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Ability of Phosphate-Solubilizing Bacteria to Enhance the Growth of Rice in Phosphorus-Deficient Soils

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Abstract

Phosphate solubilizing bacteria (PSB) are promising candidates for microbiological treatment to improve phosphorus-deficient or P-deficient soil. We have experimentally studied the growth promoting effects of PSBs on rice in P-deficient conditions. The 6 PSBs isolated from rhizosphere soil were identified by 16S rDNA sequencing as *Enterobacter asburiae* 30FPSSB1, *E. mori* NTTC11, *Priestia aryabhattai* KNB6, *P. aryabhattai* 49KNA2, *P. megaterium* 65KNA2, and *Bacillus* sp. 38DFWA. They exhibited plant growth promoting traits via phosphate solubilization and indole acetic acid (IAA) production respectively ranging within 32 - 42 mg/L and 5.3 - 340 μg/L. All the strains produced a large amount of siderophores, and some presented a broad range of antifungal activities reducing the growth of phytopathogens by 10 - 35 %. The effects of bacterial population on rice rhizosphere were evaluated on nutrient agar. Rhizobacterial treatments at high 10^5 - 10^8 CFU/mL concentrations inhibited plant growth in IAA dose dependent manner, but such effect diminished at low bacterial titers. The capacity of rhizobacteria to promote plant development under scarcity of P was investigated in pot experiments. Under axenic conditions, *Bacillus* and *Priestia* species promoted plant growth, whereas *Enterobacter* species suppressed it. Plants treated with *Bacillus* sp. 38DFWA, *P. aryabhattai* KNB6, and *P. megaterium* 65KNA2 had increased biomasses of shoots (by 33, 26 and 23 %, respectively) and roots (by 34, 48 and 48 %), which facilitated P availability and increased nutrient uptake in the plant tissues by 89, 96 and 143 %, when the inoculated plants were cultivated in insoluble P (tri-calcium phosphate, TCP) medium. In available P and TCP soil treatments, *P. aryabhattai* KNB6 and *E. mori* NTTC11 greatly increased rice plant biomass and gave the highest P accumulation in plant tissues. Interestingly, *P. aryabhattai* KNB6 strongly increased P uptake in plant tissue by 121 % and promoted rice growth by 22 % in P-deficient soil. This clearly demonstrates that *P. aryabhattai* KNB6 has great potential for use as a bio-inoculant, and is an attractive alternative to phosphate fertilizers.

Keywords: Plant growth promoting rhizobacteria, Phosphate solubilizing bacteria, PGPR, PSB, Rice, Phosphorus deficient soil, Phosphorus

Introduction

Phosphorus (P) is a macronutrient necessary for plant growth and development, because it is a structural constituent of essential biomolecules involved in the metabolic pathways of photosynthesis, biological oxidation, nutrient uptake, and cell division [1]. Most agricultural soils contain large reserves of P in both inorganic and organic forms, but the concentration of P that is available to plants is usually very low due to its poor solubility [2]. P-deficiency in agricultural soils is common worldwide, so farmers typically use chemical fertilizers containing soluble forms of P to maximize plant productivity. However, a plant can uptake only a small part of the soluble P from fertilizer, while most of it is lost to insoluble forms that depend on soil pH. Phosphorus is immobilized into insoluble forms by Al and Fe oxides in acidic soils, whereas it is fixed by calcium in alkaline soils. These 'insoluble' forms of low solubility are unavailable to support plant growth [3]. Excessive and repeated P-fertilizer use affects microbial diversity, damages soil fertility, and significantly decreases crop yields. Moreover, improper use of P-fertilizers in agriculture has increased both the costs and the environmental problems.

Bacterial biofertilizers are plant growth promoting rhizobacteria (PGPR) applied in the field to promote plant growth, reduce the need for synthetic fertilizer, and increase plant productivity. PGPR typically benefit plant growth via several mechanisms, such as symbiotic nitrogen fixation, phosphorus

dissolution, production of ammonia, siderophores, and phytohormones, diminishing plant stress by aminocyclopropane-1-carboxylate (ACC) deaminase activity, and control of phytopathogenic microorganisms through biological control agents [4]. Phosphate solubilizing bacteria (PSB) are naturally abundant rhizospheric microorganisms that can provide an alternative approach in sustainable agriculture to satisfy the P requirements for plant growth. They transform phosphate from insoluble forms into available forms, namely HPO_4^{2-} and $H_2PO_4^-$, through acidification, exchange reactions, and production of organic acids such as gluconic and citric acids [1,5]. On the other hand, PSBs can mineralize organic phosphorus to available inorganic forms by synthesis of several phosphatase enzymes [6]. Phosphate solubilization and mineralization abilities can coexist in the same bacterial strain [7]. Bacterial genera *Pseudomonas, Bacillus, Rhizobium, Agrobacterium, Burkholderia*, *Erwinia*, *Enterobacter*, along with the fungus *Penicillium,* are reported to be among the most powerful P-solubilizers in soil [7-10]. Several reports have introduced PSBs inoculants in plant rhizosphere to increase plant growth, P uptake, and plant productivity [9-12].

Rice (*Oryza sativa* L.) is the globally most important food crop, providing food for over half of the world's population. With the continuing increase in the worldwide population, the amount of rice production needs to also increase dramatically, encouraging overuse of chemical fertilizers and pesticides. This increases costs and has adverse effects on the environment and on human health. Therefore, the use of PSB bio-fertilizers to provide availability of nutrients and minerals, plant growth stimulating substances, and biological control agents, seems attractive as an economical and environmentally friendly sustainable agriculture strategy. In this study, plant growth promoting characteristics of rhizosphere bacteria and their antagonist activities against fungal pathogens were determined. The effects of bacterial rhizosphere population on plant growth were investigated. Finally, their potential to promote the growth of rice plants cultivated under P-deficient conditions was assessed. This study screened, in pot experiments that would need field validation, for an efficient strain to promote plant growth through use as a bio-inoculant and as a bio-fertilizer, especially in P-deficient soils.

Materials and methods

Microorganisms and culture conditions

The 6 bacterial strains used in this research, 30FPSSB1, NTTC11, KNB6, 49KNA2, 65KNA2, and 38DFWA, exhibiting phosphate-solubilizing activity, have previously been isolated from rhizosphere soil collected from Surat Thani and Songkhla provinces of Thailand [13]. Briefly, 5 g of soil was collected, diluted, and spread on Pikovskaya's medium (PVK). The culture plates were incubated at 30 °C for 3 days. Colonies surrounded by clear zones on this medium were selected and screened for phosphate-solubilizing activity and indole acetic acid production. The bacterial strain SM-P208 served as a reference strain for phosphate solubilizing activity; and the 5 pathogenic fungi, *Pyricularia oryzae*, *Curvalaria lunata*, *Helnimthosporium oryzae*, *Rhizoctonia solani*, and *Fusarium moniliforme* were used in antagonistic activity tests. The reference bacteria and pathogenic fungi were sourced from the Plant Protection Research and Development, Department of Agriculture, the Ministry of Agriculture and Cooperatives, Thailand. Bacterial inoculum was prepared by streaking bacteria on nutrient agar medium (NA) and incubating overnight at 30 °C; then a single colony was transferred to nutrient broth (NB) shaken at 150 rpm and incubated at the same temperature for 18 h. Bacteria were maintained in pure culture by storing in 20 % (v/v) glycerol and keeping at −80 °C until further use.

Identification of the bacterial isolates

Bacterial isolates were identified on the basis of 16S rDNA gene sequencing. The genomic DNA of isolates was extracted using the Genomic DNA mini Kit (Blood/culture cell) (Geneaid Biotech Ltd., Taiwan). The primers 20F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1500R (5′-GTTACCTTGTTACG ACTT-3′) were used for amplification of 16S rDNA gene [14]. The PCR amplification was programmed for initial denaturation step at 94 °C for 3 min; followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and elongation at 72 °C for 2 min; followed by a final amplification step at 72 °C for 3 min. The PCR product was analyzed in 0.8 % (w/v) agarose gel by electrophoresis and purified with Gel/PCR kit (Geneaid Biotech Ltd., Taiwan). Then, sequencing of the purified PCR products was performed on an ABI Prism® 3730XL DNA sequencer by a sequencing service provider. The sequences were edited by BioEdit Sequence Alignment Editor and the closest matching sequences in GenBank database were determined using BlastN at the National Center for Biotechnology Information (NCBI). The sequences from this study have been assigned GenBank accession numbers OM883933.1-OM883938.1 and OM915390.

Plant growth promoting activities

Bacterial plant growth promoting properties by phosphate solubilization, indole acetic acid (IAA) production, and siderophore production were assessed by standard methods as follows.

Phosphate solubilizing ability of each isolate was tested by culturing on the National Botanical Research Institute's Phosphate (NBRIP) growth medium containing (in $g.L^{-1}$) 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_26H_2O$, 0.25 g $MgSO_47H_2O$, 0.2g KCl, and 0.1g $(NH_4)_2SO_4$. Bacterial inoculum at 1 % (v/v) prepared by the above method was transferred to a 250 mL flask containing 50 mL NBRIP medium (pH 7.0) and incubated at 30 °C on a rotary shaker at 150 rpm for 5 days. One milliliter of cell culture was taken for determination of bacterial cell viability on standard plate count agar, and the remaining cell culture was centrifuged at 8,000 ×*g* for 10 min and passed through Whatman No.1. Quantitative determination of phosphate solubilization ability of the supernatant employed the molybdenum blue method, and the concentration of soluble phosphate was calculated from a standard curve for KH_2PO_4 [15]. The pH of filtrate culture medium was determined using a pH meter.

IAA production was analyzed by the Glickman and Dessaux method [16]. Bacteria were inoculated in NB supplemented with 0.1 % (w/v) L-tryptophan and incubated at 30 °C on a rotary shaker at 150 rpm. Cell culture was withdrawn after 48 h and centrifuged at 10,000 ×*g* for 10 min. Two mL of Salkowski's reagent was mixed with 1 mL of culture supernatant and incubated at room temperature for 25 min. The intensity of pink color was measured with a spectrophotometer at 530 nm.

Siderophore production was investigated using the O-CAS assay [17]. The overlay CAS medium was prepared according to Schwyn and Neilands method [18], modified to not have nutrients, and using agarose as the gelling agent at 0.9 % (w/v). CAS medium was applied on top of NA containing PSBs cultivated overnight. After a period of 15 min at the most, siderophore production was monitored by the development of orange halos surrounding colonies. Qualitatively siderophores were detected from the formation of an orange zone around the bacterial colony, and the intensity was categorized as low production (+), medium production (+ +), or high production (+ + +) [19].

Bacterial antagonism to fungal pathogens

Antagonistic activities of the PSBs against pathogenic fungi were tested on potato dextrose agar (PDA) plates using a dual culture technique [20]. Mycelia disc was placed in the center of a PDA plate and cultured at 30 °C for 3 days, then the exponential growth phase culture of each PSB was streaked 5 cm away from the fungal disc, and all the plates were incubated at 30 °C for 5 days. The inhibition of fungal growth was quantified by measuring the distance between the edges of bacterial and fungal colonies on the dual culture plate, and compared to the control plate (without bacteria). The zone of inhibition was calculated using the formula: Inhibition (%) = (C-T)/(C)×100, where C is the maximum growth of the fungal mycelia on control plate and T is fungal mycelia growth in dual culture.

Study of activity in promoting rice growth

Based on the PGR traits, various bacterial concentrations were explored for their potential to improve rice growth under axenic conditions. Rice seeds of the Jasmine Chiya variety were surface disinfected with 70 % (v/v) ethanol, followed by sterilization twice with 15 and 10 % (v/v) Haiter® (containing 6 % NaOCl) for 10 and 5 min, respectively. The sterilized seeds were washed several times with sterile distilled water and inoculated aseptically on half-strength Murashige and Skoog (1/2 MS) medium. Seeding cultures were maintained at 25 ± 2 °C under 16/8 h light/dark photoperiod with a light intensity of 3,000 Lux for 3 days. Bacterial solutions were prepared by cultivating the PSB strains until the mid-exponential phase in NB overnight with shaking. Cell cultures were harvested by centrifugation at 8,000 ×*g* for 10 min and the cell pellets were washed twice with sterile saline solution; then the concentrations were adjusted to the range from 10^3 to 10^8 CFU/mL prior to inoculation on rice seeds. Germinated seeds were selected, dipped in each bacterial concentration, and cultivated on MS medium that was reduced 20-fold in available phosphate. The medium was overlaid with sterilized sand on surface before plant transfer. Inoculated and non-inoculated seeds were incubated in a growth cabinet, in the conditions previously described. Plant growth characteristics were determined after 14 days of cultivation.

The suitable bacterial concentration without negative effects on plant growth was used to study PGPR ability under P-deficiency, by use of inoculated germinated seeds on medium having 2 phosphate sources: MS I (+KH_2PO_4 + TCP) was MS reduced 20-fold in the available phosphate plus 0.5 g/L of tri-calcium phosphate (TCP); and MS II (−KH_2PO_4+ TCP) was MS without available phosphate source. After 14 days of cultivation, the plants were taken out from bottles and their roots were washed with sterilized water to remove debris. Root elongation and shoot height were recorded as indicators of plant growth. Shoots and roots were oven dried at 105 °C for 24 h to determine the dry plant biomass. Oven-dried tissues were finely

ground, and root and shoot materials were mashed. The crushed samples were used to determine the total phosphorus concentrations in the roots and shoots by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).

Pot experiments

Selected PSBs that enhanced rice growth *in vitro* were tested for plant growth promotion in P-deficient soils. Sand lacking P, used as planting substrate, was prepared following [21], with some modifications. The sand was prepared by sieving (< 2 mm) and soaking in 1 M HCl for 24 h. Then, excess HCl in the sand was removed by tap water washing and it was dried at 35 °C for 7 days prior to planting. For seedlings, Jasmine Chiya rice seeds were soaked in sterilized water, placed in a sealed box with moist paper, and kept at room temperature for 48 h. Then, germinated seeds were transplanted into nursery tray containing sand without P. The seeds were grown in a greenhouse and watered daily. At 20 days after transplanting, the rice plants were pulled out and washed with sterilized water. Then, plant roots were incubated for 1 h in fresh bacterial solution prepared as previously described. The rice plants were re-transplanted into 10-inch pots that contained 4 kg of sand without P and watered daily. The plants were subjected to 3 treatments, without P, soluble P, and TCP (insoluble P) by providing nutrient solution for each treatment weekly [11]. All the treatment groups were placed in a greenhouse controlled for ambient irradiance, temperature and air humidity. All treatment groups were harvested at 60 days after transplanting. The plants were removed from pots and thoroughly washed with tap water. Then growth indicators and P concentrations were analyzed as previously described for the *in vitro* experiment.

Statistical analysis

The data were analyzed by one-way ANOVA for statistically significant differences ($p < 0.05$) between means that were *post hoc* tested by Duncan's multiple range test, and correlation coefficients were also checked using SPSS version 17.0.

Results and discussion

Plant growth promoting ability of isolated bacterial strains

Bacterial strains used in this study, isolated from rhizosphere soil, were identified as PSB strains based on halo formation on PVK medium. These isolates represented common soil bacteria belonging to the genera *Enterobacter*, *Priestia*, *Bacillus*, and *Pseudomonas* (**Table 1**). They presented a phosphate-solubilizing ability ranging from 22.3 to 42.0 mg/L and had significantly dropped pH of the liquid medium from 7.0 to 4.1 - 6.3 after 5 days of cultivation. The acidity of medium affected viability of *Enterobacter* species, but not those of *Priestia*, *Bacillus*, and *Pseudomonas* species. PSBs played an important role to plant growth and productivity through several mechanisms. One of the mechanisms is facilitating P solubility by producing organic and inorganic acids to transform insoluble phosphate to soluble phosphate. In this study, cell cultures of isolated PSBs decreased pH of the growth medium and increased soluble phosphate in liquid medium. Several reports claim that the bacterial species *Rhodococcus* sp., *Arthrobater nicotinovorans*, and *Pseudomonas* sp. could decrease pH of the culture medium and increase phosphate solubilization [11]. Similarly, Zhao *et al.* [22], observed the bacterial strain *Burkhoderia cepacia* and found a negative correlation between pH and available phosphate. This suggests that acidification of the medium could facilitate P solubilization. The release of organic acids like gluconic, ketogluconic, oxalic, succinic, etc., plays a major role in P solubilization by reducing pH of the soil, and replacing metal ions that usually form insoluble complexes, thereby releasing P in soluble form [10].

All the tested rhizobacteria were positive for IAA production. *E. asburiae* 30FPSSB1 and *E. mori* NTTC11 produced the highest concentrations of IAA (340 and 337 $\mu g.L^{-1}$) while the lowest production was seen by *Bacillus* sp 38DFWA and *Pseudomonas* sp. (5.3 and 18.1 $\mu g.L^{-1}$). *P. aryabhattai* 49KNA2, *P. megaterium* 65KNA2, and *P. aryabhattai* KNB6 were able to produce 97.0, 73.0 and 69.0 $\mu g.L^{-1}$, respectively (**Table 1**). PGPR are known to produce the plant hormone IAA, which is considered one of the most physiologically active phytohormones. IAA can improve plant growth increasing the size and weight of roots, root hairs, and lateral root numbers, allowing the plants to access more nutrients and water [12,23]. In addition, rhizobacterial IAA can help plant species to resist biotic and abiotic stress conditions [24]. The present study found that the isolated PSBs also produced other secondary metabolites, like siderophores. Siderophores are organic molecules with low molecular weight and function as ferric iron chelating agents. Siderophore producing bacteria have been reported to dissolve and reduce iron into availability for growth, and have retarded the growth of fungal pathogens by limiting their access to iron [20,25].

Antagonistic activity

The antifungal activities of isolated strains were tested against 5 rice pathogen fungi (**Table 2**). The isolates *E. asburiae* 30FPSSB1, *E. mori* NTTC11, *Bacillus* sp. 38DFWA, and *Pseudomonas* sp. SM-P208 showed a broad range of antifungal activities by inhibiting the growth of all test organisms by 10 - 35 %, but *P. aryabhattai* KNB6, *P. aryabhattai* 49KNA2, and *P. megaterium* 65KNA2 presented less antagonistic activity. We found a correlation between the siderophore production and the antagonistic inhibition in *Enterobacter* strains 30FPSSB1 and NTTC11, as well as *Pseudomonas* sp. SM-208. Pathogenic microorganisms in soil produce siderophores that act as a virulence factor. Under iron limitation, the siderophores produced by microorganisms made a ferric-siderophore complexes, but the other organism, especially phytopathogens, cannot utilize these. The producing strain can utilize this complex by a very specific receptor in its outer cell membrane, so the growth of pathogens is restricted by their lack of this receptor [25]. Prior studies have reported as advantages of siderophore producing microorganisms the control of phytopathogens, especially suppression of fungal growth and spore germination [25,26]. It is concluded that the antifungal activity and siderophore-producing performance of *Enterobacter* strains makes these bacteria potential biocontrol agents.

Table 1 The 16S rDNA sequence analysis and plant growth-promoting activities of PSBs.

Isolated strain	Isolated strains had homologous bacterial species in the GenBank database			Phosphate solubilization			IAA (μg/L)	Siderophore
	Bacterial species	Accession No.	Identity (%)	Solubilize P (mg/L)	pH	CFU× 10^6		
30FPSSB1	*Enterobacter asburiae*	OM883935.1	99.5	36.0 ± 0.1^{b}	4.2^{de}	ND	340.0 ± 20.0^{a}	+++
NTTC11	*Enterobacter mori*	OM883934.1	99.4	32.5 ± 0.2^{b}	4.1^{e}	ND	337.0 ± 7.0^{a}	+++
KNB6	*Priestia aryabhattai*	OM883933.1	98.9	36.6 ± 2.8^{b}	4.3^{d}	4.8	69.0 ± 1.0^{c}	++
49KNA2	*Priestia aryabhattai*	OM883937.1	100.0	42.0 ± 0.6^{a}	4.6^{b}	1,860	97.0 ± 5.0^{b}	++
65KNA2	*Priestia megaterium*	OM883938.1	99.9	41.1 ± 0.5^{a}	4.5^{bc}	2,380	73.0 ± 5.0^{bc}	++
38DFWA	*Bacillus* sp.	OM883936.1	99.8	22.3 ± 1.7^{c}	6.3^{a}	140	5.3 ± 0.1^{d}	++
SM-P208	*Pseudomonas* sp.	OM915390	99.0	32.6 ± 0.7^{b}	4.4^{cd}	3.25	18.1 ± 0.5^{d}	+++

The values are given as mean ± standard error of 3 replications, and different superscripts within a column denote significant differences ($p < 0.05$). Siderophore production is indicated by (+) positive/ low production; (++) medium production; or (+++) high production. ND means not detected.

Table 2 Antifungal activities of PSBs isolated from plant rhizosphere.

Bacterial strain	% Inhibition				
	P. oryzae	***H. oryzae***	***R. solani***	***F. moniliforme***	***C. lunata***
E. asburiae 30FPSSB1	22.2 ± 1.5^{a}	31.1 ± 3.0^{a}	35.0 ± 2.0^{a}	33.3 ± 1.3^{a}	34.0 ± 1.2^{a}
E. mori NTTC11	14.5 ± 1.7^{ab}	32.0 ± 1.1^{a}	32.00 ± 4.0^{a}	29.0 ± 2.5^{ab}	28.1 ± 7.0^{ab}
P. aryabhattai KNB6	0.0 ± 0.0^{c}	12.1 ± 3.2^{b}	1.3 ± 1.2^{c}	20.0 ± 5.0^{abc}	5.0 ± 5.0^{c}
P. aryabhattai 49KNA2	4.7 ± 0.5^{bc}	4.8 ± 4.7^{bc}	1.0 ± 1.0^{c}	22.1 ± 7.0^{ab}	8.0 ± 8.0^{c}
P. megaterium 65KNA2	7.5 ± 1.0^{bc}	1.8 ± 1.8^{c}	0.00 ± 0.0^{c}	7.5 ± 2.0^{c}	9.0 ± 9.0^{bc}
Bacillus sp. 38DFWA	11.7 ± 5.0^{b}	24.0 ± 2.0^{a}	10.0 ± 4.0^{b}	16.4 ± 6.0^{bc}	31.0 ± 5.0^{a}
Pseudomonas sp. SM-P208	11.0 ± 6.1^{b}	26.0 ± 3.0^{a}	13.0 ± 4.0^{b}	29.1 ± 4.1^{ab}	24.7 ± 7.0^{abc}

The values are given as mean ± standard error of 4 replications, and different superscripts within a column denote significant differences ($p < 0.05$).

Effects of bacterial concentration on plant growth

In preliminary tests an excessively high bacterial concentration inhibited root development, so a suitable bacterial concentration without plant growth inhibition was determined prior to the *in vitro* plant

growth assay. We investigated effects of all bacterial strains on rice growth promotion *in vitro* with the bacterial titers of 10^8, 10^5, 10^4 and 10^3 CFU/mL (T1 to T4, respectively) compared with sterile distilled water (control). Inoculation of seeds with a high concentration (T1) of bacterial cells had a very strong negative effect on plant growth (quantified by shoot height and root length) and on plant dry mass (**Figures 1(A) - 1(D)**). Plant growth was dramatically enhanced by treatments with medium (T3) and low (T4) bacterial cell concentrations. The present study agrees with previous reports in that a high density inoculum (10^8 CFU/mL) had adverse effects on plant growth and root development both on culture medium and in soil [27]. A higher bacterial inoculation might lead to more IAA production, which would induce ethylene production and thus inhibit root growth and development [28]. In addition, high bacterial density could produce metabolites harmful to plant growth, inhibiting root development [29], and induce plant defense mechanisms with adverse plant growth effects [30]. With any of the isolated strains, concentrations exceeding 10^5 CFU/mL were plant growth inhibitory, thus the bacterial concentration 10^4 CFU/mL was used in the *in vitro* plant growth assay.

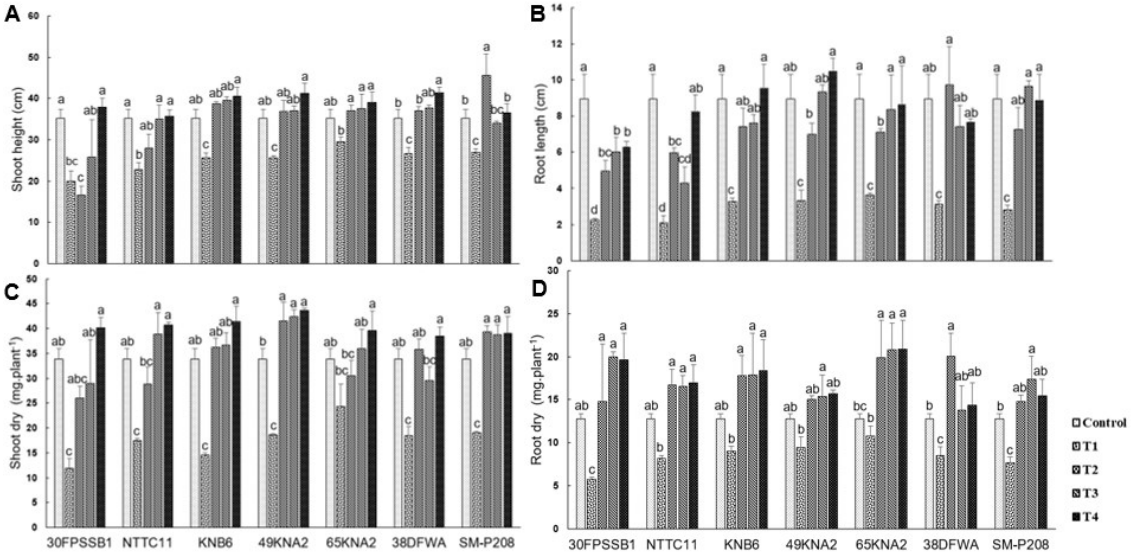

Figure 1 Plant growth indicators shoot height (A), root length (B), and shoot and root dry masses (C and D) of plants treated with the 4 bacterial concentrations 10^8, 10^5, 10^4 and 10^3 CFU/mL shown as T1, T2, T3 and T4, respectively. Data shown are given as mean ± standard error (n = 8), and different letters in one histogram denote significant differences between different concentrations tested of each bacterial strain ($p < 0.05$).

***In vitro* plant growth promotion by isolated strains**

Based on the plant growth promoting performances, all isolated strains were used in the plant assays. The effects of bacteria on rice growth when cultivated with medium having P and/or insoluble TCP are shown in **Figure 2**. A low level of phosphate did not affect plant height and root length (**Figures 2(A) - 2(B)**) but it influenced plant biomass. Shoot and root mass were reduced after a plant was cultured in only TCP (control plant) (**Figures 2(C) - 2(D)**). Inoculation of plant with some isolated bacterial strain in the presence of available P or TCP significantly improved plant biomass (shoots and roots). Soils treated with *Bacillus* sp. 38DFWA, *P. aryabhattai* KNB6, or *P. megaterium* 65KNA2 with access to soluble P gave shoot and/or root biomass increases. Interestingly, treatment of plants with these bacteria in the presence of TCP apparently induced plant growth to similar level as that reached with available P. In contrast, *Enterobacter* strains 30FPSSB1 and NTTC11 strongly inhibited plant growth, although they showed some plant growth promoting traits.

P concentrations of inoculated plants cultivated with insoluble P (MSII) were determined, to assess these isolated strains for their ability to solubilize TCP into available P found in rice tissue (**Figure 2(E)**). The data showed that a large amount of P accumulated in plant tissues when treated with strains *Bacillus* sp. 38DFWA, *P. megaterium* 65KNA2, and *P. aryabhattai* KNB6, with respective increases over the baseline by 89, 96 and 143 %, and there was more P accumulation in shoot than in root. Moreover, the *Enterobacter* species (both 30FPSSB1 and NTCC11) restricted P uptake by the plant tissue, in this plant growth assay.

Influence of PSB inoculation on rice growth in P-deficient soil

In vitro experiments showed that several isolated *Bacillus* and *Priestia* strains stimulated plant growth, while isolated *Enterobacter* species had adverse effects. So, those isolated *Bacillus* and *Priestia* strains that gave the greatest plant growth were selected and assessed under P-deficient conditions. P-deficient soil strongly affected shoot growth but had less effect on root growth, as shown in **Figure 3**. Shoot height significantly increased in soil containing P source (soluble or insoluble P), while the least shoot height was in P-deficient soil (without P) (**Figure 3(A)**). There was no effect from bacterial inoculations on shoot height, but all such treatments increased shoot mass (without P, soluble P, and TCP) (**Figure 3(C)**). Bacterial strain *Bacillus* sp.38DFWA and *P. aryabhattai* KNB6 inoculums under P-deficient conditions increased shoot dry mass by 38 and 23 %, respectively, when compared to non-inoculated plants. *P. aryabhattai* KNB6, *E. mori* NTTC11, *Bacillus* sp. 38DFWA, and *Pseudomonas* sp. SM-P208 greatly promoted shoot mass in soil with available P, with increases by 90, 80, 78 and 26 %, respectively. Interestingly, these bacteria contributed to a large amount of shoot mass in soil with TCP, with increases by 392, 416, 341 and 441 %, respectively, when compared with control ($p < 0.05$). Neither the level of phosphate in soil nor the bacterial inoculations influenced root length, which showed no differences between the P treatments (**Figure 3(B)**). While the root masses did not significantly differ, the mass likely increased when treated with *P. aryabhattai* KNB6, *E. mori* NTTC11, and *Pseudomonas* sp. SM-P208 under soluble P or TCP conditions (**Figure 3(D)**).

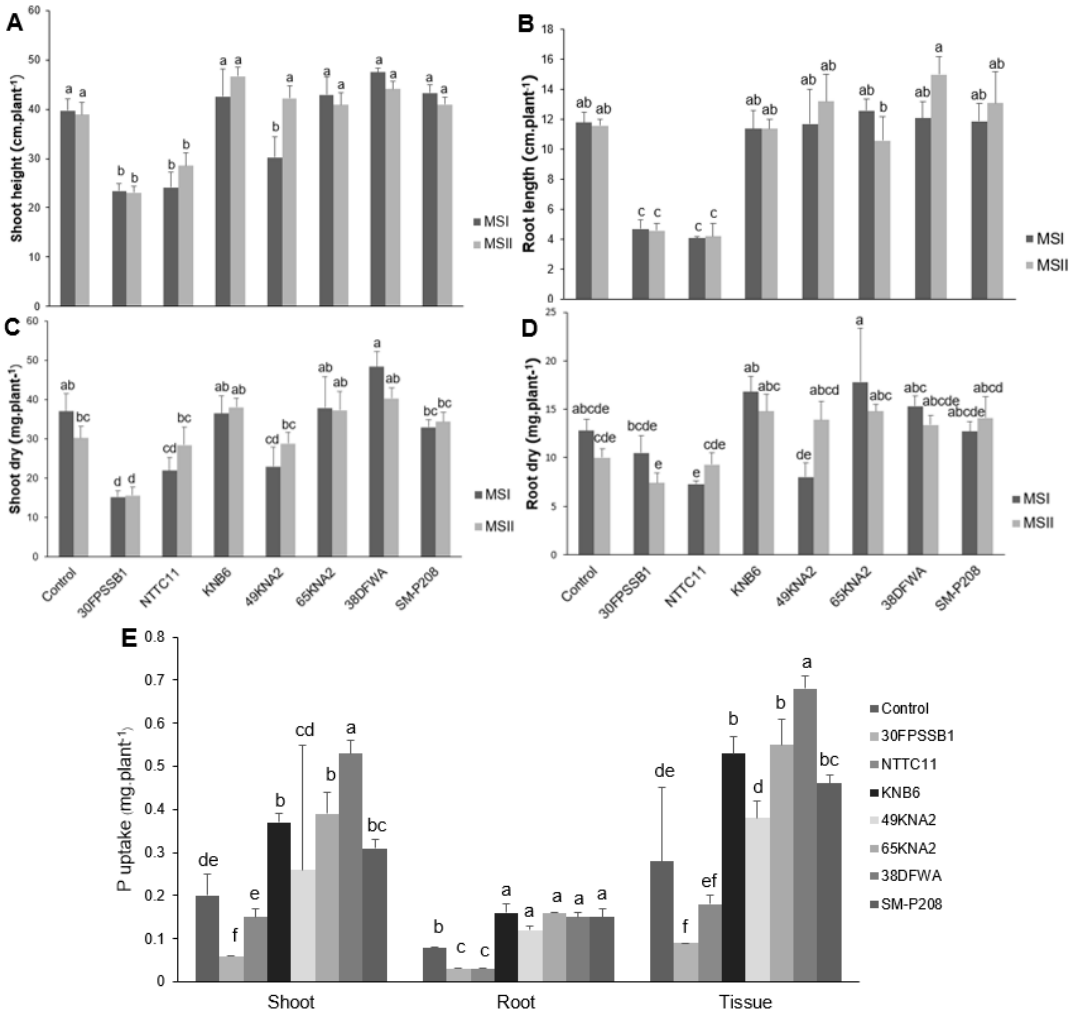

Figure 2 *In vitro* rice growth when inoculated with each isolated bacterial strain and cultured in MS I medium (+KH_2PO_4 + TCP) or in MS II medium (−KH_2PO_4 + TCP) for 14 days. Plant growth is represented by shoot height (A), root length (B), and the dry biomasses of shoots (C) and roots (D). P accumulation in plant tissue treated under MS II medium was determined (E). Data shown are given as mean ± standard error (n = 8), and different letters in each histogram denote significant differences ($p < 0.05$).

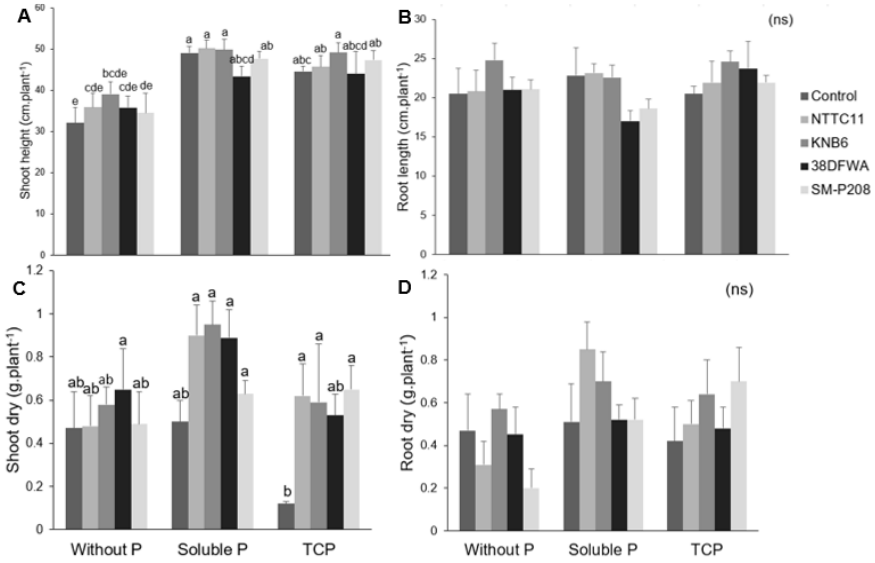

Figure 3 Rice growth in P-deficient (without P), soluble P and TCP treated soils was tested in pot experiments. Shoot height (A), root length (B), and the dry biomasses of shoots (C) and roots (D). Results are shown as mean ± standard error (n = 9), and different letters in each histogram denote significant differences ($p < 0.05$).

A low level of P (without P and insoluble P) affected shoots more than roots. P accumulation in shoots and roots was differently induced by the various bacteria ($p < 0.05$) (**Figure 4**). With available P, *E. mori* NTTC11 and *P. aryabhattai* KNB6 treated plants had P uptake in shoots increased by 167 and 151 % (**Figure 4(A)**), in roots by 65 and 91 % (**Figure 4(B)**), and in plant tissue by 120 and 123 %, respectively (**Figure 4(C**)). However, the effects of them on P concentration were greater when the plants were grown in TCP amended soil: Especially the uptake by shoots grew by 291 and 369 %, respectively. However, there was no difference in P accumulation to roots. Moreover, P uptake in plant tissue increased by 127 to 139 % when plants treated with selected rhizobacteria were grown in TCP amended soil. In without P soil, P accumulations in shoots (191 %), roots (68 %), and plant tissue (121 %) of *P. aryabhattai* KNB6 treated plants were increased, whereas the P uptake of *E. mori* NTCC11 treated plants was lower than that in soluble P and TCP amended soils.

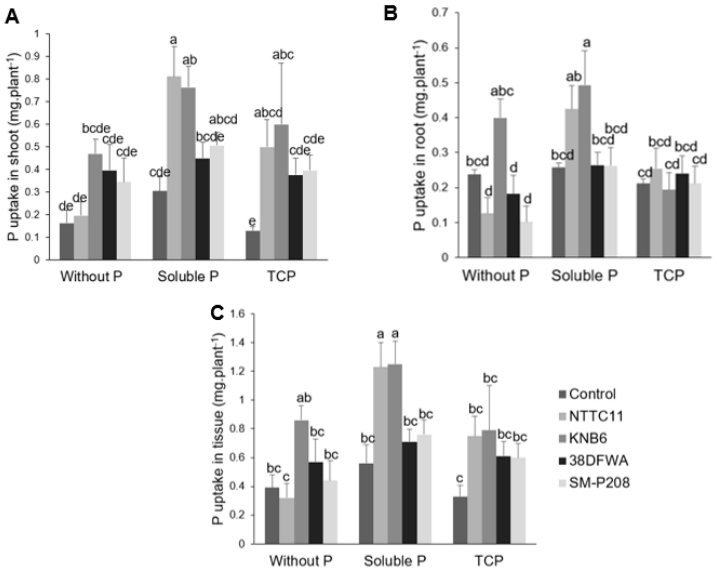

Figure 4 P concentrations in rice shoots (A), roots (B), and tissue (C) for control (without P fertilization), soluble P, and TCP treated plants. Results are shown as mean ± standard error (n = 9), and different letters in each histogram denote significant differences ($p < 0.05$).

In experimental *in vitro* TCP cultivation and TCP soil pot, *P. aryabhattai* KNB6 and *Bacillus* sp. 38DFWA solubilizing bacteria significantly increased plant biomass and P concentration in tissue ($p < 0.05$). This result matches plant growth promotion in soluble P treatment. These results possibly involved the bacterial inoculation enabling P solubilization that could increase the concentration of available P in medium and soil. They exhibited true PSB properties through improving both plant growth and nutrition. Despite the initial low levels of available P in soil, *P. aryabhattai* KNB6 inoculated plants grown in soil without P fertilizer had mainly improved rice biomass and P accumulation in shoots and roots. The enhanced rice growth with low level of P might also be associated to other bacterial PGP traits, such as bacterial N_2 fixation, ammonia and plant hormones production [30]. Previous studies have revealed the use of rhizobacteria as bioinoculant promoting growth and yield of *Zea may* and *Oryza sativa*, when grown in P-deficient soil [11,31]. The application of PSBs combined with any phosphorus fertilizer source (di-ammonium phosphate or rock phosphate) showed an increase in the amount of available phosphorus in soil and promoted the sugarcane yield [32].

In this work, all the PSB strains used as bioinoculants produced high levels of IAA, which is a plant hormone involved in root development. The root biomasses and tissue P concentrations of bacterially inoculated plants were positively correlated, indicating development of root system of rice plants when grown in P-deficient soils, even if the improvements in root length and mass were not large. The root system is of a major importance for plant growth, since it enables the uptake of nutrients and water. P uptake by plants tissue is related to root density, and improved root surface area increases the ability to absorb P from the soil [12,23]. These observations relate to the ability of inoculated strains to produce IAA, which promotes root growth and root development. Many studies have shown that wheat plant inoculation with IAA-producing and phosphate solubilizing bacteria increases root mass, root auxin concentration, and crop yield [28,33]. In contrast, IAA-deficient mutants had diminished growth promoting effects on root growth and root surface area [34]. Therefore, these auxin producing bacteria increased phosphorus uptake not only by providing more available phosphorus in the soil, but also by better root development from increased root auxin levels.

Despite *E. mori* NTTC11 promoting plant growth and P uptake in soluble P and TCP treatments, it showed strong plant growth inhibition *in vitro*. This might depend on the concentration of inoculated IAA producing bacteria and on the plant growth environment. *E. mori* NTTC11 showed the highest IAA production, so *in vitro* the plant growth culture enhanced bacterial growth, and a high accumulated concentration of IAA might stimulate ethylene production and inhibit root elongation. However, root growth inhibition might be destructive when plants grow in dry soil because endogenous IAA content is decreased in stressed plants [35]: Thereby P-deficient soil and drought stress might alter plant responses to microbial-produced auxins. Besides, IAA effects on root elongation may be explained by its dose-dependent stimulation of producing the growth inhibitor ethylene [36]. The bacterial ACC deaminase activity can be prevented by an increase of ethylene production, by this reducing ACC which is a precursor of ethylene. ACC-deaminase producing bacteria decreased soil and root ACC concentrations, lowering the plant's ethylene production [37]. The ACC deaminase mediated decrease in ethylene synthesis enhances root elongation, despite potentially inhibitory high concentrations of IAA. Thus, plant treatments with rhizobacteria that produce both auxins and ACC deaminase become especially important under unfavorable conditions. PGPR-inoculated plants, namely Para rubber (*Hevea brasiliensis*) and Jerusalem artichoke (*Helianthus tuberrosus*), experienced enhanced plant growth and maintained crop yield despite effects of salinity and drought stress [38,39].

Bacillus species is one of the most dominant rhizospheric bacteria, and has established various mechanisms to promote plant growth by increasing the availability of nutrients. Four of these isolated strains were classified *Bacillus* sp. 38DFWA and new genus *Priestia* species *P. aryabhattai* KNB6, *P. aryabhattai* 49KNA2, and *P. megaterium* 49KNA2, which were formerly *B. aryabhattai* and *B. megaterium*, respectively [40]. Among these strains, *P. aryabhattai* KNB6 was the most effective plant growth promoter of rice seedlings in all the P treatments. In prior studies, *P. aryabhattai* isolated from root of rice and grapevine rhizospheres exhibited plant growth promoting ability, pesticide-tolerance, and displayed strong phosphate solubilization under stress conditions of high pH, high salt, and elevated temperature [41,42]. Another *P. aryabhattai* isolated from agricultural soil showed potential to degrade glyphosate herbicide [43]. The above prior findings and our research together reveal that *P. aryabhattai* has diverse functions potentially supporting various applications.

Conclusions

The present study clearly indicates the potential of P-solubilizing bacterial strains, such as *P. aryabhattai* KNB6, *E. mori* NTTC11, and *Bacillus* sp. 38DFWA, as these enhanced rice plant growths by 127, 107 and 87 % under limited available phosphorus form (TCP) in greenhouse conditions. *P. aryabhattai* KNB6 exhibited the most effective plant growth promotion by increasing P uptake directly in plant tissue by 121 %, which correlated with 22 % improved plant growth in P-deficient soil. This strain is a good candidate for bio-fertilizer that could reduce fertilizer costs and improve crop yields.

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