



ISOLATION OF PHOSPHATE SOLUBILIZING BACTERIA AND THEIR USE FOR PLANT GROWTH PROMOTION IN TOMATO SEEDLING AND PLANT

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ABSTRACT

Phosphorous (P) is an essential macronutrient and most soils contain high levels of P. However, its availability to plant is limited by rapid immobilization of phosphorous compounds to insoluble forms and hence plant available forms of P in soils are found in low amounts. Phosphate solubilizing bacteria provide an eco-friendly alternative to convert insoluble phosphates into plant available forms. In the present study, three phosphate solubilizing bacterial isolates (PB-1, PB-4 and VC-01) with visually significant phosphate solubilizing abilities were isolated from tomato rhizosphere soil. In-vitro study in pikovskaya's agar revealed that isolate PB-1 had the highest phosphate solubilizing ability with a phosphate solubilizing index of 2.08 ± 0.07 followed by isolate VC-01 (1.31 ± 0.09) and PB-4 (1.24 ± 0.08). Isolates were used as bacterial inoculum to assess their ability to promote tomato (*Lycopersicon esculentum* var. Srijana) seedling and plant growth in in-vitro and greenhouse experiment respectively. Isolate PB-4 showed best growth promotion in seedling assay whereas isolate PB-1 and VC-01 also promoted seedling growth compared to control. In greenhouse experiment however, isolates VC-01 and PB-1 significantly enhanced all parameters (shoot length, root length, shoot and root dry weight) compared to uninoculated control whereas isolate PB-4 had a positive effect on all parameters except root length.

Keywords: Phosphate solubilizing bacteria, Rhizosphere, Phosphate solubilization index, Seedling vigor

INTRODUCTION

Phosphorous(P) is the second most essential macronutrient after nitrogen for plant growth and development [1]. Conventional farming system relies on heavy application of chemical phosphorous fertilizers to maintain optimum levels of phosphorous in agricultural soils. However, major portions (around 75% in some soils) of these soluble phosphorous are rapidly immobilized in soil which makes it unavailable for plants [2]. Low levels of plant available P in addition to high costs of P fertilizers have warranted the use of soil microorganisms as inoculants to mobilize poorly available phosphorous present in soil [3].



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Several bacterial species are able to solubilize insoluble inorganic phosphates in-vitro among which *Bacilli*, *Rhizobia* and *Pseudomonads* are the most studied genera [4]. This ability to dissolve insoluble calcium phosphates present in soil is termed as mineral phosphate solubilization (Mps) [5]. The basic mechanism of mineral phosphate solubilization by PSB strains entails the release of low molecular weight organic acids [5][6]. The hydroxyl and carboxyl groups of organic acids chelate the cations bound to phosphate and hence convert them to plant available forms [7]. However, P-solubilization mechanism is quite complex and depends on factors such as nutritional, physiological and growth conditions of the culture [8].

Tomato has been an economically significant crop in recent times and phosphorous nutrition is a limiting factor in maintaining its yield and quality. Environmental problems associated with the use of chemical phosphorous fertilizers pose a major challenge in maintaining its yield and quality in an eco-friendly manner [9]. The objective of the present study is to isolate phosphate solubilizing bacteria from tomato rhizosphere soil and use it as inoculants to assess the extent of growth promotion in tomato seedling and plant.

MATERIALS AND METHODS

Collection of soil samples

Rhizosphere soil samples from *Lycopersicon esculentum* var. Srijana were collected from three different organic farms of Kavre district. Tomato plants were uprooted carefully with their rhizosphere soil intact and collected in sterile plastic bags and transported to laboratory in an ice-box [10]. Plants were shaken vigorously to remove soil from the roots which was then sieved through a 2 mm sieve and collected in a sample bag and stored at 4°C until further examination [11].

Isolation, selection and maintenance of bacterial isolates

One gm rhizosphere soil was mixed in 9 ml 0.9% NaCl solution [12] and shaken vigorously for 5 minutes. 1ml of the solution was then diluted up to 10^{-6} dilution and 0.2 ml from each dilution was placed on Pikovskaya agar (PKA) [13] plates and incubated for 5 days at 30°C. Phosphate solubilization ability of the isolates is indicated by the formation of clear zones around the bacterial colonies. Isolates exhibiting distinct clear zones were visually selected and purified to obtain pure colonies. Three isolates with prominent phosphate solubilizing ability were isolated from the samples. Isolates were maintained in 40% (v/v) glycerol at -20°C for long term storage.

Phosphate solubilization index (Psi) determination

Bacterial isolates were grown in Nutrient broth (NB) for 24 hrs in a shaking incubator at $28 \pm 2^\circ\text{C}$. Two hundred microliters of bacterial cultures were taken for inoculation on PKA plates. Optical density of the culture was maintained between 0.5 - 1 at 550 nm such that each of them contained at least 10^8 cells/ml.



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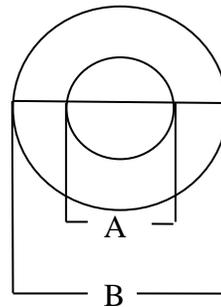
Stab inoculation was performed on PKA plates using sterilized cotton buds and the plates were incubated at 30°C for 7 days after which the phosphate solubilization index was calculated.

Phosphate solubilization index is a measure of the extent of phosphate solubilizing ability of bacterial isolates. It is determined by the formula below [14].

$$\text{Phosphate Solubilization Index (SI)} = B/A$$

Where; A = Colony diameter

$$B = \text{Total diameter (colony + halo zone)}$$



***In vitro* plant growth promotion assay**

Plant growth-promoting potential of the isolates in tomato seed germination and vigor was assessed according to Gholami [15] and Hariprasad [16] with modifications. Three replicates were taken for each treatment with ten seeds per replicate. Seeds sterilized with 1% sodium hypochlorite (NaOCl) were left to dry after washing for three times with sterilized distilled water (SDW). The seeds were then treated with 24 hours old bacterial cultures for 2 hours at $OD_{550} = (0.5-1)$ such that the culture concentration was at least 10^8 cfu/ml. Seeds were then placed in sterilized Whatmann filter paper no.1 in a petriplate. Plates were incubated at 28°C for 7 days in an incubator. Germination percentage of the seeds was recorded and the vigor index was calculated by the following formula:

$$\text{Vigor Index} = \text{Percentage of germination} \times \text{Seedling length (Shoot + Root length)} [17]$$

Plant growth promotion in green house assay

Plant growth promoting ability of isolated bacterial strains was assessed in *Lycopersicon esculentum* var. Srijana. Tomato seeds were surface sterilized with 1% sodium hypochlorite (NaOCl) [16] and grown in seedling trays until they were 3 weeks old. Seedlings were grown in a greenhouse maintaining temperatures of 28°C during day and 20°C during night. Three weeks old seedlings were then transferred to medium sized pots with 12 cm height and 9 cm diameter. Pots used for the experiment contained 600 gm of twice autoclaved sandy loam soil. Tomato seedlings were allowed to grow for two days before applying bacterial treatment.

For the preparation of bacterial treatment, isolates were first streaked on Nutrient Agar (NA) plates and incubated at 30°C for 48 h. Single colonies on NA plates were picked with an inoculating loop and transferred to 250 ml NB and grown aerobically in a rotary shaker at 160 rpm at $30 \pm 2^\circ\text{C}$ for 48h. The bacterial suspension was diluted in SDW to obtain a minimum final cell concentration of $10^8 - 10^9$



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CFU/ml at (OD = 0.5 - 1 at 550 nm). Diluted cell suspension was used to treat tomato plants in the form of soil drenching. Bacterial treatment was done twice one week apart and the temperature of the greenhouse was maintained at $30 \pm 2^\circ\text{C}$. Pots were watered twice daily with 50 ml water each day. The experiment was performed in a completely randomized block design with five replications per treatment. Uninoculated plants were used as negative control and plants treated with the phosphate solubilizing strain *Pseudomonas fluorescens* KACC 10327 was used as positive control. Plants were grown for three weeks after the last bacterial treatment and then harvested after observing the first signs of flowering. Plant height, shoot and root height along with shoot and root fresh and dry weights were measured and compared to uninoculated control.

Statistical analysis

Data generated from in vitro and green house experiments were analyzed using MS-Excel (office Package 2010).

RESULT AND DISCUSSION

Screening of phosphate solubilizing bacteria

Three bacterial isolates with distinct clear zones in PKA plates were selected for in-vitro and greenhouse experiments.

Phosphate solubilization index

The ability of isolated strains to solubilize tri-calcium phosphate (TCP) in-vitro varied significantly compared to the control strain. Isolate PB-1 exhibited maximum phosphate solubilizing index of 2.08 in in-vitro conditions. Isolates PB-4 and VC-01 showed comparable phosphate solubilizing indices of 1.24 and 1.31 respectively which was less than that of positive control *P. fluorescens* KACC 10327 which showed an index of 1.71 (Table 1). The possible mechanism for different solubilizing abilities of bacterial isolates in PKA plates is the release of organic acids and their chelating abilities [18]. However, the characterization of different organic acids produced by the isolates was not performed in the study.

Table 1. Phosphate solubilization efficiency expressed as phosphate solubilization index (PSI) of bacterial isolates in plate assay in Pikovskaya's agar

Isolates	Pi solubilizing index (PSI) ^a
<i>Pseudomonas fluorescens</i> KACC 10327	1.71±0.03
PB-1	2.08±0.07
PB-4	1.24±0.08
VC-01	1.31±0.09

^aResults expressed as the mean of three replicates \pm standard deviation.



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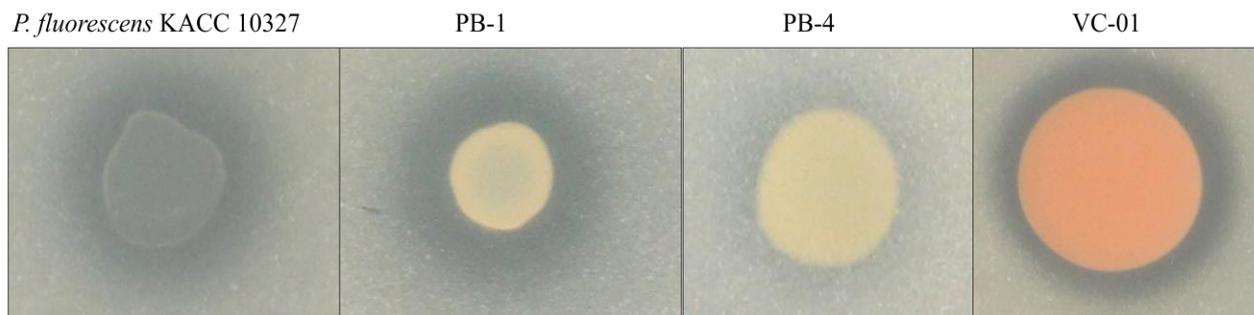


Figure 1. Clear zones (halos) produced by phosphate solubilizing bacterial isolates in Pikovskaya agar plates after 7 days incubation at $30\pm 2^{\circ}\text{C}$

In vitro plant growth promotion assay

The bacterial treatments had a positive effect on seedling vigor of tomato, compared to water control. Treatment with isolates PB-4 and VC-01 yielded the longest plumule length of 2.77 cm whereas longest radical length of 0.74 cm was observed in seeds treated with isolate PB-4 which also had the highest vigor index of 327.59. Isolates VC-01 (291.19) and PB-01 (284.65) exhibited higher vigor index compared to control (Table 2). Observed increase in seedling vigor index due to phosphate solubilizing bacteria is in accordance with the findings of [19] who reported similar increment. The underlying mechanism for the enhancement of seedling growth can be the synthesis of plant growth regulators by phosphate solubilizing bacteria.

Table 2. Effect of different treatments on germination efficiency of tomato seeds under laboratory conditions

Treatments	Radicle L (cm)	Plumule L (cm)	GP (%)	SVI
Control	0.34 ± 0.04	2.16 ± 0.24	93.33	233.33
<i>P. fluorescens</i> KACC 10327	0.34 ± 0.08	2.63 ± 0.34	83.33	247.49
<i>Bacillus subtilis</i> GBO3	0.44 ± 0.06	2.66 ± 0.09	96.67	299.68
PB-1	0.4 ± 0.09	2.65 ± 0.02	93.33	284.66
PB-4	0.74 ± 0.05	2.77 ± 0.32	93.33	327.59
VC-01	0.35 ± 0.09	2.77 ± 0.19	93.33	291.19

Note: Control – 10 mM MgSO_4 buffer; L – length; GP – Germination percentage; SVI – Seedling vigor index

ANOVA test revealed significant differences ($P < 0.05$) between mean values of treatment for plumule length. Mean radical lengths were not significantly different ($P < 0.05$).



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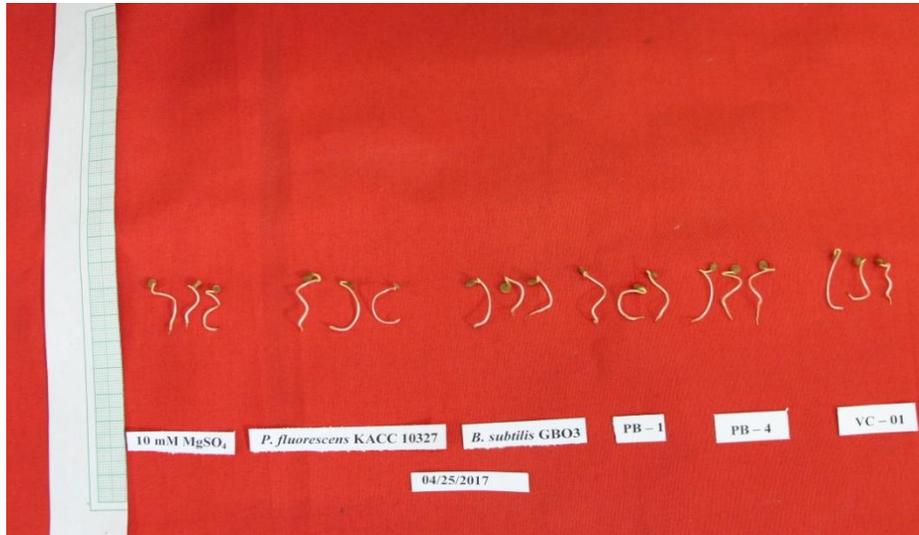


Figure 2. Germinated tomato seedlings after 7 days

Plant growth promotion in green house assay

Analysis of greenhouse experiment data revealed significant increase ($P < 0.05$) compared to control in at least five parameters due to the effect of bacterial treatment (Table 3). Among the three isolates, VC-01 showed the highest growth promoting activity in all the parameters except root dry weight and shoot length. Isolate PB-1 exhibited best effect on shoot length whereas both PB-1 and PB-4 had the best effect on root dry weight followed by VC-01. Increase in root dry weight exhibited the ability of phosphate solubilizing bacteria to promote plant growth by enhancing soil phosphate availability [20].

Shoot dry weight data analysis revealed no significant differences ($P < 0.05$) between treatments. However, all the treatments had positive effect compared to control. Positive control *P. fluorescens* KACC 10327 had the highest SDW value (6.24 g) closely followed by isolate VC-01 (6.22 g) and PB-1 (6.2 g). Plants treated with PB-4 showed mean shoot dry weight value of 5.57 g which was comparatively less than that of other isolates but still higher than that of uninoculated control.

Table 3. Effect of bacterial treatments on different growth parameters in tomato in greenhouse pot trials

Treatment	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)
Control	32.6 ± 3.21	25.72±2.13	50.45±5.8	9.03 ± 1.51	5.4±0.54	0.66±0.81
<i>P. fluorescens</i> KACC 10327	37.5±2.65	27.9±5.66	55.69±6.28	11.72±1.93	6.24±0.95	0.95±0.97
PB-1	40.9±5.55	28.9±4.52	55.91±5.66	11.65±1.49	6.20±0.75	0.96±0.98
PB-4	34.8±3.03	22.8±3.78	48.43±4.31	11.41±0.49	5.57±0.46	0.96±0.98
VC-01	39.2±4.91	34.5±8.52	57.11±5.86	12.01±1.91	6.22±0.85	0.91±0.95

Note: Control – SDW (Sterilized distilled water)

Mean values were obtained from trials which included five replicates per treatment. ANOVA test revealed significant differences ($P<0.05$) between different treatments in all of the parameters except shoot dry weight (SDW).

Figure 3. Growth enhancement in tomato roots treated with bacterial isolates

There was a significant difference ($P<0.05$) in shoot and root length between the treatments and uninoculated control. Isolate PB-1 showed the best effect (40.9 cm) on shoot length along with isolate VC-01 (39.20 cm). Furthermore, isolate VC-01 exhibited the best effect on root length (34.5) compared to control (25.72 cm). Plants treated with PB-1 had a mean root length of 28.9 cm which was significantly higher than control. However, isolate PB-4 did not have a positive effect on root length



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compared to control. Enhanced root growth can be owed to increased levels of soluble phosphates in soil [21].

CONCLUSION

In the present study, three bacterial isolates with phosphate solubilizing abilities were isolated and used as inoculants for plant growth promotion in tomato. Results indicated significant levels of growth enhancement by the isolates in seedling assay and greenhouse experiment. The positive influence can be owed to solubilization of fixed inorganic phosphates in soil as well as release of plant growth hormones, although they were not characterized in the study. Such bacterial species occur naturally in soil, however, phosphate solubilizing abilities and other plant growth promoting traits are limited by their individual microbial load. Hence, use of such isolates as inoculums in soil can exponentially increase their population, enhancing their roles in phosphate solubilization and other plant growth promoting activities. Maintaining agricultural yields is limited by phosphorous immobilization in soil which warrants the use of chemical phosphorous fertilizers. Meanwhile, increasing costs of such chemical fertilizers and the extreme negative impacts on environment associated with their use opens door for isolation and utilization of phosphate solubilizing bacteria in organic as well as sustainable agricultural practices.

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