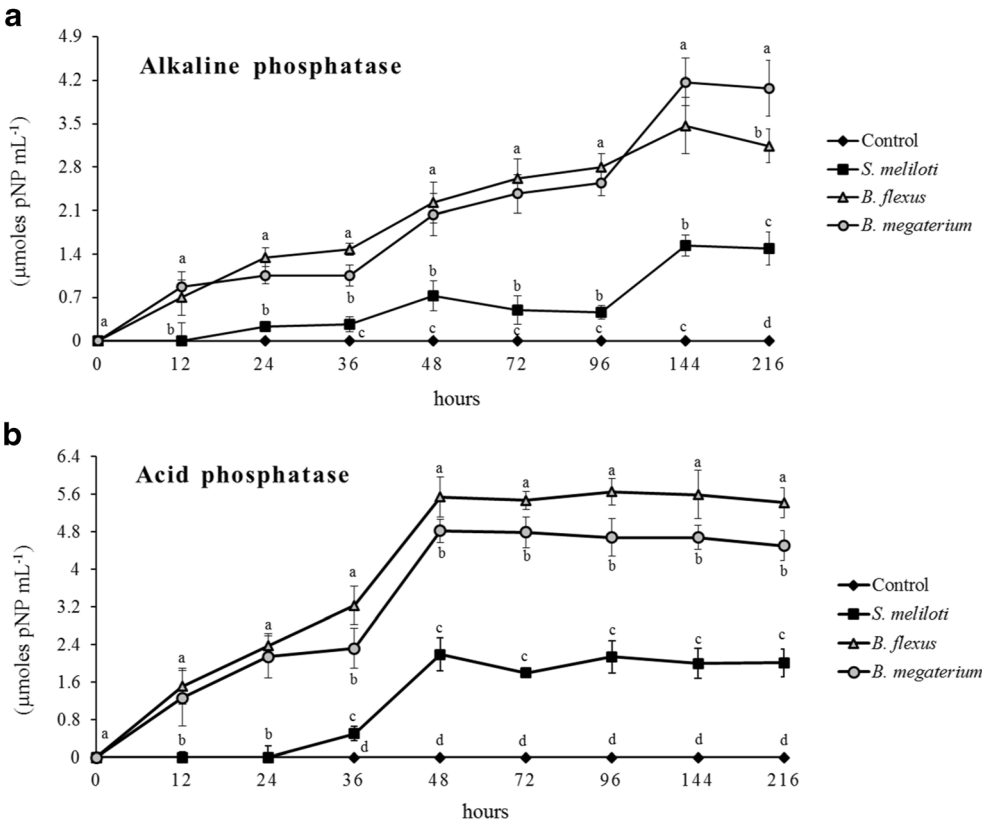

Fig. 2 Phosphatase activities secreted into the culture medium by the rhizobacteria strains *S. meliloti*, *B. flexus* and *B. megaterium*. **a**, **b** Alkaline phosphatase (**a**) and acid phosphatase (**b**) activity [in μmol *p*-nitrophenol (*p*-NP) mL^{-1} h^{-1}] in liquid culture. *Sinorhizobium meliloti* was cultured in PY broth; *B. flexus* and *B. megaterium* were cultured in LB medium. Different letters above the bars indicate a significant difference at $P < 0.05$ between treatments according to the Tukey least significant difference test

Substrate that had been fertilized in the presence of *B. flexus* or the bacterial mixture *B. flexus* and *B. megaterium* (without P) induced plant height, root and shoot biomass and shoot total P content to values similar to the control fertilized with 40 w/v P. At fertilization with 40 w/v P, the *B. flexus and B. megaterium* mixture induced plant height to levels that were similar to those of the control fertilized with 80 w/v P. Shoot dry biomass and total P content increased significantly in the treatments that included *B. megaterium* relative to the non-inoculated control, although they did not reach the values obtained with 80 w/v P (Table 1).

Inoculation with the bacterial mixture increased available P for the substrate that received fertilization without any P, as well as the substrate fertilized with 40 w/v P. Inoculation with *B. flexus* significantly increased available P, but only in the non-fertilized substrate. Non-significant differences in the substrates fertilized with 80 and 120 w/v P were observed, relative to the non-inoculated control (Table 2).

Bacterial inoculation employing a non-sterile substrate fertilized without P demonstrated a beneficial effect of strain *B. megaterium* on plant height, shoot biomass and total P content but no effect on root biomass with respect to the untreated control, while inoculation of the bacterial mixture (*B. flexus and B. megaterium*) increased only plant height and shoot biomass (Table 3). *Bacillus flexus* did not cause any significant change in any of the parameters evaluated with respect to the control.

Discussion

In this study, we observed a negative correlation between pH and P solubility in liquid cultures of rhizobacteria. A similar phenomenon was reported by Sridevi and Mallaiah (2009), who demonstrated that one particular strain of *Rhizobium* sp. decreased the pH of the growth medium from 7 to 4.05 and increased P solubilization. Soluble P concentration values of 200 μg mL^{-1} (final pH 4.46; Oliveira et al. (2009) and 96.73 μg mL^{-1} (final pH 5.8; Yu et al. (2011) have been reported in liquid cultures of strains of *Bacillus* sp. In our study, TCP solubilization in cultures of the *Bacillus* strains was accompanied by an increase in the pH of the culture medium at day 5 (*B. flexus*) and day 7 (*B. megaterium*). This slight increase in pH could be due to a decrease in the medium's carbon source (glucose) due to bacterial growth, which can inhibit the gene expression of metabolic pathways for diverse carbon sources. This in turn causes a decrease in organic acid synthesis and its secretion into the medium. Secreted organic acids chelate insoluble P to make it soluble by decreasing the medium concentration and causing a slight increase in pH (Marciano-Marra et al. 2012). A similar phenomenon has been reported for *Penicillium* spp. and *Burkholderia cepacia* (Nath et al. 2012; Zhao et al. 2014).

We demonstrated an inverse relationship between pH and P solubilization, a result in line with reports from Bianco and Defez (2010) and Collavino et al. (2010), suggesting that

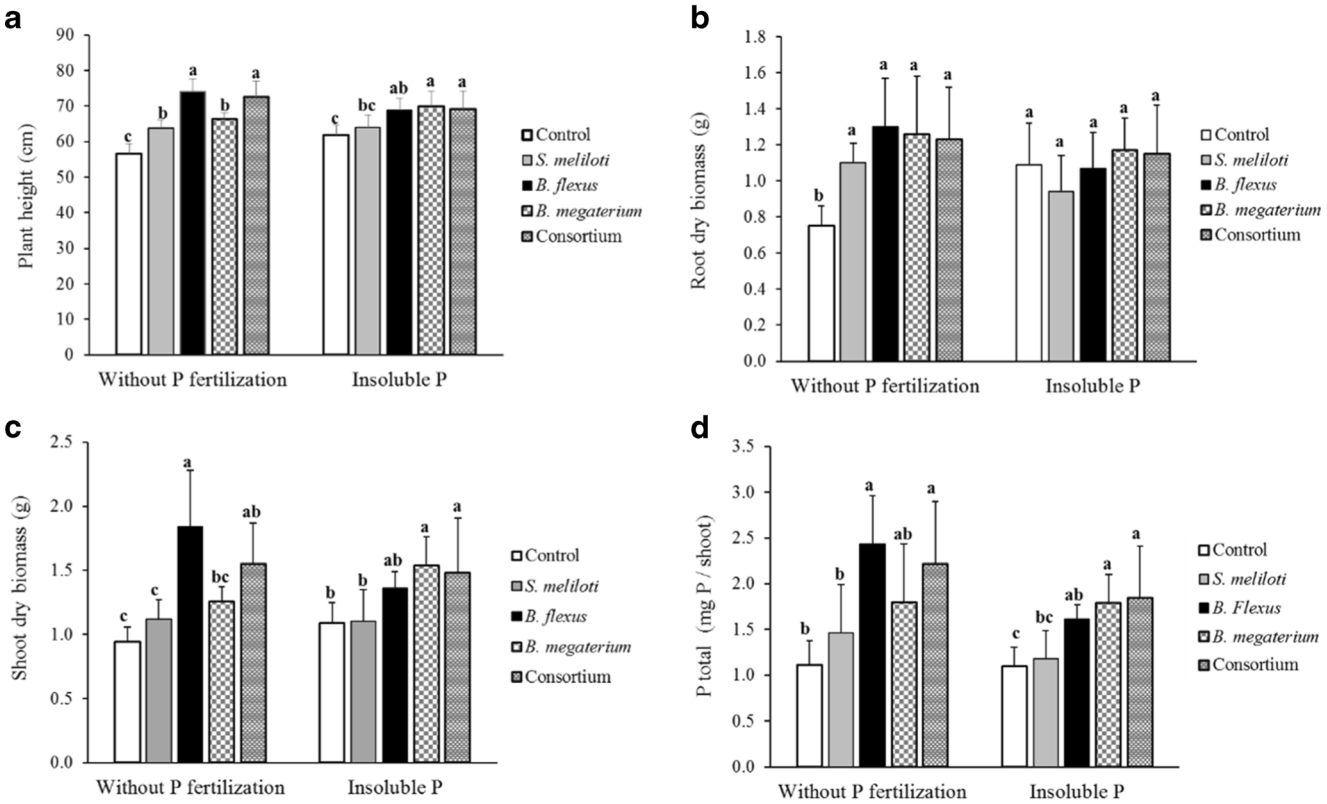

Fig. 3 Effect of inoculation by the rhizobacteria strains *S. meliloti*, *B. flexus, B. megaterium* and consortium (*S. meliloti*, *B. flexus* and *B. megaterium*) on the growth of maize plants and P nutrition. Greenhouse experiments were performed either in substrate without P fertilization or substrate fertilized with insoluble P. Parameters measured were plant height (**a**), root dry biomass (**b**), shoot dry biomass (**c**) and shoot total P content (**d**). Results are presented as the mean ± standard deviation of $n = 5$ trials). A two-way analysis of variance was performed to determine the influence of P treatments and bacterial inoculation. The control was without bacteria; all other cultures contained *S. meliloti*, *B. flexus*, *B. megaterium*, or a consortium of *S. meliloti*, *B. flexus* and *B. megaterium*. Different letters above the bars indicate significant differences at $P < 0.05$ between treatments according to Fisher's least significant difference test

medium acidification facilitates P solubilization. The secretion of organic acids by PSB plays a major role in soil P solubilization by lowering the pH and enabling the replacement (Ca^{2+}) and/or chelation (Ca^{2+}, Fe^{3+}, Al^{3+}) of the metal ions that usually form insoluble P complexes (Bashan et al. 2013).

Phosphate solubilization by bacteria native to alkaline soils, such as those from our region, has not only to be tested with TCP but also with other more insoluble forms of phosphate such as hydroxyapatite, as suggested by Bashan et al. (2013). Our findings from the testing of TCP and hydroxyapatite as more insoluble forms of phosphate are coincident with those reported by Bashan et al. (2013). We observed that our selected phosphate solubilizer bacterial strains possessed differential hydroxyapatite solubilization ability. For example, strain *B. flexus* was unable to solubilize hydroxyapatite at all, possibly due to the type of organic acids that *B. flexus* secretes into the medium. The solubilization efficiency of organic acids for chelating metal cations is strongly influenced by the number of carboxyl and hydroxyl groups, as well as the type and position of the ligand (Kpomblekou-a and Tabatabai 1994).

Phosphatase activity contributes to the solubilization of organic phosphates in the soil (Richardson 2001). Organic P generally accounts for 30–65% of total soil P content, depending on soil type and land management (Richardson et al. 2009). Bacterial strains from our study have phosphatase activities similar to those reported in the literature. For example, acid phosphatase activity can reach 8.0 μmol mL^{-1} h^{-1} in *Rhodococcus* spp. strains (Pereira and Castro 2014), while alkaline phosphatase activity was measured at 2.15 μmol mL^{-1} h^{-1} in *Xanthomonas maltophila* (De Freitas et al. 1997). Importantly, the de novo synthesis of these enzymes is stimulated when the level of inorganic P in the growth medium is limiting (Dick et al. 2011), which explains why phosphatase activity is determined in a medium without a phosphate source; otherwise, the presence of soluble P inhibits acid and alkaline phosphatase activity (Hidayat et al. 2006; Kapri and Tewari 2010).

Our results show that maize growth is promoted and P nutrition is increased in cultures inoculated with the bacterial strains when a P-deficient substrate without the addition of P fertilizer or with the addition of insoluble phosphate is used.

Table 1 Effect of bacterial inoculation under different phosphorus fertilization treatments

Treatments[a]	Plant height (cm)	Root dry biomass (g)	Shoot dry biomass (g)	Shoot total P content (mg)
0 w/v P + *B4*	73.75 ± 7.97 f	0.54 ± 0.20 f	1.18 ± 0.32 e	1.93 ± 0.51 h
0 w/v P + *B5*	56.75 ± 2.99 g	0.35 ± 0.18 h	0.53 ± 0.11 f	0.61 ± 0.14 j
0 w/v P + *B4-B5*	78.00 ± 6.48 f	0.67 ± 0.27 fgh	1.17 ± 0.28 e	1.44 ± 0.28 hi
40 w/v P + *B4*	77.25 ± 2.87 f	0.55 ± 0.03 gh	1.17 ± 0.23 e	1.41 ± 0.30 hi
40 w/v P + *B5*	86.75 ± 6.99 e	0.93 ± 0.26 ef	2.51 ± 0.22 d	3.17 ± 0.25 g
40 w/v P + *B4-B5*	95.00 ± 4.55 d	0.87 ± 0.26 fg	2.13 ± 0.26 d	2.76 ± 0.31 g
80 w/v P + *B4*	101.75 ± 3.59 cd	1.30 ± 0.27 d	4.43 ± 0.31 c	5.91 ± 0.50 e
80 w/v P + *B5*	108.50 ± 5.26 abc	1.35 ± 0.28 d	4.91 ± 0.12 b	6.71 ± 0.33 d
80 w/v P + *B4-B5*	103.75 ± 4.11 bc	1.50 ± 0.23 cd	4.54 ± 0.27 bc	4.93 ± 0.38 f
120 w/v P + *B4*	110.25 ± 2.06 ab	1.86 ± 0.31 bc	5.76 ± 0.38 a	8.63 ± 1.18 a
120 w/v P + *B5*	110.75 ± 5.06 ab	2.04 ± 0.41 ab	6.02 ± 0.48 a	7.61 ± 0.64 bc
120 w/v P + *B4-B5*	111.25 ± 4.35 a	2.35 ± 0.27 a	6.06 ± 0.41 a	7.11 ± 0.67 cd
Control 0 w/v P	60.25 ± 4.57 g	0.38 ± 0.23 h	0.65 ± 0.19 f	0.79 ± 0.2 ij
Control 40 w/v P	73.50 ± 4.73 f	0.63 ± 0.17 fgh	1.14 ± 0.32 e	1.35 ± 0.31 hi
Control 80 w/v P	101.75 ± 5.12 cd	1.28 ± 0.25 de	4.51 ± 0.26 bc	5.61 ± 0.54 ef
Control 120 w/v P	105.00 ± 4.97 abc	2.09 ± 0.48 ab	5.75 ± 0.18 a	8.07 ± 0.54 ab

Values are presented as the mean ± standard deviation (SD) (n = 4 trials). Different lowercase following the values indicate a significant between-treatment difference at $P < 0.05$ according to Fisher's least significant difference test

A generalized linear model was performed to determine the interaction between bacterial inoculation and phosphate treatment

P, Phosphorus

[a] Control: treatment without bacteria. *B4*, *B5*, *B4–B5*: treatment with *Bacillus flexus* strain *B4*, with *B. megaterium* strain *B5*, with a mixture of strains *B4* and *B5*, respectively

These results are in agreement with those obtained using other bacterial isolates in maize (Gurdeep and Reddy 2015; Hameeda et al. 2008; Pereira and Castro 2014; Zahid 2015). Inoculation with the *S. meliloti* strain did not present any significant effects on growth promotion or P nutrition in substrate fertilized with an insoluble phosphate form.

Maize fertilized with soluble P to attain a soil concentration of 80–120 w/v P or 160 w/v P (ESM Table S1) did not show any significant differences in growth or P nutrition when inoculated with bacteria. A previous study using soils with high levels of available P found that plants can bypass the energy cost required to establish an association with beneficial microorganisms (Bago et al. 2000). Under these conditions, P is acquired through the direct absorption pathway via the root hairs (Nagy et al. 2009). Therefore, it is possible that the PSB–plant root association is limited in soils with high levels of available phosphate.

Maize inoculation with the bacterial strains improved P nutrition and growth parameters at 0 and 40 w/v P when different doses of P fertilization were used; this finding is consistent with the use of the *B. flexus and B. megaterium* bacterial mixture. This beneficial effect of this synergistic bacterial mixture has previously been reported in maize (Pereira and Castro (2014), wheat (Turan et al. (2012), walnut seedlings

Table 2 Available phosphorus remaining in the substrate after harvesting of plants grown in different phosphorus fertilization treatment regimens and with different bacterial strain inoculations

Bacterial treatment	P fertilization treatment regimen			
	0 w/v P	40 w/v P	80 w/v P	120 w/v P
Control	6.06 ± 1.00 b	17.55 ± 1.1 7b	29.62 ± 1.70 a	33.50 ± 1.43 a
Bacillus flexus	7.77 ± 0.89 a	18.79 ± 1.11 ab	28.63 ± 0.97 a	32.02 ± 2.26 a
B. megaterium	5.42 ± 1.06 b	17.42 ± 1.52 b	29.64 ± 1.02 a	32.82 ± 1.43 a
B. flexus + *B. megaterium*	7.92 ± 0.59 a	19.59 ± 0.86 a	28.99 ± 0.96 a	34.41 ± 1.51 a

Values are presented as the mean ± SD (n = 4 trials)

One-way analysis of variance (ANOVA) was performed to determine the influence of different P fertilization treatments with bacterial inoculation. Different lowercase following the values indicate a significant between-treatment difference at $P < 0.05$ according to Fisher's least significant difference test

Table 3 Effect of bacterial inoculation of substrate under non-sterile conditions and when fertilized without phosphorus

Bacterial treatment	Plant height (cm)	Root dry biomass (g)	Shoot dry biomass (g)	Shoot total P content (mg)
Control without bacterial	61.00 ± 4.24 b	0.67 ± 0.12 ab	0.86 ± 0.13 b	1.42 ± 0.32 b
B. flexus	66.80 ± 5.32 ab	0.57 ± 0.19 b	0.88 ± 0.28 b	1.57 ± 0.46 b
B. megaterium	71.00 ± 4.32 a	0.73 ± 0.17 ab	1.30 ± 0.23 a	2.53 ± 0.58 a
Mixture of *B. flexus* and *B. megaterium*	69.80 ± 3.77 a	0.89 ± 0.18 a	1.22 ± 0.17 a	1.91 ± 0.36 ab

Values are presented as the mean ± SD (n = 4 trials)

One-way ANOVA was performed to determine the influence of bacterial inoculation on non-sterile substrate and fertilized without P. Different lowercase following the values indicate a significant between-treatment difference at $P < 0.05$ according to Fisher's least significant difference test

(Yu et al. (2011) and mangroves in Mexico (Rojas et al. 2001). Bioavailable P present in the substrate at the end of the bioassay indicates that *B. flexus* was able to increase the levels of soluble P in the substrate without fertilization. Furthermore, the *B. flexus* and *B. megaterium* mixture increased the P levels in the treatment without any P fertilization, or when the substrate was fertilized with 40 w/v P, relative to the non-inoculated controls. Similar results have been found using different bacterial consortia (Han and Lee 2006; Pereira and Castro 2014).

When the soil microbiota was tested in an experiment using a non-sterile substrate, we observed that the beneficial effects of *B. megaterium* and of the bacterial mixture on P nutrition and growth were not affected by competition or interaction with the native soil microorganisms; in contrast *B. flexus* did not behave well. Bacterial strains may show beneficial effects under controlled conditions, but their behavior may differ when applied to natural types of soils (Egamberdiyeva 2007; De-Bashan et al. 2010). Nevertheless, we cannot rule out the possibility that *B. flexus* may behave better in other soils. We recommend that further field trials be conducted with these strains, both independently and as a bacterial mixture.

The increase in maize growth, enhancement of shoot P content and bioavailability of P in the substrates can be attributed to the capacity of PSB for P solubilization, as well as possibly to the production of different phytohormones (López-Bucio et al. 2007; Vacheron et al. 2014). Strains *B. flexus* and *B. megaterium* have not been found to produce any auxins (Figueroa-López et al. 2016). The strains used in the present study were not thoroughly characterized for phytohormone production. However, *B. megaterium* has already been tested in the field, where it has helped reduce the severity of ear and stalk rot caused by *Fv*, in addition to inducing a significant increase in maize grain yield as compared to plants inoculated with *Fv* (Lizárraga-Sánchez et al. 2015). As soils in Sinaloa are alkaline, insoluble forms of P are expected to form with Ca^{2+}.

The abilities of the *Bacillus* strains tested in this study to dissolve TCP and hydroxyapatite, combined with their positive effects on growth promotion and P nutrition in maize, suggest that they be tested in the field as a bacterial mixture to alleviate P deficiencies in low-P soils found in regions of Sinaloa where P fertilizers are not a feasible option for low-income farmers. We propose utilizing these two strains in combination since a synergistic effect is, with one strain improving P nutrition and the other acting as a growth promoter. The inoculation of this bacterial mixture (*B. flexus* and *B. megaterium*) in maize plants combined with an integrated crop management approach in alkaline P-deficient soils could be a viable alternative to improve crop productivity, increase soil fertility and provide a sustainable strategy for the application of phosphate fertilizers.

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