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ORIGINAL PAPER

Performance of phosphate-solubilizing bacteria in soil under high phosphorus conditions

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Abstract A way to bring phosphate-saturated soils back to an environmentally safe P level is by P mining through plants. Phosphate-solubilizing bacteria (PSB) could be very useful for increasing mining efficiency over time. The goal of this research was to investigate the adaptation and performance of PSB in conditions of high total P content in soil. In the first experiment, the P-solubilizing capacity of five PSB species (three *Bacillus* spp. and two *Pseudomonas* spp.) were tested under fully controlled conditions on several growth media with different forms of insoluble phosphate ($FePO_4$, $AlPO_4$, or $(Ca)_3(PO_4)_2$) added at different rates. The colony growth after 14 days of inoculation demonstrated that all five bacteria were able to proliferate and solubilize P on each of the tested growth media, in contradiction with the normally used technique of halo determination. In the second experiment, the same bacterial species were inoculated in pure quartz sand amended with a nutrient solution and P was added separately in an insoluble form, as Fe–P, Al–P, or Ca–P. The extractable ammonium lactate ranged from 3.2 to 6.9 and 29.0 to 40.7 $mg\,kg^{-1}$ sand for the insoluble Al–P and Fe–P treatments, respectively. *Pseudomonas putida* and *Bacillus brevis* performed best as PSB at high P concentration where the P is fixed with Al or Fe. In the third experiment, *P. putida* and *B. brevis* were inoculated in an acidic sandy, P-saturated soil for 4 weeks. The inoculation of the PSB gave promising results in solubilizing P.

Keywords PSB · P saturation · P mining · Phosphorus solubilization

Abbreviations

Al	Aluminum
B.	*Bacillus*
Ca	Calcium
Fe	Iron
P	Phosphorus
P.	*Pseudomonas*
P_{lac}	Ammonium lactate-extractable P
PSB	Phosphate-solubilizing bacteria
PSD	Phosphate saturation degree
P_w	Water-extractable P

Introduction

Many agricultural soils have accumulated large P reserves as a result of excess P fertilization over the years (Fernández et al. 2007). Problems of excessive P levels are found in many countries with industrialized agriculture (Ajmone-Marsan et al. 2006; Djodjic et al. 2004; Ketterings et al. 2005; Reijneveld et al. 2010; Uusitalo et al. 2007). In Belgium, about 80 % of arable lands and 40 % of grasslands are considered fairly high to very high in soil P (Reijneveld et al. 2010).

Traditionally, P losses by erosion have been viewed as the main or even the sole source of P losses to natural waters (Kleinman and Sharpley 2003; Volf et al. 2007). However, P leaching plays an important role in acidic sandy soils with high P levels (Van Den Bossche et al. 2005). This risk of P leaching results from the generally small phosphate sorption

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capacity and high P saturation degree (PSD) of these soils (De Bolle et al. 2012). In the European Union, many of these acidic sandy soils with high PSD are subjected to strict P fertilization restrictions, which ultimately should result in P mining. However, it is felt that, with current crop rotations, it takes several decades because P mining efficiency decreases rapidly with time (Sharma et al. 2007). Ideally, efficient long-term P mining from agricultural soils would thus require methods that keep P levels (temporarily) high enough in order not to limit crop P uptake.

One such way could be to increase P availability by the addition of phosphate-solubilizing bacteria (PSB) to the soil. A number of soil microorganisms, including bacteria, have the capability of solubilizing mineral phosphates, thereby affecting the P cycle both in natural and agricultural ecosystems (Vazquez et al. 2000). In soil, PSB constitute from 0.5 up to 50 % and P-solubilizing fungi constitute from 0.05 to 0.1 % of the total respective populations (Gyaneshwar et al. 2002; Vazquez et al. 2000). Generally, the PSB outnumber the P-solubilizing fungi by 2- to 150-fold (Kucey 1983).

Especially the *Pseudomonas*, *Bacillus*, and *Rhizobium* species are widely distributed in the soil environment (Martin and Travers 1989; Thakuria et al. 2009; Wang et al. 2001). *Pseudomonas* species were reported to solubilize P under a range of temperature conditions (Trivedi and Sa 2008) and the most intensively studied species of this genus are *Pseudomonas putida* (Manna et al. 2001; Villegas and Fortin 2002), *Pseudomonas corrugata* (Pandey and Palni 1998), *Pseudomonas aeruginosa* (Musarrat et al. 2000), *Pseudomonas stuzteri* (Vazquez et al. 2000), and *Pseudomonas fluorescens* (Deubel et al. 2000). In particular, *P. putida* has been reported as an efficient PSB (Kuiper et al. 2002; Rosas et al. 2006). Within the genus *Bacillus*, *Bacillus brevis*, *Bacillus cereus*, *Bacillus circulans*, *Bacillus polymyxa*, *Bacillus thuringiensis*, and *Bacillus megaterium* species were all reported to solubilize P (de Freitas et al. 1997; Turan et al. 2007). Within the *Rhizobium* species, the research has mainly focused on *Rhizobium leguminosarum* (Rodriguez and Fraga 1999) and *Rhizobium phaseoli* (Thakuria et al. 2009).

The addition of PSB to soil to increase P availability to agricultural crops has been widely investigated (Gyaneshwar et al. 2002). To the best of our knowledge, all studies on PSB have focused on the solubilization of P in artificial growth media or in soils which were low in total P. Nearly all these studies were concerned with P solubilization from calcium phosphate sources (Arcand and Schneider 2006; Vessey 2003). We found only some studies which looked into P solubilization from $AlPO_4$ by *Pseudomonas* sp. (Henri et al. 2008; Illmer et al. 1995; Puente et al. 2004). However, these were done at low $AlPO_4$ levels.

To date, no studies have explored the potential of using PSB to increase P uptake in acidic soils rich in total P. However, such studies may prove very useful in these soils high in P where the objective is P mining, since mining efficiency is known to decrease drastically with time if no P fertilizer is added. The point of interest of this study was the solubilization of Al–P and Fe–P in soils with a high total P content, and this is compared to Ca–P solubilization. The aim of this study was threefold. We investigated (1) whether the PSB were tolerant and able to grow effectively in environments with high total insoluble P concentrations, thereby investigating if the evaluation procedure through halo determination is useful for all P forms; (2) whether PSB could solubilize the unavailable P in aluminum and iron phosphates added to a pure quartz sand substrate; and (3) whether PSB were able to solubilize P in acidic sandy soils high in total P to assess their potential for increasing P mining efficiency.

Materials and methods

Bacteria selection

Pseudomonas, *Bacillus*, and *Rhizobium* spp. are found to be the most effective PSB (Richardson and Simpson 2011; Rodriguez et al. 2006; Rodriguez and Fraga 1999). Based on previous studies (de Freitas et al. 1997; Kuiper et al. 2002), five bacteria were selected, namely, *B. brevis* (ATCC 8246), *B. polymyxa* (ATCC 842), *B. thuringiensis* (ATCC 10792), *P. corrugata* (ATCC 29736), and *P. putida* (ATCC 12633). The bacteria were obtained from DSMZ (Braunschweig, Germany) and cultured in nutrient broth (Oxoid Ltd., Hampshire, England) under shaking at 30 °C for the *Bacillus* species and at 25 °C for the *Pseudomonas* species.

This study was carried out in a three-stage approach. In a first stage, PSB growth was monitored on media where an insoluble P source was applied as Al–P, Fe–P, or Ca–P. In the second stage, the P-solubilizing capacity of the PSB was tested in an experiment using sand as a substrate, thus creating more realistic conditions, but which still allowed having maximum control over P dynamics, which is more difficult to achieve in a real soil environment. In the third stage, the PSB were tested in real acidic sandy soil with a high total P concentration.

Growth media experiment

The growth media were based on the National Botanical Research Institute's phosphate growth medium (Nautiyal 1999), but with some modifications. Each growth media was composed of glucose, 10.0 gL^{-1}; $MgCl_2 \cdot 6H_2O$, 5 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.25 gL^{-1}; and KCl, 0.2 gL^{-1} plus variable amounts of N and insoluble P sources (Table 1) together with a sufficient amount of bacteriological agar (agar no. 1, Oxoid Ltd., Hampshire, England). The pH was adjusted to 5.0 for the growth media with insoluble

Table 1 The amount (in grams per liter) of the insoluble P, N in each growth media, and the pH of the different growth media

	Fe–P			Al–P			Ca–P	
P amount	Low	Low	High	Low	Low	High	Low	High
N amount	Low	High	Low	Low	High	Low	Low	Low
$FePO_4$	5.0	5.0	15.0	–	–	–	–	–
$AlPO_4$	–	–	–	5.0	5.0	15.0	–	–
$Ca_3(PO_4)_2$	–	–	–	–	–	–	2.5	15.0
$(NH_4)SO_4$	0.1	0.5	0.1	0.1	0.5	0.1	0.1	0.1
Agar	10.0	10.0	15.0	15.0	15.0	20.0	10.0	10.0
pH	5.0	5.0	5.0	5.0	5.0	5.0	7.5	7.5

Al–P or Fe–P sources and to 7.5 for the growth media with insoluble Ca–P source (Table 1), so as to achieve similar pH conditions as found in soils with these respective P sources.

The growth media were sterilized using an autoclave (SANOclav, Bad Überkingen-Hausen, Germany) at 121 °C for 25 min and then transferred aseptically into sterilized Petri plates. Per plate, a bacterial strain was stabbed in quadruplicate on the plate using sterile toothpicks. For each growth medium and each bacterial strain, four replicate plates were used. The plates were incubated at 25 °C for 14 days. The colony growth and, if possible, the halo zone were measured at the 3rd, 7th, and 14th days of inoculation using a microscope (OPTIKA stereo microscope, ×6.7 magnification, Italy).

Experiment on sand medium

Quartz sand (0.05–0.5 mm diameter) was washed consecutively with 0.5 % NaOH, distilled water, and 5 % HCl in a 1:1 sand/solution ratio under shaking for 1 h, in order to remove all nutrients. Finally, the sand was washed with demineralized water until electrical conductivity was smaller than 3 μs and then oven-dried (105 °C).

Firstly, the insoluble P sources were thoroughly mixed with 40 g of sand and brought into polypropylene tubes (diameter of 3.4 cm, height of 6.7 cm). The amount of insoluble P added to the sand (500 $mg\,P\,kg^{-1}$) was based on the typical P content of acidic sandy soils in Flanders, namely, 500–1,000 $mg\,P_{ox}\,kg^{-1}$. Secondly, the bacteria were mixed with the nutrient solution (Table 2) and this solution was added to the sand, as a bacterial inoculum of 2.2×10^8 colony-forming units $(CFU)\,g^{-1}$ of sand, based on the population size of PSB in soils as found by Hu et al. (2009). The nutrient solution, without P, was prepared with sucrose as the main C source. We prepared several solutions separately, which were afterwards mixed in various specified ratios so as to ensure that all elements were equally available in all treatments. Per tube, 6.3 ml of this solution was added. Per treatment and per extraction day, there were three replications. Finally, all tubes were covered with perforated Parafilm to reduce water loss but still allow sufficient oxygen supply and stored in a closed incubator at 20 °C for 10 days. Samples were taken at the 5th and 10th days of incubation. Immediately after sampling, the samples were dried in the oven for 2 h at 60 °C to stop all biological activity.

Experiment with acidic sandy soil

We selected two acidic sandy soils (further referred to as soil 1 and soil 2), located in Oostkamp, Belgium, with a total P content of 1,078 and 1,267 $mg\,kg^{-1}$. The soil texture of both soils was sand (United States Department of Agriculture

Table 2 The composition (in milligrams per liter) of the nutrient solutions added to the sand

Solution 1		Solution 2	
Nutrient	Amount ($mg\,L^{-1}$)	Nutrient	Amount ($mg\,L^{-1}$)
$CaCl_2{\cdot}2H_2O$	1,175.31	$MnSO_4{\cdot}H_2O$	310.00
K_2SO_4	348.40	$ZnSO_4{\cdot}7H_2O$	90.00
$MgSO_4{\cdot}7H_2O$	492.62	$CoCl_2{\cdot}6H_2O$	0.80
$CuSO_4{\cdot}5H_2O$	249.55		
KNO_3	404.41	Fe and Al solution	
H_3BO_3	309.06	Element	Amount ($mg\,L^{-1}$)
Na_2CO_3	211.98	$Al_2SO_4{\cdot}8H_2O$	119.00
Ammonium acetate	332.20	Fe	27.92
Sucrose	14,850		
$H_{24}Mo_7N_6O_{24}$	0.18		

classification) with a composition of 87.1 % sand, 10.0 % silt, and 2.90 % clay for soil 1 and 87.9 % sand, 9.0 % silt, and 3.10 % clay for soil 2. In the top layer (0–30 cm), a PSD of 77.8 and 106.1 % for soil 1 and soil 2 was found, respectively. For both soils, there was no free $CaCO_3$ and the organic matter content was 4.5 and 3.6 %, respectively. The pH was 5.1 and 4.3 for soil 1 and soil 2, respectively. In this study, *B. brevis* and *P. putida* were inoculated separately and in combination (dual inoculation) in the same concentration as in the sand experiment. The PSB, in an inoculum of 2.2×10^8 CFU g^{-1}, were mixed with 80 g of preincubated soil. Each tube was then covered with the Parafilm and holes were made to create aerobic conditions. There were three replicates per treatment per sampling date. The samples were randomly stored in a closed incubator at 20 °C for 5 and 10 days.

Measurements

The pH, the ammonium lactate-extractable P (P_{lac}), and the water-extractable P (P_w) was measured both in the quartz sand (after 5 and 10 days) and in the soil (at weeks 2 and 4). The pH was measured potentiometrically in a 1:2.5 soil/KCl extract (pH–KCl). The P_{lac}, which is considered as the plant-available P pool in these soils (Van Den Bossche et al. 2005), was measured at both sampling occasions by extracting the soil with ammonium lactate (extraction ratio, 1:20; Otabbong et al. 2009) in dark polyethylene bottles that were shaken for 4 h on a rotational shaker. The P concentration in the filtered extract was measured colorimetrically at 700 nm (spectrophotometer, Varian, Cary 50) according to Scheel (1936).

The P_w, which is considered the available P pool, was measured as reported by Self-Davis et al. (2009). Two grams of dry sand/soil and 20 ml of distilled water were put in a centrifuge tube and shaken for 1 h on a rotational shaker. The soil slurries were then centrifuged at 3,220×*g* followed by filtration (Whatman ashless filter, 589/3). The P_w in the filtrate was determined colorimetrically at 882 nm according to Murphy and Riley (1962).

Calculation and statistical analysis

The solubilization index, the sum of the diameter of the colony and the halo zone divided by the diameter of the halo zone (Premono et al. 1996), was calculated for the Ca–P growth media. The results gathered from the three experiments were statistically analyzed using the statistical software PASW 18 package (SPSS version PASW 18, SPSS Inc., USA). The growth diameters of the PSB were statistically compared (analysis of variance [ANOVA]) between the different P sources as well as between the different PSB. In the sand experiment, we carried out a comparison between the different P sources as well as between the PSB. The results were statistically analyzed with a paired-sample *T* test. For the sand and the acidic sandy soil experiment, a one-way ANOVA (with a *T* test) was done for the pH, the P_{lac}, and the P_w. The results were also compared between dates with a paired-sample *T* test. Pearson correlation coefficients were calculated between the pH, P_w, and P_{lac} for the soil experiment for each PSB and for both soils.

Results

Growth media experiment

The colony growth diameters, after 14 days, indicated that all five PSB were able to grow on each of the respective growth media, with varying soluble N and insoluble P concentrations (Fig. 1). For all growth media, the colony growth

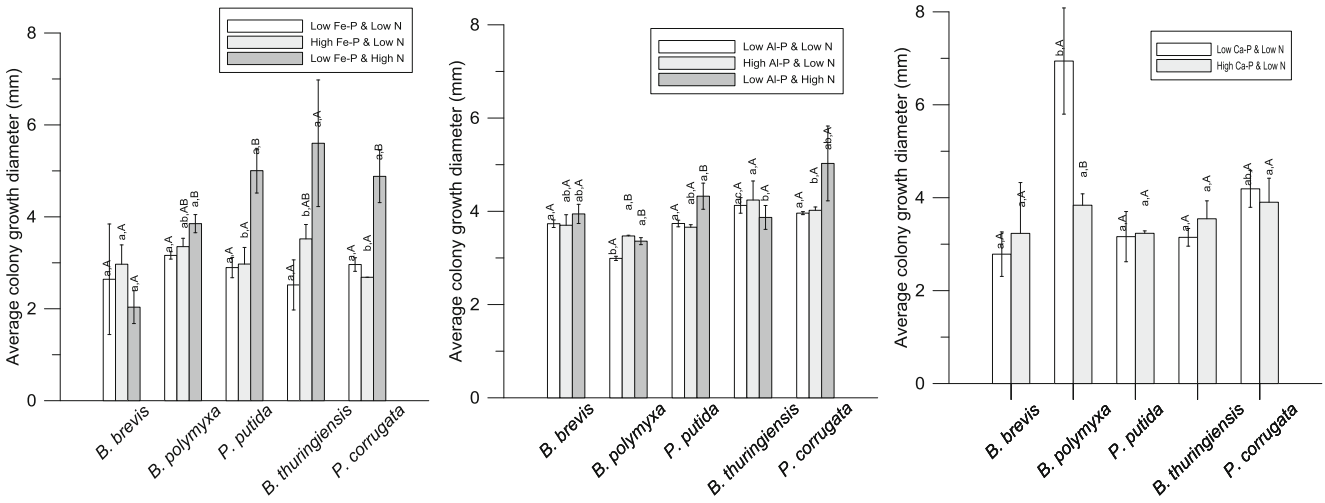

Fig. 1 The average colony growth diameter (in millimeters) at the end of the incubation (14 days at 25 °C) for the Fe–P treatments (**a**), Al–P treatments (**b**), and Ca–P treatments (**c**) ($p<0.05$, significance between PSB is marked in *lowercase letters* and significance between treatments is marked in *uppercase letters*)

diameter was found to be in the same order of magnitude, varying between 2 and 8 mm.

The colony diameter for all PSB, with the exception of *P. corrugata*, tended to increase with increasing insoluble Fe–P, from 5 to 15 gL^{-1}, but this was not statistically significant ($p<0.05$). All PSB, except *B. brevis*, showed a significant increase in the colony growth diameter with an increasing N concentration, from 0.1 to 0.5 g $NH_4(SO_4)L^{-1}$, at a constant insoluble Fe–P concentration of 5 gL^{-1}.

The colony growth diameter for all PSB tended to be in the same order of magnitude, irrespective of the insoluble Al–P concentration. For *B. polymyxa*, however, a significant increase ($p<0.05$) was found in the colony growth diameter with increasing Al–P concentration. An increasing growth diameter of all PSB, except *B. thuringiensis*, was found when the N concentration increased; for *B. polymyxa* and *P. putida*, a significant increase was even found. However, the effect of increasing P concentration was greater for the Fe–P treatment (maximum increase of 3.08 mm) than for the Al–P treatment (maximum increase of 1.06 mm).

An increase in Ca–P concentration reduced the colony growth diameter significantly for *B. polymyxa* (from 6.94 to 3.84 mm; Fig. 1c). Also, the solubilization index calculated for the Ca–P growth media decreased significantly ($p<0.05$) with increasing Ca–P concentration.

Sand experiment

The solubilization effect was dependent on the type of P compounds and the type of PSB added to the sand (Table 3). Large differences were found between the P solubility in the controls of the treatments. In the Al–P treatment, only *B. brevis* showed a trend of P solubilization, namely, a P_w value of 4.2 $mgPkg^{-1}$ compared with 3.2 $mgPkg^{-1}$ for the control treatment. The P_w value found with *B. thuringiensis*-, *P. putida*-, and *P. corrugata*-inoculated samples decreased significantly ($p<0.05$) in comparison with the control treatment. All PSB were able to solubilize P when P was under the form of Fe–P. The P_w values for PSB-inoculated samples varied from 0.7 to 1.6 $mgPkg^{-1}$ sand in comparison with 0.6 $mgkg^{-1}$ sand for the control treatment. The samples inoculated with *B. brevis*, *P. putida*, and *P. corrugata* resulted in a significant ($p<0.05$) P solubilization effect for the Fe–P treatment in comparison with the control. A higher P_w value was observed for *P. putida* and *B. thuringiensis* for the Ca–P treatment in comparison with the control. However, only *P. putida* (44.8 $mgPkg^{-1}$ sand) was significantly different ($p<0.05$) to the control (35.4 $mgPkg^{-1}$ sand), thereby proving to be an effective PSB in solubilizing P in a high Ca–P environment.

Table 3 Mean P_w values (in milligrams per kilogram sand±standard deviation) measured after 10 days of incubation for the Al–P, Fe–P, and Ca–P treatments in the sand experiment

Treatment	Al–P	Fe–P	Ca–P
Control	3.2±1.03	0.6±0.22	35.4±6.22
B. brevis	4.2±1.72	1.3±0.75*	30.7±0.88
B. polymyxa	2.6±0.60	1.1±0.77	33.6±2.4
P. putida	0.6±0.16**	1.5±0.14*	44.8±3.10*
B. thuringiensis	0.6±0.15**	0.7±0.11	39.9±7.95
P. corrugata	0.6±0.17**	1.6±0.52*	34.6±3.15

*$p<0.05$, significantly higher than the control; **$p<0.05$, significantly lower than the control

When P was under the form of Al–P, the P_{lac} values for the tubes inoculated with PSB ranged between 3.2 and 5.4 $mgPkg^{-1}$ measured on the fifth day and between 2.9 and 6.9 $mgPkg^{-1}$ measured on the tenth day (Table 4). Significantly higher P_{lac} values were found for *B. brevis*, *P. putida*, and *P. corrugata* in comparison with the P_{lac} value of the control. For the Fe–P treatment, the results for P_{lac} for the PSB-inoculated samples varied between 29.5 and 35.4 $mgPkg^{-1}$ measured on the fifth day and between 38.8 and 40.7 $mgPkg^{-1}$ measured on the tenth day. When comparing these results with the results for the control sample, no significant effects were found with PSB inoculation for the Fe–P treatment. PSB inoculation in the insoluble Ca–P treatments resulted in P_{lac} values between 177.3 and 183.1 $mgPkg^{-1}$ on the fifth day and between 220.7 and 228.8 $mgPkg^{-1}$ on the tenth day. Within the Ca–P treatments, significantly higher P_{lac} values than the control were found on the tenth inoculation day for *P. putida*, *B. thuringiensis*, and *P. corrugata*.

The largest differences in pH, both between sampling dates and between the control and PSB treatments, were observed in the Al–P treatments (Fig. 2). In the Fe–P treatments, much smaller differences in pH were found between the control and the PSB treatments. However, for *P. putida* and *P. corrugata*, which were the most effective PSB, a decrease in the pH was found compared to the control. No significant decreases in pH were observed for the PSB-inoculated Ca–P treatments compared to the control, although a decreasing trend was noticed for *B. brevis*, *P. putida*, and *P. corrugata*.

Soil experiment

The P_{lac} ranged between 351.3 and 385.4 $mgPkg^{-1}$ for soil 1 and between 460.2 and 533.0 $mgPkg^{-1}$ for soil 2 (Table 5). Significantly higher P_{lac} values were found for the inoculation with *B. brevis* than in the control for both soils. For soil 2, a significantly higher P_{lac} value was found with *P. putida*. The P_w values ranged between 18.1 and 19.9 $mgPkg^{-1}$ for soil 1 and between 33.8 and 38.1 $mgPkg^{-1}$ for soil 2 (Table 5). Significantly higher P_w values than the control

Table 4 Mean P_{lac} (in milligrams per kilogram sand±standard deviation) measured on the fifth and tenth incubation days for the Al–P, Fe–P, and Ca–P treatment

	Al–P		Fe–P		Ca–P	
	5th day	10th day	5th day	10th day	5th day	10th day
Control	2.9±0.8	5.8±1.1[a]	31.8±2.2	39.9±2.8[a]	181.5±3.5	220.6±2.2[a]
B. brevis	5.4±0.5*	6.9±1.6	35.4±3.2	39.0±2.1	183.1±0.9	220.7±3.4[a]
B. polymyxa	3.2±0.6	2.9±0.1	29.5±3.0	39.9±2.6[a]	175.8±2.0	223.6±2.6[a]
P. putida	5.2±0.7*	6.8±0.8[a]	31.9±2.8	39.3±3.2[a]	181.0±1.1	228.2±2.3[a]
B. thuringiensis	3.2±0.3	3.7±0.2	32.5±3.7	40.7±2.3[a]	177.3±1.1	228.8±6.0[a]
P. corrugata	4.0±0.7*	4.9±0.4[a]	29.0±2.2	38.8±1.6[a]	178.3±1.3	228.3±5.9[a]

*$p<0.05$, significantly higher than the control

[a]Significantly different between the measuring dates

for soil 1 (after week 2) were found with the single and dual inoculations of *B. brevis* and *P. putida*. We observed a small but significant decrease in pH in week 4 in all PSB-inoculated soils (Table 5).

Discussion

Growth media experiment

Several methods have been used for screening the efficiency of PSB to solubilize P, but to our knowledge, almost all of these used low amounts of the insoluble P source in solid or liquid media. Additionally, all these studies used Ca–P as the sole source of P at a pH of 7 (Mehta and Nautiyal 2001; Nautiyal 1999; Rosas et al. 2006). In one study, the solubilizing efficiency of *Burkholderia* spp. was investigated on $AlPO_4$ (Delvasto et al. 2008), and in another study, the toxicity of Al to *B. megaterium* was investigated (Davis et al. 1971). Here, we assessed for the first time the performance of PSB on insoluble Fe–P and Al–P and thus in media of acidic nature. Moreover, we used total added P concentrations that were much larger than in previous studies because we wanted to mimic conditions in soils with high levels of phosphate saturation.

Traditionally, the clearing/halo zone around the colony is used as an indicator for P solubilization (Mehta and Nautiyal 2001). However, it has been reported that many fungi or PSB that did not produce any halo zone on agar plates (Collavino et al. 2010; Delvasto et al. 2008) were able to solubilize insoluble inorganic phosphates in liquid medium (Leyval and Berthelin 1989; Mehta and Nautiyal 2001). Because the evaluation of P solubilization with the plate

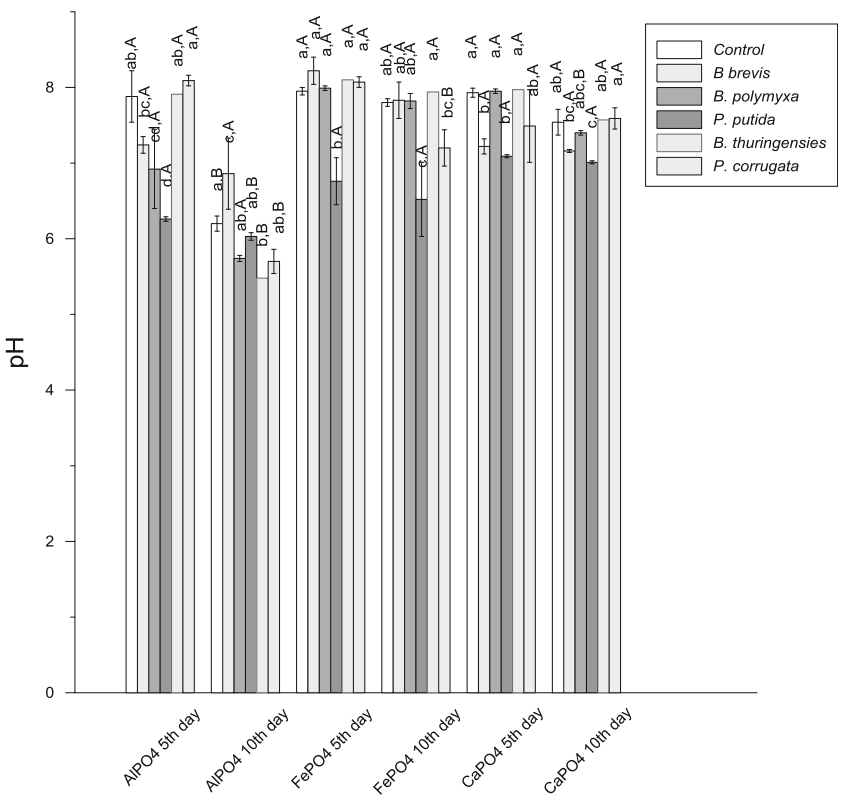

Fig. 2 Changes in pH–KCl measured on the fifth and tenth incubation days in a sand medium for the control and the inoculated samples for the Al–P, Fe–P, and Ca–P treatments ($p<0.05$, significance between the control and PSB is marked in *lowercase letters* and significance between days in treatments is marked in *uppercase letters*)

Table 5 P_{lac}, P_w, and pH (mean±standard deviation) at the end of incubation weeks 2 and 4 for soil 1 and soil 2

Treatment		P_{lac} (mg kg^{-1} soil)		P_W (mg kg^{-1} soil)		pH–KCl	
		Week 2	Week 4	Week 2	Week 4	Week 2	Week 4
Soil 1	Control	370.4±8.3	365.8±7.3	18.1±0.6	19.9±0.5	5.01±0.03	5.04±0.02
	B. brevis	384.4±5.9*	360.1±4.3	19.3±0.2*	19.6±0.3	5.03±0.01	4.99±0.01*
	P. putida	371.4±2.3	359.4±8.9	19.2±0.7*	19.9±0.3	4.96±0.04	4.93±0.01*[a]
	B. brevis+*P. putida*	385.4±3.2*	351.0±5.1	19.8±0.5*	19.6±0.2	5.00±0.01	4.94±0.01*
Soil 2	Control	466.1±9.2	463.3±7.6	35.3±1.4	38.1±0.3	4.23±0.08	4.14±0.01
	B. brevis	477.0±0.3*	472.2±2.7*	35.4±0.5	36.5±0.6	4.15±0.01*	4.09±0.01*
	P. putida	480.7±6.3*	479.8±6.1**	35.7±0.2	37.5±0.8	4.15±0.02	4.07±0.01*
	B. brevis+*P. putida*	533.0±4.6**	460.2±3.4	33.8±2.2	36.2±0.7	4.17±0.03	4.09±0.01*

*$p<0.05$, **$p<0.01$; significantly higher than the control

[a] Significantly different between two sampling dates

assay by halo zone is not always conclusive and because Malboobi et al. (2009) found that the active growth of bacteria correlates well with P solubilization, the colony growth diameter of the PSB should be viewed merely as a first indication of the P solubilization potential, thereby proving that the halo zone is not the best indicator for P solubilization and more interest is given on the effect that the treatment had on the colony growth diameter. An increase in the insoluble P concentration tended to have no negative effects on the colony growth diameters in the Fe–P and Al–P treatments. For the Ca–P treatment, a significant decrease in colony growth was found for *B. polymyxa*, suggesting that this species is not an effective PSB under high Ca–P conditions. *B. brevis*, *P. putida*, and *B. thuringiensis* had higher colony growth diameters under high Ca–P conditions, showing that they were able to adapt and solubilize P in high Ca–P environments. Problems of P saturation and P leaching are mostly found in acidic sandy soils with high amounts of P associated with Al and Fe compounds. If PSB are to be effective in increasing P mining efficiency in such soils, it is important that they are able to solubilize Al–P and Fe–P and that they continue to be effective under conditions of high total P. Our results show that PSB were equally efficient in solubilizing Al–P and Fe–P as in solubilizing Ca–P. *B. polymyxa* performed even better under high insoluble Al–P and Fe–P treatments than under high insoluble Ca–P treatments. The ability of these PSB to grow and solubilize P under the specific conditions as imposed in this experiment is an important finding with respect to increasing mining efficiency in P-saturated soils.

The increase in colony diameter following an increase in N concentration as observed for most of the PSB, indicating that environments with high N content are more conducive to the proliferation of these PSB, which contradicts the findings of Nautiyal (1999). Exceptions to this were *B. brevis* in the Fe–P environment and *B. thuringiensis* in the Al–P environment. The improved performance of the PSB under higher N conditions would be a clear advantage for using them in P-saturated soils. Indeed, such soils mostly have an overall high chemical fertility, including high mineral N availability as a result of, e.g., mineralization.

Sand experiment

To our knowledge, PSB efficiency without plants has never been tested in a sand medium. The sand medium provides an intermediary situation between the growth media (more realistic than growth media) and natural soil (sand medium allows more control over the experiment). The analysis of P_w provides a close approximation of the actual P concentration in the soil solution (i.e., directly plant-available P), without creating an acidic environment and thereby avoiding an overestimation of the P solubilization for the insoluble Ca–P treatments. The P_w values for the Ca–P treatments were one order of magnitude higher than the P_w values for the Al–P and Fe–P treatments, which can be explained by the differences in solubility products of these P sources. The solubility product of Fe–P is 5 times smaller than that of Al–P and 60 times smaller than that of Ca–P. *B. brevis*, *P. corrugata*, and *P. putida* prove to be most effective PSB in solubilization of fixed P to Al or Fe out of the P_w results. The most effective PSB here are consistent with the ones that were most efficient in the growth medium experiment.

The inoculation with PSB did not always increase P_{lac} values as compared to the uninoculated control samples. This could be explained by a temporal P immobilization by the PSB, which would imply that the P is solubilized, yet not in the soil solution. The fact that *B. polymyxa* and *B. thuringiensis* are solubilizing less P in the Al treatment could be explained by Al toxicity for these PSB, which is in agreement with the results of an Al toxicity test done on

Bacillus sp. (Davis et al. 1971). This indicates that *Pseudomonas* sp. are able to survive in media with high Al amounts, in agreement with the findings of Illmer and Schinner (1999). The results from the growth media and sand medium experiment prove that the selected PSB are efficient for P mining in high total P conditions. Almost always, an increase in P_{lac} was found over time, indicating the continuity of the P solubilization efficiency of the PSB. *P. putida*, *P. corrugata*, and *B. brevis* proved to be the most efficient PSB for all insoluble P treatments, which is in agreement with results from other experiments using Ca–P (Kuiper et al. 2002; Manna et al. 2001; Villegas and Fortin 2002). These PSB were most efficient for both experiments, growth medium and sand, thereby confirming that the colony growth diameter of the PSB is an indicator for testing the P solubilization efficiency of the PSB.

A decrease in the pH after inoculation with PSB has been considered as one of the mechanisms by which the PSB transform the insoluble Ca–P into a plant-available P form (Gyaneshwar et al. 2002; Illmer et al. 1995; Nautiyal 1999). It has been found that *P. putida* solubilize P by lowering the pH as a result of organic acid production (Vyas and Gulati 2009). This was also confirmed with a significant decrease in pH for the samples with inoculation of *P. putida* for Al–P and Ca–P treatments. However, no correlation was found between the pH and the P solubilization for the Fe–P treatments. The absence of correlation in the case of Fe–P might indicate that other mechanisms, such as chelation and/or ligand exchange, were more important than a decrease in pH (Gyaneshwar et al. 2002; Whitelaw 2000).

Soil experiment

To the best of our knowledge, P solubilization by PSB has never been studied in acidic soils with high P concentrations. An increasing trend in plant-available P (P_{lac}) was found for both of the acidic sandy soils with PSB inoculation compared to the control, except after week 4 for soil 1, but the effects were more pronounced in soil 2 which had a higher total P content. This confirms that PSB are able to solubilize P in soils rich in total P. The relatively higher values of P_{lac} for soil 2 inoculated with *P. putida* compared to the control can be explained by the better adaption of *P. putida* to lower pH conditions (Villegas and Fortin 2002).

The dual inoculation of *P. putida* and *B. brevis* was more efficient in P solubilization than the control and mostly more efficient than separate inoculation. Our results are in agreement with other research where dual inoculations (mainly using PSB and fungi or N-fixing bacteria) are reported to perform better and have a higher P availability than found for separate inoculation (Kim et al. 1998; Rosas et al. 2006).

The efficiency of the PSB to solubilize P was found for both methods P_w and P_{lac}. This proves that the PSB can be used for P mining since P_w is an indication of the easily available P in the soil and P_{lac} indicates P availability for the plant, two indicators which are important for P mining of soils.

PSB are known for lowering the pH of the inoculated media by producing organic acids (Arcand and Schneider 2006; Rodriguez and Fraga 1999), which was also found in our results. However, the differences in the soil experiment were smaller than in the sand experiment because of the buffering capacity of the soil. The absence of a significant correlation effect between lowering of pH and increase in P_{lac} indicates that the PSB solubilize P in these acidic sandy soils through chelation or ligand exchange as a result of organic acid production, which is in agreement with the results for sand experiment for Fe treatment and with previous studies (Arcand and Schneider 2006; Vyas and Gulati 2009).

Further research is currently ongoing to evaluate the effect of PSB in bigger pot and field experiments. Preliminary results from two pot experiments confirm the conclusions from this research. In one pot experiment, PSB (a mixture of *B. brevis*, *P. putida*, and *P. corrugata*) were tested on two acidic sandy soils (one with high total P content and one with low total P content) without plants. In both soils, the amount of easily available P (P_w) was higher in the treatments with PSB addition than in the untreated controls (data not shown). In a second bigger pot experiment, using three soils with different total P content and cropped with grass, inoculation with the same PSB increased the P availability to the growing crop (data not shown), demonstrating the potential of PSB inoculation for increasing P depletion rates in soils high in P.

Conclusions

The present study examined the P solubilization efficiency of PSB under high insoluble P conditions. In a growth media experiment, all five tested PSB species were able to grow on all the different growth media, i.e., the tested PSB were able to adapt to high insoluble P conditions. When the same PSB were inoculated in sand, to create more realistic conditions, they proved again that they could solubilize P in high P conditions. The addition of *B. brevis*, *P. putida*, and *P. corrugata* resulted in significantly higher available P concentrations than in the control. Especially *B. brevis* and *P. putida* exhibited the most promising capacities to solubilize P in high insoluble P conditions. This was especially true in the Al–P and Fe–P treatments, which indicates their ability to solubilize P in acidic sandy soils with a high total P content. Further research is needed to evaluate the effect of PSB in bigger pot and field experiments.

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