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REGULAR ARTICLE

Isolation and characterization of phosphate solubilizing rhizobacteria to improve plant health of tomato

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Abstract In the present study, 43 isolates of Phosphate solubilizing rhizobacteria (PSRB) were isolated from 37 rhizospheric soil samples of tomato collected from tomato growing regions of Karnataka. Among the 43 isolates, 33 were found to be positive for solubilizing both inorganic and organic forms of phosphorous. The isolates were analyzed for their ability to colonize roots of tomato and to increase the seed quality parameters under laboratory conditions. On the basis of above criteria, 16 isolates were selected for further studies. Organic acids from PSRB isolates were analyzed and phytase zymogram for two isolates *viz.,* PSRB21 and 31 was prepared. Under greenhouse conditions, all selected isolates showed increased shoot length, root length, fresh weight, dry weight and phosphorous content of tomato seedlings to various extent with respect to control. Analysis of pH and available phosphorous in rhizosphere soil samples of 30 day-old-seedlings revealed that the available phosphorous content was high in rhizospheric soil samples of plants raised from seeds bacterized with PSRB isolates over control. Even though all selected PSRB's were able to increase the plant growth, only few of them showed protection against fusarium wilt and none of them against early blight.

Keywords Phosphorous · Phosphate solubilizing rhizobacteria · Organic acid · Phytase · Fusarium wilt · Early blight

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Introduction

Phosphorus is the second most plant nutrient available in soil after nitrogen. Though soils usually contain high amount of total phosphorous, most of the phosphorous occur in insoluble form as iron and aluminum phosphates in acidic soils and calcium phosphates in alkaline soils. Some of them appear after the application of chemical fertilizers. Seventy five percent of the soluble phosphate fertilizers added to crops may be converted to sparingly soluble form by reaction with the free Ca^{2+} ions in high pH soils or with Fe^{3+} or Al^{3+} in low pH soils (Goldstein 1986). Plants are unable to utilize precipitated form of phosphorous. However, organic matter, on the other hand, is an important reservoir of immobilized phosphate that accounts for 20–80% of soil phosphorous (Richardson 2001) and only a small portion (~0.1%) is available to plants. Conversion of the insoluble forms of phosphorous to a form accessible

by plants, like orthophosphate, is an important trait of Phosphate solubilizing rhizobacteria (PSRB) in increasing growth and yield of crop plant.

Microorganisms are important components of soil and directly or indirectly influence the soil's health through their beneficial or detrimental activities. Rhizosphere microorganisms mediate soil processes such as decomposition, nutrient mobilization and mineralization, nitrogen fixation and denitrification (Kang et al. 2002; Pradhan and Sukla 2005). Furthermore, solubilization of phosphorus in rhizosphere is the most common mode of action implicated in plant growth promoting rhizobacteria (PGPR) that increase the nutrient availability to the host plant (Richardson 2001).

The ability of phosphate solubilizing microorganisms to solubilize phosphorous complexes has been attributed to their ability to reduce pH of the surroundings, either by releasing organic acids or protons. Organic acids such as citrate, lactate, succinate etc. secreted by PSRB contribute for phosphate solubilization in the rhizosphere. The organic acids secreted can either directly dissolve the mineral phosphates as a result of anion exchange or can chelate both Fe and Al ions associated with phosphate (Bajpai and Rao 1971). Finally, the insoluble form of phosphorous is converted into soluble monobasic (H_2PO_4) and dibasic (HPO_4^{2-}) ions, a process referred to as mineral phosphate solubilization. This leads to an increase in the availability of phosphorous to plants and in turn the plant uptake (Gyaneshwar et al. 2002).

The enzyme phytase, belongs to a special class of phosphomonoesterases (myo-insitol hexakisphosphate 3-phosphorylase, EC 3.1.3.8 and Myo-inositol hexakisphosphate 6-phosphorylase, EC 3.1.3.26) is known to secreted by several soil microorganisms. It is involved in the stepwise degradation of phytate to lower phosphate esters. Plants are known to produce phytase (Greiner and Alminger 2001), which display low activity in roots and other plants organs. This suggests that plant roots poorly or may not posses an innate ability to acquire phosphorous directly from soil phytate. The present study has been carried out to isolate and characterize an effective PSRB isolate which has an ability to solubilize both forms of phosphate in soil and subsequently advantage of its use over PSRB isolates solubilize only one form of phosphorous in improving plant growth. The second objective was to asses the ability of selected isolates to protect tomato plants against Fusarium wilt and early blight disease under greenhouse conditions

Materials and methods

Plant materials

Seeds of tomato (*Lycopersicon esculentum* Mill.) cultivar PKM-1 were obtained form local seed agencies. Seeds were surface-sterilized with 1% sodium hypochlorite for 30 s and then rinsed in sterile distilled water, air dried and used for the experiments.

Soil samples

Thirty seven rhizosphere soil samples were collected from tomato plants of various agro climatic regions of Karnataka during June–September, 2006. Rhizosphere soil samples were collected from healthy field-grown plants. For greenhouse studies, a sandy loam soil was collected from the experimental plot of Department of Applied Botany and Biotechnology, University of Mysore, India. The soil was dried and sieved to 2 mm before mixing it with cow dung manure (3:1 v/v). The potting mixture was analyzed for its physical and chemical characters (Murugesan and Rajakumari 2006) (Table 1).

Microorganisms

Fusarium oxysporum f. sp. *lycopersici* and *Alternaria solani* were obtained from the culture collection of the Department of Applied Botany and Biotechnology, University of Mysore, India and maintained on potato dextrose agar (PDA) slants at 4°C till further use. Inoculum was prepared by culturing the fungus on PDA medium for 7 days in petriplates. Conidial suspension was prepared by pouring 20 ml of sterile distilled water in each petriplate. The concentration of microconidia for *F. oxysporum* was adjusted to 1,000 conidia/ml (Ramamoorthy et al. 2002). Similarly for *A. solani*, the conidial concentration was adjusted to 5×10^4 conidia/ml and used for greenhouse experiments (Vloutoglou and Kalogeraksi 2000).

In order to isolate PSRB's, rhizosphere soil samples were serially diluted and suitable dilutions were spread plated onto Pikovskaya's agar medium (Pikovskaya 1948). Plates were incubated at 28±2°C,

Table 1 Chemical and physical characteristics of soil used in greenhouse studies

Soil type	pH	% of organic mater	Available elements (Kg/ha)					WHC (%)
			N	P	K	Mg	Ca	
Sandy loam	6.9	3.63	474.2	135.6	225.3	328	3,004	61.7

N nitrogen, *P* phosphorus, *K* potassium, *Mg* magnesium, *Ca* calcium, *WHC* water holding capacity

analyzed for zone of clearance up to 10 days. The bacterium which showed zone of clearance on repeated subculture onto Pikovskaya's medium was selected for further studies.

Phosphate solubilization

Phosphate solubilization index was calculated by inoculating the PSRB onto Pikovskaya's agar medium and measuring the diameter of halo zone and bacterial colony. The solubilization index [= the ratio of the total diameter (colony + halo zone) to the colony diameter] was calculated by formula of Edi Premono et al. (1996). The bacteria, found to be positive for calcium phosphate solubilization were further analyzed for their ability to solubilize the calcium phosphate in liquid medium following the standard procedures (Alikhani et al. 2006).

All the PSRB's were analyzed for their phytase activity on phytase screening medium (PSM) containing (% w/v): Calcium phytate 0.5; Glucose, 1.0; $(NH_4)_2SO_4$, 0.03; $MgSO_4$, 0.05; $CaCl_2$, 0.01; $MnSO_4$, 0.001; $FeSO_4$, 0.001; and agar, 2.0, pH 7.0 (Howson and Davis 1983). The isolates showing clear zones on PSM were further screened on liquid production medium of following composition (%; w/v): pea flour, 0.5; sucrose, 1.0; asparagine, 0.1; $MgSO_4.7H_2O$, 0.05; KCl, 0.05; $FeSO_4$, 0.001; and $MnSO_4$, 0.001, pH 5.5. The sterile medium (50 ml) in 250 ml conical flasks was inoculated with 24-h-old cultures grown on Luria–Bertain (LB) medium at 2% (v/v), and incubated under shaking conditions (120 rpm) at 45°C for 24 h. The fermented broth was clarified by centrifugation at 10,000 rpm for 10 min and assayed for phytase activity (Gulati et al. 2007). All PSRB isolates were maintained in 40% glycerol at −70°C for further use.

Identification and characterization of PSRB

Biochemical tests for the identification of PSRB's were done following the standard procedures (Cuppuccino and Sherman 2004). Confirmation of selected rhizobacterial isolates was done by Fatty acid methyl ester analysis.

(i) **Gram reaction**: A smear of each organism was prepared from 12 to 24-h-old cultures, and the standard procedures for Gram staining were used.
(ii) **Root colonization bioassay**: This was carried out following standard procedures of Silva et al. (2003). The bacterium that colonized the root even after repetition was selected for further studies.
(iii) **Indole acetic acid (IAA):** A modified method of (Patten and Glick 2002) was used to screen IAA producing rhizobacteria using LB broth supplemented with L-tryptophan (500 µg/ml).
(iv) **Siderophore:** Siderophore production was determined as described by Schwayn and Neilands (1987) using blue indicator dye, chrome azurol S. Bacterial isolates exhibiting an orange halo after 5 days of incubation at 28±2°C were considered positive for the production of siderophores.
(v) **Chitinase and β-1, 3 glucanase:** Production of the two enzymes determined as described by Renwick et al. (1991) on a defined medium.
(vi) **Cyanogenesis:** Hydrogen cyanide (HCN) production was determined on slants of TSA (10%) amended with Glycine (4.4 g/l) and $FeCl_3.6H_2O$ (0.3 mM) (Bakker and Shippers 1987; Castric 1975).
(vii) **Cellulase:** Production of cellulase was determined in basal medium supplemented with Carboxymethyl cellulose (CMC) (10 g/l) (Cattelan et al. 1999).

In vitro antagonism assay

Antagonistic nature of PSRB against *F. oxysporum* and *A. solani* was determined by employing dual culture technique. Briefly, bacterial isolates were

seeded at the edges of 90 mm petriplates containing PDA and incubated for 36 h at 28±2°C. A 9 mm diameter plug of fungus was placed on the centre of the circle. Plates were incubated at 28±2°C for 7 days. The radii of the fungal colony towards and away from the bacterial colony were measured. The percentage of growth inhibition was calculated using the formula,

$$\% \text{ inhibition } = (R - r)/R \times 100$$

Where r is the radius of the fungal colony opposite the bacterial colony and, R is the maximum radius of the fungal colony away from the bacterial colony (Idris et al. 2007).

Analysis of pH variation and organic acids in culture filtrate

The culture filtrates (Pikovskaya's broth) of 72 h-old-cultures inoculated with selected PSRB's were analyzed for the presence of gluconic acid, oxalic acid, citric acid, malic acid, succinic acid, lactic acid and fumaric acid by thin-layer chromatography. After checking pH, 50 ml of culture filtrate was concentrated completely by lyophilization followed by dissolving it in minimal quantity of distilled water. The suspension was acidified to pH 2.0 with 0.1 N HCl and then applied, along with standard solutions to precoated cellulose plates (10 by 10 cm; thickness, 0.1 mm). The plates were developed with diethyl ether–formic acid–water (70:20:10) and organic acids were visualized with 0.045% bromophenol blue in 95% ethanol. The detection limit of the method was 10 μg/ml for all the acids analyzed (Altomare et al. 1999).

Phytase assay

Culture filtrate was assayed for phytase activity by incubating 150 μl with 600 μl of substrate solution [0.2% (w/v) sodium phytate in 0.1 M sodium acetate buffer, pH 5.0] for 30 min at 39°C (Shimizu 1992). The reaction was stopped by adding 750 μl of 5% trichloroacetic acid solution and free phosphate was determined by a modification of the method of Fiske and Subbarow (1925). Color reagent (750 μl), prepared by mixing four volumes of 1.5% (w/v) ammonium molybdate in a 5.5% (v/v) sulfuric acid solution and one volume of a 2.7% (w/v) ferrous sulfate solution, was added to the sample solution (750 μl) and the production of phosphomolybdate was measured spectrophotometrically at 700 nm (Hitachi U-2000, Tokyo, Japan).

Phytase zymogram analysis

Phytase zymogram analysis was done for isolates PSRB21 and 31. Lyophilized culture filtrate was mixed with sample loading buffer [62.5 mM Tris buffer (pH 6.8) containing 0.05% bromophenol blue, 0.72 M 2-β-mercaptoethanol, 10% glycerol and 2% sodium dodecyl sulfate (Laemmli 1970) in a micro-centrifuge tube and the tubes were placed in boiling water bath for 5 min. The denatured samples were resolved by SDS-PAGE on a 10% separating gel topped with a 4% stacking gel. Following electrophoresis, the gels were either stained for proteins or phytase activity. Phytase zymograms were prepared by soaking the gels first in 1% Triton X-100 for 1 h at room temperature and then in 0.1 M sodium acetate buffer (pH 5.0) for 1 h at 48°C. The enzyme activity was detected by incubating the gels for 16 h in a 0.1 M sodium acetate buffer (pH 5.0) containing 0.4% (w/v) sodium phytate. Active bands were visualized by immersing the gel in 2% (w/v) aqueous cobalt chloride solution. After 5 min incubation at room temperature, the cobalt chloride solution was replaced with a freshly prepared solution containing equal volumes of 6.25% (w/v) aqueous ammonium molybdate solution and 0.42% (w/v) ammonium metavanadate solution. Phytase activity was visualized as zones of clearance on an opaque background (Bae et al. 1999).

Effect of PSRB seed bacterization on seed germination and seedling vigor under laboratory conditions

Seed bacterization

Twenty five milliliters of bacterial inoculum containing 1×10^8 cfu/ml was added to 100 ml Erlenmeyer conical flask. One hundred mg of CMC was added as adhesive material. Ten gram of seeds was soaked in bacterial suspension for 12 h on a rotary shaker at 150 rpm. The bacterial suspension was drained off and the seeds were dried overnight aseptically in laminar air flow. Seeds soaked in distilled water amended with CMC served as control.

Plant growth promotion

Germination test was carried out by the paper towel method according to the standard procedures of International Seed Testing Association (ISTA 2005) using four replicates of 100 seeds for each treatment. Seedling vigor was analysed using the method of Abdul Baki and Anderson (1973). To asses the vigor, the length of the root and shoot of an individual seedling was measured. The vigor index (VI) was calculated using the formula: $VI = (\text{mean root length} + \text{mean shoot length})(\%\text{germination})$.

Greenhouse studies

Plant growth promotion

The plants were grown in greenhouse under a day/night cycle of 16/8 h and 28/20°C and 65% relative humidity. Potting soil (soil: decomposed cow dung manure at 3:1 v/v) was autoclave-sterilized for 1 h on two consecutive days and was filled into pots (20 cm diameter). Bacterized seeds of tomato were sown thickly and seedlings were thinned after 10 days of sowing leaving 8 seedlings per pot. Thirty day-old-seedlings were sampled and analyzed for shoot length, root length, fresh weight, dry weight and total phosphorous content. Seedlings were air dried, grounded and digested in 15 mL $HClO_4$ and 5 mL HNO_3. The spectrophotometric vanado-molybdate method was used to measure phosphorous (Babana and Antoun 2006). 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 phosphorous content by following Olsen's method (Murugesan and Rajakumari 2006). For each treatment, six replicates were maintained with six pots per replicate.

Efficacy of PSRB seed bacterization on fusarium wilt and early blight incidence

Seedlings were raised as explained earlier. Fifteen day-old-seedlings were challenge inoculated with conidial suspension (1,000 microconidia/ml) of *F. oxysporum*. Wilt incidence was recorded up to 30 days after challenge inoculation. Conidial suspension (5×10^4 conidia/ml) was sprayed onto leaf of 25 day-old-seedlings until runoff for early blight disease. Appearance of typical leaf spot symptom was recorded up to 20 days after inoculation. For each experiment, four pots per replication were maintained with eight replications.

Statistical analysis

Data from laboratory and greenhouse experiments were analyzed separately for each experiment and were subjected to arcsine transformation and analysis of variance (ANOVA) (SPSS, version II). Significant effects of treatments were determined by the magnitude of F value ($P\leq0.05$). Treatment means were separated by Tukey's HSD test.

Results

Isolation and characterization of selected PSRB

Thirty seven rhizosphere soil samples were screened for the presence of PSRB's on Pikovskaya's medium. Forty three PSRB were isolated, of which 33 isolates were found to solubilize both inorganic (calcium phosphate) and organic (calcium phytate) form of phosphorous. Only 16 isolates were selected for further studies based on their ability to colonize tomato root and promote plant growth under laboratory conditions (Table 2).

Bacterial characterization

All the selected PSRB isolates were positive for in vitro root colonization and negative for cellulase and HCN production. Except isolates PSRB27, 28, 31, 32 and 41, all other isolates were positive for IAA production. Only four isolates (PSRB6, 21, 27 and 32) were found to be producing siderophores. Isolates PSRB12 and 27, and PSRB14 and 38 were found positive for chitinase and β-1, 3 glucanase respectively (Table 2). Not all the selected PSRB isolates were able to inhibit the growth of *F. oxysporum* and *A. solani*. Isolate PSRB1 showed highest inhibition of 65 and 61% to *F. oxysporum* and *A. solani* respectively. Where as isolates PSRB2, 8, 12, 14, 19, 20, 21, 28, 31 and 41 were unable to inhibit the fungal growth (Table 2).

Table 2 Selected physiological and biochemical traits of PSRB's isolated from rhizospheric soil of tomato

Isolates	Gram's test	P solubilization		IAA	Sid	Chi	Glu	Dual culture assay (% mycelial inhibition)	
		CP	Phy					*F. oxysporum*	*A. solani*
B. subtilis PSRB1	+	+	+	+	−	−	−	65±3.46	61±3.46
Unknown PSRB2	+	+	+	+	−	−	−	−	−
P. putida PSRB6	−	+	+	+	+	−	−	50±2.30	54±2.30
Azatobacter sp. PSRB7	−	+	+	+	−	−	−	43±1.73	49±4.04
B. amyloliquefaciens PSRB8	+	+	+	+	−	−	−	−	−
Azatobacter sp. PSRB12	−	+	+	+	−	+	−	−	−
Unknown PSRB14	−	+	−	+	−	−	+	−	−
Enterobacter sp. PSRB19	−	+	+	+	−	−	−	−	−
B. subtilis PSRB20	+	+	−	+	−	−	−	−	−
P. fluorescens PSRB21	−	+	+	+	+	−	−	−	−
P. fluorescens PSRB27	−	+	−	−	+	+	−	62±4.04	50±2.88
Serratia sp. PSRB28	−	+	+	−	−	−	−	−	−
B. subtilis PSRB31	+	+	+	−	−	−	−	−	−
P. fluorescens PSRB32	−	+	+	−	+	−	−	31±1.73	30±2.30
B. megaterium PSRB38	+	+	+	+	−	−	+	22±1.15	8±1.15
Pseudomonas sp. PSRB41	−	+	+	−	−	−	−	−	−

All isolates were positive for root colonization and negative for Cellulase and HCN production

IAA indole acetic acid, *P* phosphate solubilization, *CP* calcium phosphate, *Phy* phytate, *Sid* siderophore, *Chi* chitinase, *Glu* β-1, 3 glucanase

In vitro phosphate solubilization

Under in vitro conditions maximum solubilization index of 3.1 and 3.0 was shown by isolates PSRB1 and 14 respectively. But in liquid culture, isolates PSRB7 and 19 showed phosphate solubilization of 143 and 141 μg/ml respectively which were significantly ($P \leq 0.05$) higher over control and other PSRB isolates (Table 3). Almost all selected PSRB isolates lowered the pH of culture up to a little extent over uninoculated control (pH 6.0). In contrast, organic acid was not detected in all culture filtrates. It may be due to the limitation of the methodology we used. The predominant organic acid found was Gluconic acid in culture filtrates of PSRB1, 2, 7, 8, 19 and 32 (Table 3). Of the selected 16 isolates, only three (PSRB14, 20 and 27) were negative for phytase production. Highest phytase activity was shown by the isolate PSRB31 (0.0068 katals ml^{-1}) followed by PSRB41 (0.0067 katals ml^{-1}) and least activity of 0.0022 katals ml^{-1} was shown by the isolate PSRB12 (Table 3). The phytase zymogram analysis of PSRB21 and 31 revealed that the molecular weight of enzyme was around 35 kDa protein.

In vitro plant growth promotion

All selected bacterial isolates showed increased seed germination and varied seedling vigor when compared to control upon seed bacterization. Isolate PSRB38 significantly increased seed germination (88%) and seedling vigor (1,522) followed by PSRB28. Where as in the control germination and VI was found to be 71% and 859 respectively (Table 4).

Greenhouse experiments

Analysis of root length, shoot length, fresh weight and dry weight of 30 day-old-seedlings revealed that, seedlings raised form the PSRB bacterized seeds showed increased shoot and root length. Of the 16 isolates, PSRB19 showed significant increase in shoot length (14.0 cm), root length (18.0 cm), fresh weight (0.762 g/seedling) and dry weight (0.110 g/seedling) followed by isolate PSRB8. In the control, shoot length, root length, fresh and dry weight was found to be 9.0 cm, 12.5 cm, 0.225 and 0.041 g/seedling respectively (Table 5).

Table 3 In vitro phosphate solubilization

Isolates	Calcium phosphate solubilization				Phytase activity (Katals ml^{-1})
	Index* in agar assay (cm)	In broth Assay			
		P (μg/ml)	pH	Organic acids	
Control	0.00 ± 0.00^{h}	00 ± 0.00^{j}	6.0 ± 0.00^{a}	–	0.00 ± 0.00^{g}
PSRB1	3.1 ± 0.125^{a}	126 ± 4.33^{b}	4.0 ± 0.1155^{de}	Gl	0.0043 ± 0.011^{d}
PSRB2	1.2 ± 0.033^{efg}	90 ± 2.30^{def}	4.6 ± 0.057^{bc}	Gl	0.0052 ± 0.005^{c}
PSRB6	2.4 ± 0.089^{abcd}	100 ± 11.55^{cd}	4.5 ± 0.1039^{bc}	Ci	0.0035 ± 0.003^{e}
PSRB7	2.0 ± 0.089^{bcdef}	143 ± 4.63^{a}	3.6 ± 0.063^{e}	Gl, Ox	0.0064 ± 0.003^{a}
PSRB8	1.8 ± 0.036^{defg}	82 ± 1.73^{efg}	4.7 ± 0.098^{b}	Gl	0.0054 ± 0.008^{bc}
PSRB12	1.1 ± 0.033^{fg}	106 ± 3.46^{cd}	4.6 ± 0.011^{bc}	ND	0.0022 ± 0.009^{f}
PSRB14	3.0 ± 0.158^{a}	92 ± 3.46^{cdef}	4.7 ± 0.057^{b}	Ci	0.0000 ± 0.000^{g}
PSRB19	1.9 ± 0.089^{cdefg}	141 ± 11.54^{ab}	3.7 ± 0.086^{e}	Gl	0.0022 ± 0.010^{f}
PSRB20	1.4 ± 0.063^{efg}	109 ± 4.33^{c}	4.7 ± 0.028^{b}	Ib	0.0001 ± 0.001^{g}
PSRB21	2.4 ± 0.033^{abcd}	71 ± 4.61^{g}	$4.5\pm.1443^{bc}$	ND	0.0059 ± 0.013^{b}
PSRB27	2.7 ± 0.089^{abc}	98 ± 4.04^{cde}	4.2 ± 0.080^{cd}	uk	0.0000 ± 0.000^{g}
PSRB28	1.5 ± 0.036^{efg}	100 ± 3.46^{cd}	$4.6\pm.1559^{bc}$	La	0.0053 ± 0.005^{c}
PSRB31	1.5 ± 0.063^{efg}	73 ± 4.61^{g}	4.7 ± 0.023^{b}	ND	0.0068 ± 0.019^{a}
PSRB32	2.8 ± 0.158^{ab}	52 ± 4.05^{h}	$4.5\pm.1039^{bc}$	Gl	0.0032 ± 0.004^{e}
PSRB38	2.1 ± 0.125^{bcde}	79 ± 2.30^{fg}	4.5 ± 0.051^{bc}	ND	0.0058 ± 0.017^{b}
PSRB41	1.2 ± 0.125^{efg}	31 ± 2.30^{i}	6.0 ± 0.023^{a}	ND	0.0067 ± 0.017^{a}

Values are the mean with in column sharing the same letters are not significantly different according to Tukey's HSD at $P\leq0.05$

*Solubilization index = Total diameter (Colony + halo zone)/colony diameter

Gl gluconic acid, *La* lactic acid, *Ox* oxalic acid, *Ci* citric acid, *Ib* isobutyric acid, *uk* unknown organic acids, *ND* not detected

The selected PSRB isolates were found to solubilize complex form of phosphorous in the rhizosphere under greenhouse conditions and the orthophosphate released by these PSRB's was utilized by growing seedlings and was evident by the increased level of phosphorous in plant tissue in comparison with untreated control. Significantly ($P\leq0.05$) higher level of phosphate accumulation of 1.57 mg/g dry weight was noticed in seedlings raised from seeds bacterized with isolate PSRB21 followed by PSRB1 (1.55 mg/g dry weight). When rhizospheric soil samples were analyzed for variation in pH, only isolate PSRB12 and 19 were found reduced the pH up to 5.4 in comparison with control (6.0). Analysis of available phosphorous in rhizospheric soil samples revealed that soil samples collected from the seedlings raised from seeds bacterized with isolate PSRB1 showed significantly ($P\leq0.05$) higher level of available phosphorous (85 μg/g soil) followed by isolate PSRB8 (83 μg/g soil). Where as the rhizospheric soil samples collected from control seedlings showed least available phosphorus of 30 μg/g soil (Table 5).

Results of greenhouse experiments demonstrated that PSRB27 significantly ($P\leq0.05$) protected the tomato plants against fusarium wilt with disease incidence over control. Isolate PSRB1, 6, 19, and 38 also showed a decreased level of disease incidence ranging from 27 to 35% against Fusarium wilt. But none of the PSRB isolates were found to suppress early blight significantly. Control, seedlings showed 86 and 92% of fusarium wilt and early blight incidence respectively (Fig. 1).

Discussion

The indiscriminate and excessive application of chemical fertilizers has lead to health and environmental hazards. Agronomists are desperate to find alternative strategies that can ensure competitive yields while protecting the health of soil. This new approach to agriculture often referred to as sustainable agriculture requires agricultural practices that are friendlier to the environment and that maintain the

Table 4 Effect PSRB seed bacterization on seed germination and seedling vigor under laboratory conditions

Isolates	MRL (cm)	MSL (cm)	Germination (%)	VI
Control	6.2 ± 0.115^{f}	5.9 ± 0.063^{g}	71 ± 2.30^{hi}	859 ± 16.74^{i}
PSRB1	8.6 ± 0.167^{bcd}	8.0 ± 0.092^{a}	76 ± 1.15^{defgh}	$1{,}262\pm23.67^{cd}$
PSRB2	7.5 ± 0.138^{def}	6.8 ± 0.080^{cde}	84 ± 1.73^{ab}	$1{,}201\pm20.20^{cdef}$
PSRB6	7.3 ± 0.086^{def}	6.5 ± 0.071^{e}	79 ± 2.30^{bcde}	$1{,}090\pm28.86^{gh}$
PSRB7	8.2 ± 0.109^{bcde}	7.0 ± 0.086^{bcd}	74 ± 0.57^{efghi}	$1{,}125\pm46.18^{fgh}$
PSRB8	8.0 ± 0.179^{bcde}	7.1 ± 0.057^{bc}	77 ± 1.15^{cdefg}	$1{,}163\pm28.86^{efgh}$
PSRB12	7.9 ± 0.161^{bcde}	6.6 ± 0.109^{de}	79 ± 3.46^{bcde}	$1{,}145\pm19.62^{efg}$
PSRB14	8.2 ± 0.115^{bcde}	7.3 ± 0.028^{b}	83 ± 0.57^{abc}	$1{,}286\pm24.84^{c}$
PSRB19	7.6 ± 0.121^{cdefg}	6.4 ± 0.069^{ef}	76 ± 2.88^{defgh}	$1{,}064\pm36.95^{gh}$
PSRB20	8.0 ± 0.462^{bcde}	6.8 ± 0.128^{cde}	73 ± 2.02^{fghi}	$1{,}080\pm56.58^{gh}$
PSRB21	10.7 ± 0.519^{a}	7.4 ± 0.023^{b}	70 ± 0.57^{i}	$1{,}267\pm28.86^{cd}$
PSRB27	7.6 ± 0.288^{cdefg}	6.4 ± 0.051^{ef}	74 ± 1.73^{efghi}	$1{,}036\pm21.36^{h}$
PSRB28	9.2 ± 0.635^{ab}	8.1 ± 0.057^{a}	82 ± 2.30^{abc}	$1{,}419\pm42.14^{b}$
PSRB31	8.0 ± 0.346^{bcde}	6.8 ± 0.075^{cde}	78 ± 1.73^{bcdef}	$1{,}154\pm25.40^{efg}$
PSRB32	8.3 ± 0.317^{bcde}	7.1 ± 0.109^{bc}	80 ± 2.88^{bcde}	$1{,}232\pm53.11^{cde}$
PSRB38	9.1 ± 0.1790^{bc}	8.2 ± 0.051^{a}	88 ± 1.15^{a}	$1{,}522\pm35.79^{a}$
PSRB41	6.9 ± 0.075^{ef}	6.0 ± 0.086^{fg}	72 ± 1.73^{ghi}	929 ± 10.96^{i}

Values are the mean with in column sharing the same letters are not significantly different according to Tukey's HSD at $P\leq0.05$

MRL mean root length, *MSL* mean shoot length, *VI* vigor index

long-term ecological balance of the soil ecosystem. In this context, PSRB have been considered as one of the possible alternatives for chemical phosphate fertilizers (Richardson 2001; Vessy 2003; Thakuria et al. 2004). Reyes et al. (2006) studied the biodiversity of phosphate-solubilizing microorganisms in rhizosphere and bulk soils collected form rock phosphate in Tachira, Venezuela. According to them, large numbers of phosphate solubilizing microorganisms were found in the rhizospheric than in the bulk soil.

In our studies, 37 rhizosphere soil samples from tomato plants were screened for the presence of phosphate solubilizing bacteria. Among the 43 isolates, 33 were found positive for solubilization of both organic and inorganic forms of phosphorous. Based on the root colonization assay and in vitro plant growth promoting ability, 16 PSRB isolates were selected for further studies. Similar studies have been conducted by Alikhani et al. (2006), where different isolates of *Rhizobia* from Iranian soils were tested for their ability to dissolve inorganic and organic phosphates. They also analyzed the drop in pH of the culture filtrate with the release of soluble orthophosphate which indicated the importance of organic acid production in the mobilization process.

Similarly in our studies too the decrease in the pH of culture filtrate was observed and well correlated with the increased level of orthophosphate in culture filtrate. Analysis of lyophilized culture filtrates by TLC revealed the presence of, gluconic, oxalic, citric, lactic and isobutyric acids. Which indicated that the solubilization of phosphate and decrease in pH were in relation to the production of organic acids. Lowering of pH, owing to the production of organic acids such as gluconic, 2-keto gluconic, lactic, isovaleric, isobutyric, acetic, oxalic, citric acid was reported by several early researchers (Omar 1998; Rodriguez and Fraga 1999; Thakuria et al. 2004; Alikhani et al. 2006; Islam et al. 2007).

Soil with Ca–P as a major phosphorous source also has high buffering capacity (Ae et al. 1991). But several early reports suggests that the low buffering capacity of the screening media would lead to the isolation and designation of any bacteria that can lower the pH of the medium as phosphate solubilizing microorganisms do. But the bacteria probably will not be able to do this in soil because the soil is buffered. In the soil, 20–80% of phosphate is in organic form (Richardson 2001) and plant may poorly/not posses an innate ability to acquire phosphorus directly from soil phytate (Greiner and Alminger 2001). Hence,

Table 5 Effect of PSRB seed bacterization on plant growth and rhizosphere soil under greenhouse conditions

Isolates	MSL (cm)	MRL (cm)	FW (g/s)	DW (g/s)	Total P (mg/g dry weight)	pH of RS	Available P in RS (μg/g soil)
Control	09.0±0.16^{c}	12.5±0.34^{de}	0.225±0.007^{h}	0.041±0.0011^{d}	1.26±0.121^{bc}	6.0±0.057^{ab}	30±1.15^{i}
PSRB1	14.0±0.57^{ab}	17.0±0.57^{ab}	0.640±0.017^{b}	0.0425±0.0013^{d}	1.55±0.080^{ab}	5.9±0.173^{ab}	85±2.88^{a}
PSRB2	13.5±0.52^{ab}	17.0±0.98^{ab}	0.550±0.005^{cdef}	0.0565±0.0018^{bcd}	1.43±0.046^{abc}	6.0±0.115^{ab}	56±7.12^{cdef}
PSRB6	11.0±0.52^{bc}	15.0±0.52^{bcd}	0.479±0.011^{f}	0.0439±0.0023^{cd}	1.41±0.046^{abc}	5.5±0.057^{ab}	50±1.15^{defg}
PSRB7	13.0±1.11^{ab}	17.0±0.86^{ab}	0.572±0.018^{bcd}	0.0585±0.0013^{bcd}	1.49±0.059^{abc}	5.9±0.260^{ab}	60±2.30^{cde}
PSRB8	13.5±0.34^{ab}	18.0±0.46^{a}	0.724±0.011^{a}	0.098±0.0051^{a}	1.49±0.103^{abc}	5.9±0.115^{ab}	83±1.73^{ab}
PSRB12	09.0±0.40^{c}	11.0±0.17^{e}	0.231±0.012$_{h}$	0.046±0.0034^{bcd}	1.39±0.069^{abc}	5.4±0.057^{c}	48±3.46^{efgh}
PSRB14	14.0±0.99^{ab}	16.5±0.40^{abc}	0.489±0.013^{ef}	0.059±0.0075^{bcd}	1.22±0.075^{c}	5.5±0.173^{ab}	39±0.57^{ghi}
PSRB19	14.0±0.40^{ab}	18.0±0.57^{a}	0.762±0.007^{a}	0.110±0.0063^{a}	1.50±0.144^{abc}	5.4±0.173^{c}	65±2.88^{cd}
PSRB20	15.0±0.98^{a}	15.0±0.23^{bcd}	0.609±0.011^{bc}	0.065±0.0028^{bc}	1.27±0.040^{bc}	5.7±0.000^{ab}	34±2.30^{hi}
PSRB21	14.0±0.81^{ab}	15.5±0.22^{abc}	0.579±0.013^{bcd}	0.059±0.0046^{bcd}	1.57±0.063^{a}	5.9±0.057^{ab}	71±2.30^{abc}
PSRB27	13.0±0.52^{ab}	15.0±0.40^{bcd}	0.559±0.015^{cde}	0.056±0.0023^{bcd}	1.24±0.051^{c}	5.8±0.000^{ab}	36±1.73^{ghi}
PSRB28	12.5±0.29^{ab}	16.5±0.23^{abc}	0.496±0.023$_{ef}$	0.054±0.0034^{bcd}	1.50±0.121^{abc}	5.8±0.115^{ab}	75±3.33^{abc}
PSRB31	13.0±0.34^{ab}	17.0±0.57^{ab}	0.527±0.011^{def}	0.059±0.0051^{bcd}	1.31±0.046^{abc}	5.5±0.173^{ab}	68±4.04^{bc}
PSRB32	14.5±0.75^{a}	16.0±0.71^{abc}	0.639±0.020^{b}	0.068±0.0057^{b}	1.38±0.034^{abc}	6.0±0.057^{ab}	38±2.88^{ghi}
PSRB38	13.5±0.46^{ab}	14.0±0.17^{cde}	0.541±0.006^{cdef}	0.052±0.0040^{bcd}	1.30±0.051^{abc}	6.1±0.057^{a}	44±1.15^{fghi}
PSRB41	11.0±0.52^{bc}	12.5±0.29^{de}	0.329±0.008^{g}	0.040±0.0051^{d}	1.41±0.161^{abc}	5.9±0.173^{ab}	41±2.88^{ghi}

Values are the mean two repeated experiment. Mean values with in column sharing the same letters are not significantly different according to Tukey's HSD at $P \leq 0.05$

MRL mean root length, *MSL* mean shoot length, *FW* fresh weight, *DW* dry weight, *P* phosphorous, *RS* rhizosphere soil sample

these isolated PSRB's were further analyzed for their phytase activity which degrades the soil phytate to lower phosphate esters which are available to plants.

In our study, among the 43 isolates studied, 33 were found to solubilize calcium phytate, and among them PSRB31 predominantly produced higher level of phytase. Phytase has been isolated and characterized earlier from a few Gram-positive and Gram-negative soil bacteria e.g. *Bacillus subtilis* (Kerovuo et al. 1998), *B. amyloliquefaciens* PS11 (Kim et al. 1997), *B. laevolacticus* (Gulati et al. 2007), *Klebsiella terrigena* (Greiner et al. 1997), *Pseudomonas* spp. (Richardson and Hadobas 1997) and *Enterobacter* sp. (Yoon et al. 1996). Idriss et al. (2002) reported that the extracellular phytase from *B. amyloliquefaciens* FZB45 promotes growth of maize seedlings under in vitro conditions.

PSRB can exert a direct effect on plant growth other than the mechanism of phosphate solubilization like, production of phytohormones, biological nitrogen

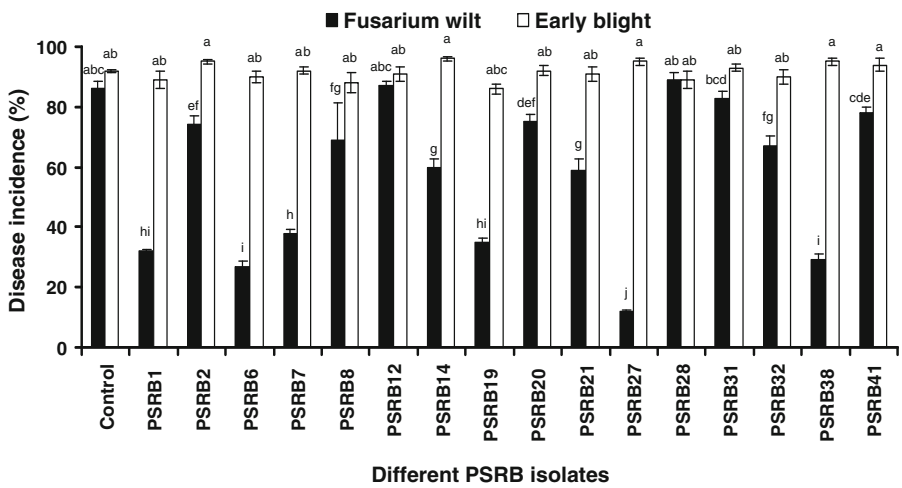

Fig. 1 Effect of PSRB seed bacterization on fusarium wilt and early blight incidence of tomato under greenhouse conditions. Percentage protection is mean from two repeated experiments. Mean designated with the same *letter* are not significantly different according to Tukey's HSD at $P \leq 0.05$

fixation, enhancing the availability of other trace elements, increased iron nutrition through iron-chelating siderophores and volatile compounds that affects the plant signaling pathways. Additionally, by antibiosis, competition for space and nutrients, and induction of systemic resistance in plant against a broad-spectrum of root and foliar pathogens (Leinhos and Nacek 1994; Arshad and Frankenbergar Jr 1998; Khan and Khan 2001; Gyaneshwar et al. 2002). Characterization of different traits of rhizobacteria was the common procedure employed while screening and selecting PGPR's (Cattelan et al. 1999). In our studies too, PSRB's were characterized for different traits like, root colonization, IAA production, siderophore, HCN, chitinase, β-1, 3 glucanase, cellulase, antibiotics production and its plant growth promoting ability.

The results of plant growth promotion studies under laboratory and greenhouse conditions did not correlate and may be due to the varied ability of PSRB isolates to perform under different environmental conditions. In both conditions, the selected PSRB isolates were effective in promoting plant growth when compared uninoculated control. This may be due to their ability to stimulate plant growth directly, production of IAA along with phosphate solubilization. When rhizosphere soil samples of 30 day-old-seedlings were analyzed, not much variations in pH was observed with all PSRB isolates. Seedlings raised from seeds bacterized with PSRB isolates that were found negative for phytase showed accumulation of phosphorus content which was almost equal to uninoculated control. This was well correlated with the stabilized pH in the rhizosphere soil sample and may be due to the buffering capacity of soil which reduce the phosphate solubilizing ability of PSRB (Ae et al. 1991). All other isolates showed increased level of phosphorus accumulation in seedlings, among them isolate PSRB21 treatment showed significant increase in accumulation of phosphorous in comparison with uninoculated control and other PSRB isolates.

Isolate PSRB21 showed significant increase in phosphorus accumulation in seedlings. But isolate PSRB19 was found to significantly increase fresh weight and dry weight of seedlings over PSRB21. Phosphorus solubilization is one of the important mechanisms through which PSRB isolates promote plant growth, but this is not only the way of plant growth promotion. There are several other mechanisms like direct stimulation, production of gibberellins, cytokinin, ACC deaminase, volatile compounds that are reported previously (Podile and Kishor 2006) which was not characterized in the present study. It was assumed that one or many of these traits may be involved in the plant growth promoting activity by isolate PSRB19.

When seedlings raised from PSRB's treated seeds were challenge inoculated with pathogens like *F. oxysporum* and *A. solani*, all selected PSRB isolates afforded some degree of resistance against fusarium wilt. Among them, isolates which showed antagonism against *F. oxysporum* under in vitro experiments provided higher degree of resistance over the isolates not inhibiting the fungal growth. But in plants raised from seeds bacterized with PSRB19, a reduced incidence (35%) of fusarium wilt was noticed even the isolate was not inhibiting the fungal growth in dual culture experiments. The protection offered by isolate PSRB19 was may be due to induced systemic resistance. Unfortunately, in the current study we have not come across a potential PSRB which has the ability to reduce the early blight incidence significantly by inducing systemic resistance. Even PSRB isolates found to inhibit the fungal growth in dual culture experiments failed to reduce the early blight incidence, due to the fact that the pathogen and PSRB isolates were spatially separated.

The present study provides strong evidence that in nature phosphate solubilization is a complex process and much of the phosphate solubilizing bacteria posses more than one mechanism to solubilize phosphate available in different forms. The results obtained from this study also reveals that usage of biofertilizers with only phosphate solubilizing ability should be avoided, and be replace with PSRB's having other beneficial mechanisms for plant health improvement as biofertilizers to improve plant health. Therefore, finding agricultural inoculants with high phosphate solubilizing ability and protecting plants from wide range of plant pathogens would be of immense interest for improving plant health and reducing phosphorous pollution in the soil.

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