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ORIGINAL PAPER

Isolation of phytase-producing bacteria from Himalayan soils and their effect on growth and phosphorus uptake of Indian mustard (*Brassica juncea*)

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Abstract Phytase-producing bacteria (PPB) is being investigated as plant growth promoting rhizobacteria (PGPR) to improve the phosphorus (P) nutrition and growth of plants grown in soil with high phytate content. Phytate is dominant organic P forms in many soils and must be hydrolyzed to be available for plants. Indian mustard (*Brassica juncea*) is a plant with economic importance in agriculture and phytoremediation, therefore biotechnological tools to improve growth and environmental stress tolerance are needed. In this study, we isolated and characterized PPB from Himalayan soils and evaluated their effect on growth and P uptake by *B. juncea* under greenhouse conditions. Sixty five PPB were isolated and based on phytate hydrolysis, three efficient PPB were chosen and identified as *Acromobacter* sp. PB-01, *Tetrathiobacter* sp. PB-03 and *Bacillus* sp. PB-13. Selected PPB showed ability to grow at wide range of pH, temperature and salt concentrations as well as to harbour diverse PGPR activities, such as: solubilization of insoluble Ca-phosphate (193–642 μg ml^{-1}), production of phytohormone indole acetic acid (5–39 μg ml^{-1}) and siderophore. *Tetrathiobacter* sp. PB-03 and *Bacillus* sp. PB-13 showed 50 and 70 % inhibition of phytopathogen *Rhizoctonia solani*, respectively. Greenhouse potting assay also showed that the bacterization of *B. juncea* seeds with *Tetrathiobacter* sp. PB-03 and *Bacillus* sp. PB-13 significantly increased the biomass and P content in 30 days old seedlings. This study reveals the potential of PPB as PGPR to improve the growth of *B. juncea*.

Keywords Himalayan soil · Mustard · PGPR · Phytase · Phosphorus · Rhizosphere

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Introduction

In the rhizosphere, bacteria play a pivotal role in the transformation and mobilization of macro- and micronutrients in soils, with the subsequent improvement of the nutritional status of plants (Jha et al. 2012). Among various macronutrients for plants, P is present in soils as insoluble inorganic and organic forms with poor availability to plants (Richardson et al. 2009). In the rhizosphere, the conversion of the insoluble forms of inorganic P to a form accessible by plants is achieved by phosphate-solubilizing bacteria (PSB) which release phosphates meanly by organic acids releasing. Thus, PSB belonging to *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Kluyvera*, *Streptomyces*, *Pantoea* and *Pseudomonas* genera, have been widely studied for increase the growth and yield of crop plants (Hariprasad and Niranjana 2009; Martínez-Viveros et al. 2010; Kumar et al. 2011). However, organic P forms, particularly phytates, are predominant in most soils (10–50 % of total P) (Turner et al. 2002; Mullen 2005) and must be mineralized

by phytases (*myo*-inositol-hexakisphosphate-phosphohydrolases) to be available P for plants (Hayes et al. 1999; Richardson 2001). In this context, bacteria with both activities, production of organic acids to solubilise inorganic P and production of phytase to mineralise phytate, have been isolated from rhizosphere (Jorquera et al. 2008, 2011) and proposed as potential PGPR to be used in soil with high content of organic P. Studies have also revealed that PPB not only harbour the ability to mineralize phytate but also other PGPR activities, such as the production of indole acetic acid, siderophore, volatiles and ammonia (Martínez et al. 2011; Saharan and Nehra 2011).

Indian mustard is an important plant in food and environmental science with ability to growth in both tropical and sub-tropical regions of the world (Dileep Kumar 1999). *B. juncea* is also considered to be one of the most promising species for phytoremediation to remove heavy metals from contaminated soils (Saxena et al. 1999). In contaminated soils and other harsh environments (deserts), factors that limit plant growth include arid conditions, low water supply and nutrient deficiency (including P). Therefore, improvement of plant growth under stressed conditions is crucial for optimum phytoremediation performance of *B. juncea* (Belimov et al. 2005). In this context, a recent study describes the occurrence of PGPR carrying phytase gene associated to ancient plants in the Mohave desert, USA (Jorquera et al. 2012). Thus, PPB containing diverse PGPR activities represents a potential biotechnological tool to be considered in the improving of yield and tolerance of economical relevant plant grown in diverse agro-ecosystems.

The objectives of present study were to isolate and characterize PPB from Himalayan soils and evaluate their effect on growth and P uptake of Indian mustard under greenhouse conditions.

Materials and methods

Isolation of phytate-hydrolyzing bacteria

Phytase-hydrolyzing bacteria were isolated from Himalayan soil samples taken from Uttarakhand region, northern India (Badrinath, 30.73°N 79.48°E). One gram of soil sample was added to 9 ml of sterile saline solution, vigorously shaken for 20–30 min and serial dilutions were plated onto Luria–Bertani broth (LB; 10 g l^{-1} D-glucose, 5 g l^{-1} yeast extract, 10 g l^{-1} tryptone) supplemented with 10 mM Na-phytate ($Na_{12}C_6H_6O_{24}P_6$) and incubated at 37 °C for 48 h. Sixty five isolates were screened for their ability to hydrolyze phytate on the phytase screening medium agar (PSM; 10 g l^{-1} D-glucose, 4 g l^{-1} Na-phytate, 2 g l^{-1} $CaCl_2$, 5 g l^{-1} NH_4NO_3, 0.5 g l^{-1} KCl, 0.5 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.01 g l^{-1} $FeSO_4{\cdot}7H_2O$, 0.01 g l^{-1} $MnSO4{\cdot}H_2O$) described by Kerovuo et al. (1998). In vitro phytase activity of isolates was assayed according to counterstaining technique described by Yanke et al. (1998). Briefly, the technique involved flooding of the plate with 2 % cobalt chloride for 5 min, after which the solution was replaced with freshly prepared colouring reagent containing equal volumes of 6.25 % (w/v) aqueous molybdate and 0.42 % (w/v) ammonium vanadate solution and incubated for 5 min at room temperature. Colonies exhibiting clearing zones after removal of the chromogen were separated and stored on slants for further study in liquid cultures. Thus, based on phytate hydrolyzing activity, estimated according to the clear halo zone (diameter) onto PSM media, three efficient phytase-producing strains (PB-01, PB-03 and PB-13) were selected for characterization tests, including both phosphate solubilising and phytate mineralizing activity, and their plant growth promotion potential.

Characterization of selected isolates

Identification based on morphological, physiological and biochemical characteristics of selected bacterial isolates were carried out by Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Additionally, Gram staining was performed using Himedia Gram Stains-Kit (K001-1Kt) and growth in LB broth at different temperatures (from 10 to 55 °C), pH (from 5 to 11) and salinities (from 0.5 to 8.5 % NaCl) was examined by spectrophotometry at 600 nm.

Genetic characterization based on 16S rRNA gene sequence was also done. Briefly, genomic DNA from selected isolates was extracted as described by Neumann et al. (1992) and PCR amplification of 16S rRNA gene was carried out by using the primers: RDNA-1A (5′-AGA GTT TGA TCC TGG CTC AG-3′) and RDNA-1B (5′-AAG GAG GTG ATC CAG CCG CA-3′). The PCR was done as follow: a hot-start of 94 °C for 3 min followed by 35 cycles of 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1.5 min, and a final extension for 10 min at 72 °C. Amplified PCR products were purified with QIAquick Gel Extraction kit (Qiagen, Germany) and sequenced in an automated DNA sequencer (Applied Biosystems 3730) at DNA Sequencing Facility, University of Delhi (South Campus), New Delhi, India. The sequences obtained were compared with sequences in the GenBank database from the National Centre for Biotechnology Information (NCBI) using blastn program (http://blast.ncbi.nlm.nih.gov/) and then deposited in GenBank under accession number of JN616417, JQ727431 and JN616416 for PB-01, PB-03 and PB-13 strains, respectively. The phylogenetic affiliations of selected isolates in relation to some PPB deposited in GenBank was analyzed using MEGA5 program (http://www.megasoftware.net/).

Phytase production and phosphate-solubilising activities

Extracellular phytase production was checked in bacterial culture grown in PSM and determined according to the method described by Engelen et al. (1994). The reaction mixture contained 1 ml of crude enzyme preparation and 2 ml of 5 mM Na-phytate (in 0.1 M citrate buffer, pH 5.5). Reaction was carried out at 37 °C for 30 min and stopped by adding 2 ml freshly prepared colour reagent containing ammonium-molybdate. The colour developed from the phytase activity (P released) was determined at 415 nm in a spectrophotometer. One unit of phytase is defined as amount of enzyme liberating 1 μM P min^{-1}.

Qualitative plate assay for phosphate-solubilising activity by selected isolates was estimated by method of Pikovskaya (1948). Isolates were separately picked up from colony, inoculated on Pikovskaya's agar (0.5 g l^{-1} Yeast extract, 10 g l^{-1} Dextrose, 5 g l^{-1} Ca-phosphate, 0.5 g l^{-1} $(NH_4)_3PO_4$, 0.2 g l^{-1} KCl, 0.1 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.0001 g l^{-1} $MnSO_4 \cdot H_2O$, 0.001 g l^{-1} $FeSO_4 \cdot 7H_2O$, 15 g l^{-1} Agar, pH 6.8) plates and incubated at 28 °C for 7 days. The plates were then examined for halo zone around bacterial culture and solubilization Index (SI) was calculated as: SI = (colony diameter + halo zone diameter)/colony diameter (Edi-Premono and Vleck 1996). Additionally, phosphate-solubilising efficiency of selected isolates was measured in National Botanical Research Institute Phosphate (NBRIP; 10 g l^{-1} D-glucose, 5 g l^{-1} Ca-phosphate, 5 g l^{-1} $MgCl_2 \cdot 6H_2O$, 0.25 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.2 g l^{-1} KCl, 0.1 g l^{-1} $(NH_4)_2SO_4$, 15 g agar; Nautiyal 1999) cultures. Phosphorus content in supernatant was estimated at 24, 48 and 72 h of incubation by using ammonium-molybdate method as described above.

Screening for other PGPR activities

Siderophore, indole acetic acid, hydrogen cyanide, organic acids and ammonia production

Siderophore production was determined by using blue indicator dye and chrome azurol S agar (Schwyn and Neilands 1987). Bacterial isolates exhibiting orange halo zone on chrome azurol S agar after 5 days of incubation at 28 °C were considered positive for the production of siderophores. Indole acetic acid (IAA) production by bacterial isolates was determined in LB broth supplemented with L-tryptophan (500 μg ml^{-1}) at 24, 48 and 72 h according to described by Patten and Glick (2002). For this, bacterial cells were removed by centrifugation at 10,000 rpm for 5 min at 4 °C. One ml of the supernatant was mixed with 4 ml of Salkowski's reagent in the ratio of 1:4 and incubated at room temperature for 20 min. Development of a pink colour indicated indoles. The absorbance of supernatant mixture (supernatant + Salkowski's reagent) for indole production was measured at 530 nm and quantity of indoles was determined by comparison with a standard curve using an IAA standard graph.

For hydrogen cyanide (HCN) production the methodology described by Bakker and Schippers (1987) was used. Isolates were grown on plates of tryptic soy agar (10 %) amended with glycine (4.4 g l^{-1}) and $FeCl_3 \cdot H_2O$ (0.3 mM). A change from yellow to orange, red, brown, or reddish brown was recorded as an indication of weak, moderate, or strongly cyanogenic potential, respectively. Organic acid production potential of different isolates was analyzed using thin layer chromatography (TLC) on Silica-G (Merck chemicals) gel plates using different solvent systems.

Finally, ammonia production test was performed by growing selected isolated in peptone water for 72 h at 30 °C. Change in colour after addition of 1 ml Nessler's reagent (K_2HgI_4; 1.4 %) in each tube was observed. The presence of faint yellow colour indicates small amount of ammonia and deep yellow to brownish colour indicates maximum ammonia production.

Biocontrol activity

Biocontrol activity was assayed by employing dual culture technique and *Rhizoctonia solani* as phytopathogen model. Briefly, bacterial isolates were seeded at the edges of Petri plates containing potato dextrose agar (PDA; HiMedia Chemicals) and incubated for 72 h at 28 °C. After incubation, plates were inoculated with fungal spores and newly incubated at 28 °C for 7 days. The radii of the fungal colony towards and away from the bacterial colony were measured. The percentage of growth inhibition PI was calculated as: PI = (R–r)/R × 100; where r is radius of the fungal colony opposite the bacterial colony and, R is maximum radius of the fungal colony away from the bacterial colony (Idris et al. 2007).

Greenhouse experiment

Seeds and soils

Seeds of Indian mustard were surface-sterilized in laminar air flow with 1 % sodium hypochlorite for 30 s and then rinsed 2–3 times in sterile distilled water, air dried and used in the pot experiment. Soil was collected from the experimental plot of the university farm, dried at room temperature, sieved (2 mm mesh) and then mixed with cow dung manure (3:1 soil:manure). The soil mixture was autoclaved for 1 h and used in pot experiments. The chemical characteristics of soil mixture were: silty clay loam, 1.5 % organic acids content, pH 6.7, cation exchange capacity

0.17 $cmol_{(+)}$ kg^{-1}, K 235 kg ha^{-1}, N 200.7 kg ha^{-1}, P_{Olsen} 28.2 kg ha^{-1}.

Seed bacterization

Twenty five millilitres of bacterial inocula in LB media containing 1×10^8 c.f.u. ml^{-1} was added to 100 ml Erlenmeyer flasks with 100 mg of carboxy methyl cellulose (CMC) as an adhesive material. Ten gram seeds were soaked in bacterial suspension for 12 h on a rotary shaker under shaking (150 rpm). The bacterial suspension was drained off and the seeds were aseptically dried overnight in a laminar air flow chamber. Seeds soaked in sterile LB media amended with CMC served as control.

Pot experiments

Bacterized seeds were transfer to pots (20 cm diameter) and the plants were grown under greenhouse at 20–25 °C with a 16:8 h day:night regime and 60 % relative humidity. Thirty days old seedlings were sampled and analyzed for shoot length (SL), root length (RL), fresh weight (FW), dry weight (DW) and total P content. Seedlings were air dried, grinded and digested in 15 ml $HClO_4$ and 5 ml HNO_3. The spectrophotometric vanado-molybdate method was used to measure P (Engelen et al. 1994). Rhizosphere soil samples were collected from each treatment by uprooting the plants carefully without damaging the root system. Roots were shaken gently to remove loosely bound soil and rhizosphere soil samples from each treatment were then analyzed for variation in pH and available P content by Olsen's method (Murugesan and Rajakumari 2005). The pots were arranged in completely randomized block design with five replications in each treatment and each treatment repeated thrice.

Data analysis

Data from laboratory and greenhouse experiments were analyzed separately for each experiment and subjected to analysis of variance (ANOVA) (SPSS, version 7.5). Significant differences of treatments were determined by the magnitude of F value ($P \leq 0.05$).

Results

Characterization of selected isolates

Based on biochemical tests (Table 1) and analysis of 16S rDNA gene sequence (Fig. 1), the isolates were identified as *Achromobacter* sp. (isolate PB-01), *Tetrathiobacter* sp. (isolate PB-03) and *Bacillus* sp. (isolate PB-13). The three isolates showed motility and the ability to grow at wide range of temperature (10–42 °C) and pH (5–11), and produce catalase and acid from dextrose and fructose. Particularly, *Achromobacter* sp. PB-01 was the only isolate able to utilize salicin whereas *Tetrathiobacter* sp. PB-03 was the only isolate able to produce gelatinase and acid from lactose, dulcitol and cellobiose. In relation to *Bacillus* sp. PB-13, this strain showed fluorescence under UV and ability to produce endospore, urease and ornithine decarboxilase and utilize trehalose, xylose and starch. It is noteworthy that *Tetrathiobacter* sp. PB-03 and *Bacillus* sp. PB-13 were able to tolerate high NaCl concentrations (8.5 %).

Screening for PGPR activities

As described above, the three isolates were firstly chosen based on efficient phytase activity on PSM agar respect to other environmental isolates, and enzymatic assay showed a higher phytase activity in *Bacillus* sp. PB-13 (0.24 U ml^{-1}) compared with *Achromobacter* sp. PB-01 (0.14 U ml^{-1}) and *Tetrathiobacter* sp. PB-03 (0.15 U ml^{-1}) (Table 2). Assays on Pikovskaya's agar also showed that the three isolates were able to solubilise Ca-phosphate with a maximum solubilization index (2.0) by *Tetrathiobacter* sp. PB-03 followed by *Achromobacter* sp. PB-01 and *Bacillus* sp. PB13. In NBRIP broths (Fig. 2), higher P releasing in the supernatants was produced by *Tetrathiobacter* sp. PB-03 (582 and 642 μg ml^{-1}) and *Achromobacter* sp. PB-01 (601 and 626 μg ml^{-1}) at 48 and 72 h of incubation. In contrast, *Bacillus* sp. PB-13 showed the lower P releasing (227 and 193 μg ml^{-1}) at the same incubation time.

All the selected isolates were found positive for siderophores, IAA and ammonia production whereas negative for HCN production (Table 2). The higher IAA production in liquid cultures was obtained in LB broths inoculated with *Tetrathiobacter* sp. PB-03 (29 and 39 μg ml^{-1}) whereas the lower IAA production was observed with *Achromobacter* sp. PB-01 (<10 μg ml^{-1}) at 48 and 72 h of incubation (Fig. 2). The IAA production by *Bacillus* sp. PB-13 ranged between 15 and 20 μg ml^{-1}. Organic acid production was not detected in any culture filtrates as analyzed by TLC (data not shown). Respect to inhibition assay (Table 2), *Bacillus* sp. PB13 showed the highest inhibition percent (71 %) of *R. solani* followed by *Tetrathiobacter* sp. PB-03 (53 %), whereas isolate *Achromobacter* sp. PB-01 showed the lowest inhibit fungal growth (9 %) (Table 2).

Greenhouse experiment

In general, the inoculation with the three isolates resulted in an increase of all plant growth parameters measured in relation to uninoculated seedlings (Table 3). The inoculation of plants with *Tetrathiobacter* sp. PB-03 showed higher

Table 1 Morphological, physiological and biochemical characteristics of selected phytase-producing bacteria

Test	Isolates			Test	Isolates		
	PB-01	PB-03	PB-13		PB-01	PB-03	PB-13
Colony morphology				Utilization/assimilation of			
Shape	Oval	Oval	Oval	Glucose	−	−	−
Margins	Entire	Entire	Wavy	Arabinose	−	−	−
Surface	Smooth	Rough	Rough	Galactose	−	−	−
Color	Cream	Cream	Cream	Rhamnose	−	−	−
				Adonitol	−	−	−
Cell morphology				Sucrose	−	−	−
Shape	Rods	Rods	Rods	Raffinose	−	−	−
Size	Short	Moderate	Moderate	Dextrose	+	+	+
Arrangement	Single/pairs	Rods in chain	Single	Fructose	+	+	+
				Lactose	−	+	−
Growth				Dulcitol	−	+	−
Temperature	10–42 °C	10–42 °C	15–55 °C	Cellobiose	−	+	−
pH	5–11	5–11	5–11	Mannitol	+	−	+
NaCl (%)	0.5–2.5	0.5–8.5	0.5–8.5	Sorbitol	+	−	+
MacConkey agar	+[†]	−	−	Maltose	+	−	+
				Inositol	+	−	+
Production of				Salicin	+	−	−
Endospore	−	−	+	Citrate	+	+	−
H_2S	−	−	−	Trehalose	−	−	+
Lysine decarboxilase	−	−	−	Xylose	−	−	+
Arginine dihydrolase	−	−	−	Starch	−	−	+
Catalase	+	+	+				
Cytochrome oxidase	+	−	−	Gram Stain	−	+	+
Caseinase	−	−	−	Motility	+	+	+
Urease	−	−	+	Fluorescence (UV)	−	−	+
Ornithine decarboxilase	−	−	+	Nitrate reduction	+	−	+
Gelatinase	−	+	−	Voges-Proskauer reaction	−	−	+
				Methylred test	−	±	−

[+] Positive reaction; [−] negative reaction; [†] non lactose fermenting

weight (fresh and dry) and length (shoot and root) compared with other isolates and uninoculated seedlings. However, in relation to P content in plant tissues, the highest values (19 mg g^{-1}) were obtained in seedlings inoculated with *Bacillus* sp. PB-13 compared with untreated seeds. It is noteworthy, that analysis of available phosphorus in rhizospheric soil samples revealed higher P content in inoculated pots (8.9–10.5 mg kg^{-1}) compared with uninoculated pots (6.7 mg kg^{-1}). There were no significant changes in pH of soil samples collected from different seedlings.

Discussion

In recent past, rhizobacteria have been studied as biofertilizers and recognized as a potential alternative to chemical fertilizer (Martínez-Viveros et al. 2010). Considering the fact that plants are not able to utilize P directly from organic P, studies has been conducted with the objectives of determining the effect of PPB on plant growth (Idriss et al. 2002; Yadav and Tarafdar 2003; Hariprasad and Niranjana 2009; Singh and Satyanarayana 2010). In this study, we isolated diverse PPB strains from Himalayan soils and three efficient PPB were chosen and identified as member of *Burkholderiales* (*Achromobacter* sp. PB-01 and *Tetrathiobacter* sp. PB-03) and *Bacillales* (*Bacillus* sp. PB-13) order. A wide variety of PBB (*Bacillus*, *Paenibacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Serratia* and *Staphylococcus*) have commonly been isolated from the rhizosphere (Yoon et al. 1996; Richardson and Hadobas 1997; Hussin et al. 2007; Shedova et al. 2008; Jorquera et al. 2011). In relation to biochemical characteristics of selected, all selected PPB showed (a) a wide range of growth at different temperature and pH, (b) motility, (c) the production of catalase, and

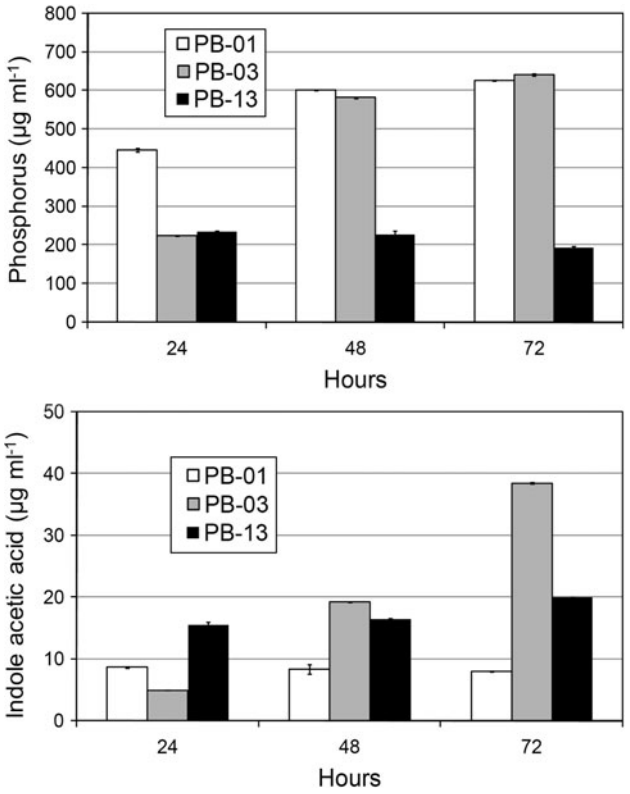

Fig. 1 Phosphorus solubilization and IAA production by selected isolates at different time intervals

(d) utilization of dextrose and fructose. However, they also showed differences in the majorities of tests analysed with a wide variety of characteristics. Production of enzymes and utilization/assimilation of diverse sugars by phytate-utilizing rhizobacteria have been reported by Jorquera et al. (2008) and Acuña et al. (2011).

The selected PPB strains showed diverse PGPR activities, such as production of siderophore, IAA and ammonia. Jorquera et al. (2008) isolated P-utilizing bacteria from the rhizospheres of five pasture plants (*Lolium perenne*, *Trifolium repens*, *Triticum aestivum*, *Avena sativa*, *Lupinus luteus*), which presented more than one mechanism for utilizing insoluble forms of phosphorus suggesting phosphate solubilization as a complex process. The production of IAA in vitro ranged between 8 and 34 µg ml^{-1}. Previous studies have reported IAA productions between 9 and 48 µg ml^{-1} in *Bacilli* rhizospheric strains (Lebuhn et al. 1997; Ali et al. 2010; Acuña et al. 2011). However, IAA production could also be influenced by diverse factors such as medium conditions (pH), availability of substrates, presence of organic acids and metals and growth stage (Frankenberger and Arshad 1995; Martínez-Viveros et al. 2010; Jha et al. 2012). In relation to P releasing, the phytase activity of selected PPB ranged between 0.14 and 0.2 U ml^{-1}. Acuña et al. (2011) reported phytase activity of 0.14 U mg^{-1} proteins with *Bacillus* sp. MQH-19 isolated from rhizosphere of pasture plants. The P solubilization in NBRIP media also depends on availability of substrate, strain used, phosphates activity and pH of media. Analysis of pH variation of soil samples collected from pots under study after 30 days did not show any significant change in pH between soil with treated and untreated seedlings. Alkaline pH of soil used in this study favoured two factors i.e., phytase activity and siderophores production, as bacterial phytases are more specific towards phytate and works well under neutral to alkaline conditions (Lei and Porres 2003; Oh et al. 2004), and production of siderophores by bacteria occurs in response to iron deficiency which normally occurs in neutral to alkaline pH soils, due to low iron solubility at elevated pH (Sharma and Johri 2003). The P content of soil samples was positively correlated with phytase activity of different isolates reveals their survival or growth abilities under stabilized pH.

Table 2 PGPR activities of selected phytase-producing bacteria

Test	Isolates		
	PB-01	PB-03	PB-13
Phytase activity (U ml^{-1})	0.14 ± 0.02	0.15 ± 0.00	0.24 ± 0.01
Solubilization/mineralization of			
Ca-phosphate	+	+	+++
Na-phytate	+	+	++
Index solubilization	1.66	2.0	1.25
Production of			
Siderophore	+	+++	+++
Indole acetic acid	+	+	+
Hydrogen cyanide	−	−	−
Ammonia	+	++	++
Rhizoctonia solani inhibition (%)†	8.4 ± 0.33	50.67 ± 0.57	69.24 ± 0.88

+ Positive reaction; − negative reaction; † in relation to 83.33 % of *R. solani* inhibition with 50 mg kg^{-1} of fungicide (Mancozeb)

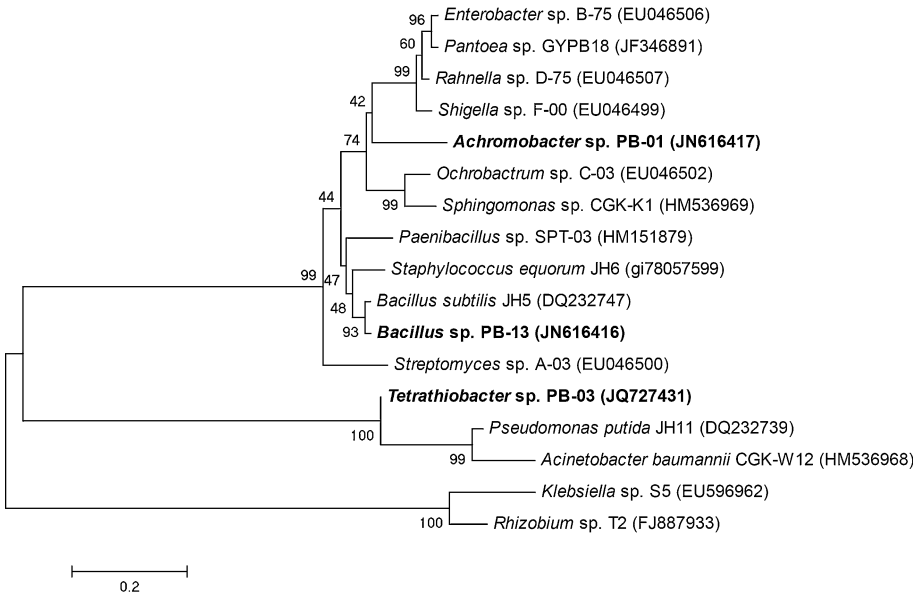

Fig. 2 Phylogenetic tree showing the phylogenetic affiliation of the selected phytase-producing bacteria (PPB) in relation to representative 16S rRNA gene sequences of culturable phytate-hydrolyzing bacteria taken from GenBank. The neighbor-joining tree was constructed using MEGA5. The bar indicates 20 % sequence divergence and a bootstrap analysis was performed with 1,000 trials. The *bold characters* indicate PPB genes identified in this study

Table 3 Effect of phytase-producing bacteria inoculation on plant growth parameters of seedlings after 30 days in greenhouse

Parameter	Uninoculated*	Inoculation*		
		PB-01	PB-03	PB-13
Fresh weight (mg)	182 ± 3^{d}	757 ± 9^{b}	$1{,}520 \pm 17^{a}$	240 ± 10^{c}
Dry weight (mg)	39 ± 20^{d}	86 ± 1^{b}	378 ± 1^{a}	48.8 ± 2.3^{cd}
Shoot length (cm)	6.7 ± 0.1^{c}	8.4 ± 0.1^{bc}	13.4 ± 0.1^{a}	9.6 ± 0.2^{b}
Root length (cm)	3.5 ± 0.02^{c}	4.2 ± 0.1^{bc}	6.7 ± 0.1^{a}	3.5 ± 0.1^{c}
Phosphorus content (mg g^{-1})	14.3 ± 2.0^{c}	17.4 ± 1.4^{ab}	16.1 ± 5.5^{bc}	19.1 ± 0.9^{a}

* Values are the mean with in row sharing the same letters are not significantly different according to Tukey's HSD at $P \leq 0.05$

Irrespective of in vitro P solubility potential of different isolates, it is reported that due to buffering capacity of soil, stabilization of pH reduces solubilization potential by bacteria (Ae et al. 1991; Hariprasad and Niranjana 2009), hence the phosphate solubilization under these conditions might depends mainly on phytase production. In addition, Martinez et al. (2011) recently observed significant differences of bacterial growth and P releasing as affected by N sources (urea and ammonium sulphate) used in NBRIP medium.

Interesting, two isolates (*Tetrathiobacter* sp. PB-03 and *Bacillus* sp. PB-13) showed significant inhibition of phytopathogen *R. solani*. Generally, *R. solani* is eradicated from the seeding substrate by fumigation with methyl bromide, but this toxic fumigant is gradually prohibited throughout the world (Dhingra et al. 2004), thus, the use of selected PPB might be a safe prophylaxis alternative for *R. solani*.

In relation to greenhouse experiment, higher plant growth and P content in tissues was scored in seedlings raised from bacterized seeds with selected PPB compared with uninoculated controls. Unno et al. (2005) isolated diverse *Burkholderia* strains from white lupin rhizosphere and some of them significantly promoted the growth of lupin. Moreover, *Bacilli* strains with varying P solubilization and IAA production abilities have also been shown to promote the growth of maize and wheat (Beneduzi et al. 2008; Trivedi and Pandey 2008). The inoculation of cucumber plant with *Pseudomonas* strain (YC-A18) carrying phytase gene resulted in higher plant biomass respect to control when plants were grown in sterile soils (Jorquera et al. 2012). It is noteworthy that taken in account all parameters evaluated (P releasing, production of IAA, *R. solani* inhibition, increase of biomass and P content in seedlings), *Tetrathiobacter* sp. PB-03 and *Bacillus* sp. PB-13were found to be most effective in relation to *Achromobacter* sp. PB-01.

Conclusions

Selected phytase-producing bacteria from Himalayan soils showed ability to grow at wide range of pH, temperature and salt concentrations as well as to harbour diverse PGPR activities, including production of siderophore and indole

acetic acid, releasing of P from insoluble inorganic phosphates and inhibition of phytopathogen *R. solani*. Also, the inoculation of *Brassica juncea* seeds with two isolates (*Tetrathiobacter* sp. PB-03 and *Bacillus* sp. PB-13) increased the biomass and P content in 30 days old seedlings. All these characteristics suggest their potential use as PGPR under various agro-climatic conditions, particularly to improve the growth and stress tolerance of *Brassica juncea* a plant with economic importance in agriculture and phytoremediation.

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References

Acuña JJ, Jorquera MA, Martínez OA, Menezes-Blackburn D, Fernández MT, Marschner P, Greiner R, Mora ML (2011) Indole acetic acid and phytase activity produced by rhizosphere bacilli as affected by pH and metals. J Soil Sci Plant Nutr 11:1–12

Ae N, Arihara J, Okada K (1991) Phosphorus response of chickpea and evaluation of phosphorus availability in Indian Alfisols and Vertisols. Phosphorus nutrition of grain legumes in the semi-arid tropics. Johansen C, Lee KK, Sahrawat KL (eds) Patancheru 502(324):33–41

Ali B, Sabri AN, Hasnain S (2010) Rhizobacterial potential to alter auxin content and growth of Vigna radiata (L.). World J Microbiol Biotechnol 26(8):1379–1384

Bakker AW, Schippers B (1987) Microbial cyanide production in the rhizosphere in relation to potato yield reduction and Pseudomonas SPP-mediated plant growth-stimulation. Soil Biol Biochem 19(4):451–457

Belimov A, Hontzeas N, Safronova V, Demchinskaya S, Piluzza G, Bullitta S, Glick B (2005) Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). Soil Biol Biochem 37(2):241–250

Beneduzi A, Peres D, da Costa PB, Bodanese Zanettini MH, Passaglia LM (2008) Genetic and phenotypic diversity of plant-growth-promoting bacilli isolated from wheat fields in southern Brazil. Res Microbiol 159:244–250

Dhingra OD, Costa MLN, Silva GJ Jr, Mizubuti ESG (2004) Essential oil of mustard to control *Rhizoctonia solani* causing seedling damping off and seedling blight in nursery. Fitopatologia Brasileira 29(6):683–686

Edi-Premono MM, Vleck PLG (1996) Effect of phosphate solubilizing *Pseudmonas putida* on the growth of maize and its survival in the rhizosphere. Indonasian J Crop Sci 11:13–23

Engelen AJ, Van der Heeft F, Randsdorp P, Smit E (1994) Simple and rapid determination of phytase activity. J AOAC Int 77(3):760

Frankenberger WT, Arshad M (1995) Phytohormones in soils: microbial production and function, vol 43. CRC, Boca Raton, FL

Hariprasad P, Niranjana S (2009) Isolation and characterization of phosphate solubilizing rhizobacteria to improve plant health of tomato. Plant Soil 316(1):13–24

Hayes JE, Richardson AE, Simpson RJ (1999) Phytase and acid phosphatase activities in extracts from roots of temperate pasture grass and legume seedlings. Funct Plant Biol 26(8):801–809

Hussin ASM, Farouk AEA, Greiner R, Salleh HM, Ismail AF (2007) Phytate-degrading enzyme production by bacteria isolated from Malaysian soil. World J Microbiol Biotechnol 23(12):1653–1660

Idris HA, Labuschagne N, Korsten L (2007) Screening rhizobacteria for biological control of Fusarium root and crown rot of sorghum in Ethiopia. Biol Control 40(1):97–106

Idriss EE, Makarewicz O, Farouk A, Rosner K, Greiner R, Bochow H, Richter T, Borriss R (2002) Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. Microbiology 148(7):2097

Jha CK, Patel B, Saraf M (2012) Stimulation of the growth of Jatropha curcas by the plant growth promoting bacterium Enterobacter cancerogenus MSA2. World J Microbiol Biotechnol 28(3):891–899

Jorquera MA, Hernandez MT, Rengel Z, Marschner P, De la Luz MM (2008) Isolation of culturable phosphobacteria with both phytate-mineralization and phosphate-solubilization activity from the rhizosphere of plants grown in a volcanic soil. Biol Fertil Soils 44(8):1025–1034

Jorquera MA, Crowley DE, Marschner P, Greiner R, Fernández MT, Romero D, Menezes-Blackburn D, De La Luz MM (2011) Identification of β-propeller phytase-encoding genes in culturable *Paenibacillus* and *Bacillus* spp. from the rhizosphere of pasture plants on volcanic soils. FEMS Microbiol Ecol 75(1):163–172

Jorquera MA, Shaharoona B, Nadeem SM, de la Luz Mora M, Crowley DE (2012) Plant growth-promoting rhizobacteria associated with ancient clones of creosote bush (*Larrea tridentata*). Microbial Ecol. doi:10.1007/s00248-012-0071-5

Kerovuo J, Lauraeus M, Nurminen P, Kalkkinen N, Apajalahti J (1998) Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis*. Appl Environ Microbiol 64(6):2079–2085

Kumar Dileep (1999) Fusarial wilt suppression and crop improvement through two rhizobacterial strains in chick pea growing in soils infested with *Fusarium oxysporum* f. sp. ciceris. Biol Fertility Soils 29(1):87–91

Kumar V, Singh P, Sangwan P, Verma AK, Agarwal S (2011) Phytase producing bacteria from Himalayan soil and their tricalcium phosphate solubilizing abilities. Ann Biol 27(2):121–128

Lebuhn M, Heulin T, Hartmann A (1997) Production of auxin and other indolic and phenolic compounds by *Paenibacillus polymyxa* strains isolated from different proximity to plant roots. FEMS Microbiol Ecol 22(4):325–334

Lei XG, Porres JM (2003) Phytase enzymology, applications, and biotechnology. Biotechnol Lett 25(21):1787–1794

Martínez OA, Jorquera MA, Crowley DE, de la Luz MM (2011) Influence of nitrogen fertilisation on pasture culturable rhizobacteria occurrence and the role of environmental factors on their potential PGPR activities. Biol Fertil Soils 47(8):875–885

Martínez-Viveros O, Jorquera M, Crowley D, Gajardo G, Mora M (2010) Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. J Soil Sci Plant Nutr 10(3):293–319

Mullen M (2005) Phosphorus in soils: biological interactions. Encycl Soils Environ 3:210–215

Murugesan A, Rajakumari C (2005) Techniques in biotechnology. In: Murugesan AG, Rajakumari C (eds) Environmental science and biotechnology-theory and techniques. MJP Publishers, Chennai, p 371

Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170(1):265–270

Neumann B, Pospiech A, Schairer HU (1992) Rapid isolation of genomic DNA from gram-negative bacteria. Trends Genet TIG 8(10):332

Oh BC, Choi WC, Park S, Kim YO, Oh TK (2004) Biochemical properties and substrate specificities of alkaline and histidine acid phytases. Appl Microbiol Biotechnol 63(4):362–372

Patten CL, Glick BR (2002) Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. Appl Environ Microbiol 68(8):3795–3801

Pikovskaya R (1948) Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Mikrobiologiya 17:362–370

Richardson AE (2001) Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. Funct Plant Biol 28(9):897–906

Richardson A, Hadobas P (1997) Soil isolates of *Pseudomonas* spp. that utilize inositol phosphates. Can J Microbiol 43(6):509–516

Richardson AE, Barea JM, McNeill AM, Prigent-Combaret C (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321(1): 305–339

Saharan B, Nehra V (2011) Plant growth promoting rhizobacteria: a critical review. Life Sci Med Res 2011:1–30

Saxena P, Krishnaraj S, Dan T, Perras M, Vettakkorumakankav N (1999) Phytoremediation of metal contaminated and polluted soils. Heavy metal stress in plants-From molecules to ecosystems. Springer, Heidelberg, pp 305–329

Schwyn B, Neilands J (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160(1):47–56

Sharma A, Johri B (2003) Growth promoting influence of siderophore-producing Pseudomonas strains GRP3A and PRS9 in maize (*Zea mays* L.) under iron limiting conditions. Microbiol Res 158(3):243–248

Shedova E, Lipasova V, Velikodvorskaya G, Ovadis M, Chernin L, Khmel I (2008) Phytase activity and its regulation in a rhizospheric strain of *Serratia plymuthica*. Folia Microbiol 53(2):110–114

Singh B, Satyanarayana T (2010) Plant growth promotion by an extracellular HAP-phytase of a thermophilic mold *Sporotrichum thermophile*. Appl Biochem Biotechnol 160(5):1267–1276

Trivedi P, Pandey A (2008) Plant growth promotion abilities and formulation of *Bacillus megaterium* strain B 388 (MTCC6521) isolated from a temperate Himalayan location. Indian J Microbiol 48:342–347

Turner BL, Papházy MJ, Haygarth PM, McKelvie ID (2002) Inositol phosphates in the environment. Philos Trans R Soc Lond B Biol Sci 357(1420):449–469

Unno Y, Okubo K, Wasaki J, Shinano T, Osaki M (2005) Plant growth promotion abilities and microscale bacterial dynamics in the rhizosphere of Lupin analysed by phytate utilization ability. Environ Microbiol 7(3):396–404

Yadav R, Tarafdar J (2003) Phytase and phosphatase producing fungi in arid and semi-arid soils and their efficiency in hydrolyzing different organic P compounds. Soil Biol Biochem 35(6):745–751

Yanke L, Bae H, Selinger L, Cheng K (1998) Phytase activity of anaerobic ruminal bacteria. Microbiology 144(6):1565–1573

Yoon SJ, Choi YJ, Min HK, Cho KK, Kim JW, Lee SC, Jung YH (1996) Isolation and identification of phytase producing bacterium, Enterobacter sp. 4, and enzymatic properties of phytase enzyme. Enzyme Microb Tech 18:449–454