Phosphate solubilizing bacteria isolated from the rhizosphere soil and its growth promotion on black pepper (*Piper nigrum* L.) cuttings

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Abstract

Bacterial isolates from the rhizosphere soil and root cuttings of bush black pepper (*Piper nigrum* L.) (pepper raised by laterals) exhibiting high phosphate solubilizing ability *in-vitro* is described in this paper. Microbial phosphorus solubilization (MPS) trait was analyzed by determining the P solubilization efficiency E (E = Diameter of bacterial growth/Diameter of clearing zone × 100). The highest P solubilization efficiency was demonstrated by the isolate PB-21 followed by the isolate PB-16: which was identified as *Pseudomonas* sp. All isolates under study released inorganic phosphate from tricalcium phosphate (TCP) indicating the potential of these strains to release soluble inorganic phosphates from fixed phosphate sources for plant uptake. The isolate $PIAR_{6-2}$ was able to solubilize 20.01% of P and also fix atmospheric nitrogen, which was later identified as the nitrogen fixing *Azospirillum* sp. Greenhouse trials using two systems: viz; Soil:Terracare (composted coir pith) and Sand:Soil:FYM with three experimental sets such as rock phosphate (RP as an external P source), PSB isolate in combination with VAM and PSB isolate alone, all against their respective control sets showed very clearly the growth promoting activity of phosphate solubilizing bacteria. Field studies were also carried out using these isolates and some promising results were obtained. Further studies are required to analyze these strains to confirm its plant growth promoting properties. The *in-vitro* analyses and greenhouse studies of these bacteria reflect their potentiality as efficient P solubilizer in black pepper growing soils.

Introduction

The soil environment surrounding plant roots is the zone of intense microbial activity. The existence of soil microbes capable of transforming soil phosphorus and fixing nitrogen from the atmosphere to forms available to the plants has been recorded by many investigators. It has been observed that a high proportion of phosphate solubilizing microorganisms are concentrated in the rhizosphere of plants (Whipps and Lynch, 1986). The recognition in mid-70s that biological nitrogen fixation offers the most promising supplement to chemical nitrogenous fertilizers led to a wide array of studies on associative, free living and symbiotic nitrogen fixation by rhizosphere micro flora. These microbes are commonly found in association with the roots of diverse plants (Brown, 1975).

The main problem in applying P as a plant nutrient in P fixing tropical soils is its conversion

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to unavailable P in soil up to 85%. Therefore, for making P available to plants, several microorganisms are used as P solubilizers. Isolation of indigenous micro flora capable of phosphorus solubilization and nitrogen fixation is an important procedure when studying their inherent capacity to benefit crops probably because of their superior adaptability to the environment than the introduced strains. The aim of the present study was to record the phosphate solubilizing capacities of some indigenous bacteria isolated from rhizosphere of bush black pepper (*Piper nigrum* L.) raised by rooting lateral shoots and to select promising strains for further experiments to determine the effectiveness of these strains as plant growth promoting rhizobacteria (PGPR).

Materials and methods

Isolation and evaluation of phosphate solubilizing bacteria (PSB)

Tricalcium phosphate (TCP) is regarded as a model compound for measuring the potential or relative rates of microbial solubilization of insoluble inorganic phosphate compounds. In addition, the insoluble calcium phosphate forms a major portion of insoluble phosphate in soil (Devi and Narasimhan, 1978). Solubilization of precipitated TCP in unbuffered solid agar medium plates has been used widely as the initial criterion for the isolation of phosphate solubilizing microorganisms (Pikovskaya, 1948). Microorganisms on precipated calcium phosphate agar produces clear zones around their colonies if they are capable of solubilizing calcium phosphate.

Suitable dilutions (10^{-4}) of serially diluted rhizosphere soil suspension were poured plated on Pikovskaya's Agar (glucose – 10 g, $Ca_3(PO_4)_2$ – 5 g, $(NH_4)_2SO_4$ – 0.5 g, KCl – 0.2 g, $MgSO_4$ – 0.1 g, $MnSO_4$ – traces, $FeSO_4$ – traces, Yeast Extract – 0.5 g, Agar – 15 g, Distilled water – 1 L, pH – 7.0) and the plates were incubated at 30 ± 5 °C for 48–96 h. Phosphate solubilization is indicated by the formation of a solubilization or a clear zone around the bacterial colonies. Single bacterial colonies having clear solubilization zones were isolated separately on to fresh Pikovskaya's agar plates, incubated at 30 ± 5 °C for 10 days and an analysis of the MPS trait was made by measuring the zone of solubilization around the colony growth. The solubilization efficiency (E) of these isolates was calculated based on the relation,

$$\text{Solubilization efficiency(E)} = \frac{\text{Solubilization diameter (S)}}{\text{Growth diameter (G)}} \times 100$$

(Nguyen et al., 1992).

The release of soluble P from TCP was determined by the method described by Jackson (1967). Single colonies of these isolates grown on nutrient agar were inoculated into 0.05 L of nutrient broth and 0.0005 L of these 24 h grown cultures were inoculated into 0.05 L of Pikovskaya's broth and incubated over shaker at 29 ± 3 °C and 100 rpm for 5 days. The drop in pH of the medium was measured by a pH meter. The nitrogen-fixing bacteria isolated from secondary roots of bush pepper, *Azospirillum* sp. $PIAR_{6-2}$ has also been tested for its P solubilizing efficiency.

To evaluate the P release and growth promoting efficiency of the phosphate solubilizing bacteria PB-21, a nursery experiment on black pepper was conducted at IISR Experimental Farm, Peruvannamuzhi, Calicut, Kerala. The single node black pepper cuttings (cultivar. Karimunda) were planted in 0.20 × 0.15 m poly bags (1.5 kg mixture per bag) with two potting media viz., (1) Potting mixture (1:1:1, Sand:Soil:FYM) and (2) Soil:TC (TC – Terracare Coir pith compost marketed by M/s. Marson Biocare Ltd., Mumbai, India) (80:20 w/w). The treatments imposed were Control (C), Potting media added with rock phosphate (RP), Phosphate solubilizing bacteria (PSB), RP + PSB mixture, VAM and VAM + PSB combined inoculation. The PSB culture PB-21 that is identified as efficient strain from *in-vitro* studies was used for growth promotion studies. The VAM culture isolated and identified (*Glomus fasiculatum*) by the Division of Crop Protection was used to evaluate the growth promotion and P utilization in combination with PB-21.

The VAM culture was multiplied on sorghum and soil with root bits at 50 g per bag containing 500–600 propagules mixed thoroughly with the mixture. The rock phosphate was applied at 0.1% P per bag and mixed thoroughly. PSB was applied at 0.05 L culture concentrate (with 10^6 cfu/mL) per bag. The cuttings were grown for 3 months and the observations on shoot length, total root length, length of finer roots with < 1 mm diameter (GS root, v5.2-GS root is a software used for the root length measurement studies and its version is 5.2), total dry matter and P uptake were recorded. The P availability in the medium was also measured after 3 months (Jackson, 1967). The population of PSB was also monitored.

Results and discussion

On Pikovskaya's agar, strains PB-21, PB-16, PB-19c and PB-13 showed very distinct clearing zone with PB-21 giving a very large, clear and transparent solubilization zone and highest percentage of P solubilization in Pikovskaya's broth (Table 1). Though the isolate $PIAR_{6-2}$ did not give any detectable solubilization zone in plate assay in Pikovskaya's broth about 20.01% of P was solubilized by the same strain (Figure 1). All isolates released inorganic phosphate from TCP indicating the potential of these strains to release inorganic soluble phosphate from fixed phosphates sources for plant uptake. Highest P solubilization efficiency (E) was demonstrated by PB-21 (Table 1).

An interesting feature of the *Azospirillum* isolate $PIAR_{6-2}$ is that it is also a phosphate solubilizer bringing about 20% of phosphate solubilization in Pikovskaya's broth containing TCP as phosphate source (Table 1). Katznelson and Bose (1959) found that rhizosphere bacteria has greater metabolic activity and suggested that they might contribute significantly to the phosphate economy of the plant. Laboratory studies reviewed by Kucey et al. (1989) have shown that the microbial solubilization of soil phosphate in liquid medium studies has often been due to the excretion of organic acids as a result of which a decrease in pH was effected.

Table 1. Phosphate solubilization by some bacterial isolates from rhizosphere soil of bush pepper with TCP as P source

Isolate	Fall in pH	Solubilization zone (mm)	% P solubilized	Solubilization efficiency (E)
PB-21	1.56	33	40.43	412.5
PB-16	1.72	12	39.08	133.3
PB-19C	0.30	25	30.43	192.3
PB-13	2.01	15	32.91	125.0
$PIAR_{6-2}$	1.15	ND[a]	20.01	NC[b]

[a]ND, Not Detectable; [b]NC, Not Calculated.

A few reports have indicated the phosphate solubilizing activity of some nitrogen fixers (Mahesh Kumar et al., 1999: Seshadri et al., 2000). A detailed study of the organism would throw light on the phosphate solubilizing properties that could be incorporated in agriculture as the same strain ($PIAR_{6-2}$) offers traits for nitrogen fixation also.

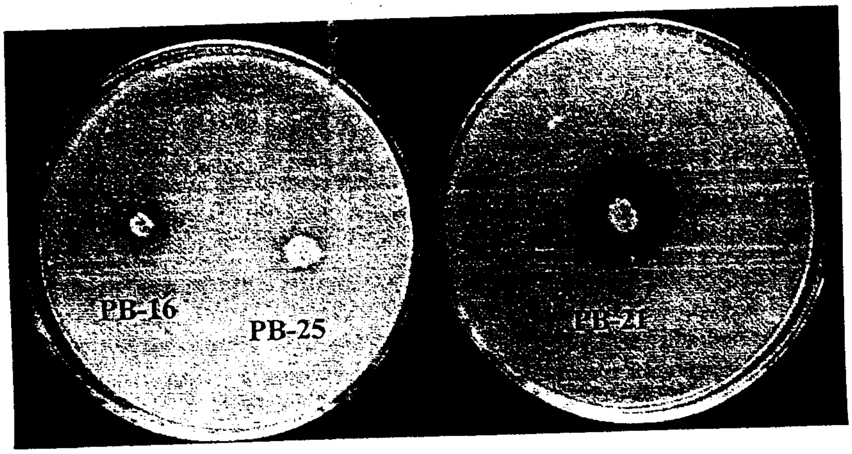
Growth promotion

The maximum and significantly high shoot length was observed in VAM treated cuttings on par with VAM + PSB treatment (Figure 2).

The inoculation of PSB alone and PSB + Rock phosphate has also increased the shoot growth (Table 2). The control plants on both the potting media have recorded the lowest shoot length. The dry matter production in 3 months also observed a similar trend where VAM, VAM + PSB inoculations produced highest dry matter of black pepper followed by PSB + RP & RP treatments. Dry matter in inoculation of PSB alone and PSB + RP was on par. Similar studies by Ahmed et al. (1999) also showed that combining *phosphorene* (as a source of phosphate solubilizing bacteria) at 0.1% with phosphate fertilizers had an incrementally beneficial effect on growth and P uptake on olive seedlings. Combined inoculation of organisms has given a better growth of the plant. The total and finer root length measured was significantly highest in VAM and combined inoculation of VAM + PSB, followed by PSB alone (Table 2).

Similar synergistic interaction between VAM and PSB was observed on black pepper (Kandiannan et al., 2000) and tomato (Kim et al., 1998). The inoculation of PSB + RP also recorded significantly high root length over RP application alone. The control plants produced only very less root mass. Production of finer roots (< 1 mm diameter) was maximum in microbial inoculated treatments. All VAM, PSB and combined inoculations recorded significant finer root production over RP and control treatments.

There was not much difference due to the media that was used to raise the cuttings on



Phosphate Solubilizing Bacteria

Figure 1. Phosphate solubilizing bacterial isolates from bush black pepper rhizosphere.

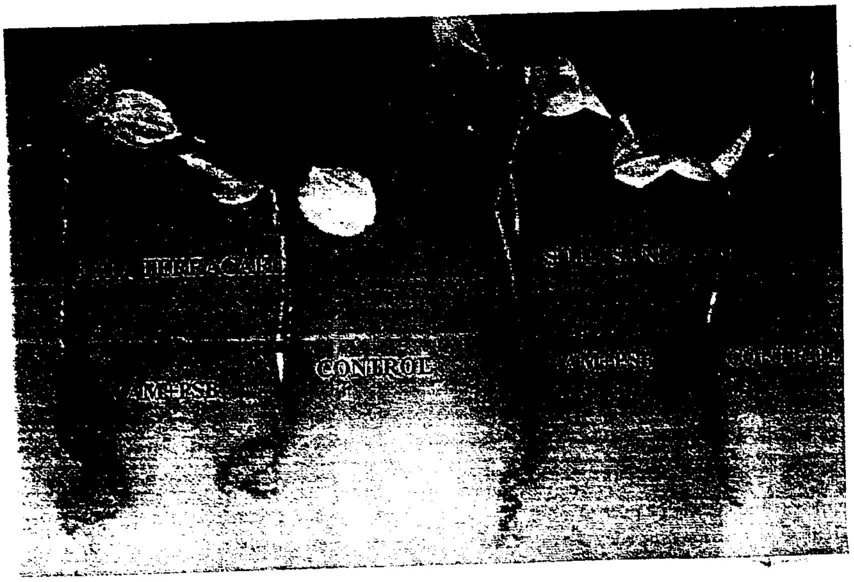


Figure 2. Effect of VAM + PSB isolate PB-21 on the growth of black pepper.

Table 2. Effect of PSB and VAM on P availability, uptake and growth promotion. of black pepper cuttings

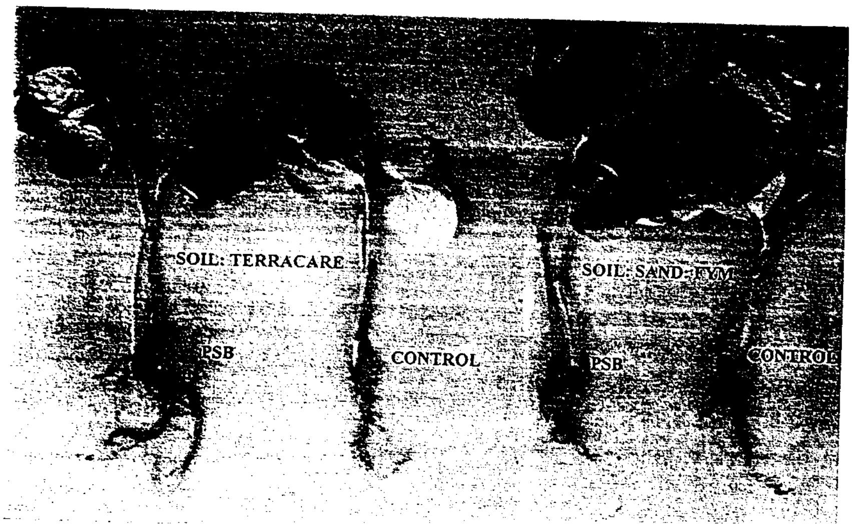
Treatment	Shoot length (mm)	Total root length (mm)	Root (< 1 mm dia.) length (mm)	Dry matter (g/bag)	P uptake (mg/plant)	Avail. P (mg/kg)	PSB (×10^4 cfu/g)
C	11.33	170.5	93.45	4.43	6.22	19.25	8.3
RP	15.56	211.3	170.6	6.58	9.02	40.03	11.5
PSB	14.83	668.3	540.1	7.23	9.39	48.86	17.0
RP & PSB	15.57	575.5	520.2	6.91	8.98	57.16	13.0
VAM	21.13	731.7	561.8	8.17	10.75	30.47	8.2
VAM + PSB	18.83	696.5	567.4	7.98	9.82	43.31	14.0
CD 5%	3.84	39.1	36.2	1.04	1.67	2.97	2.2

shoot length and dry matter production of black pepper cuttings over 3 months. Even though soil:sand:FYM medium recorded highest soil available Bray's P (56.89 ppm) over soil:TC medium (22.81 ppm), the shoot length and dry matter production were on par. But the root length measured was significantly high in soil:TC media as regard to total and finer root lengths (Table 3). As the medium was porous and provided very suitable condition for root penetration. the soil:TC produced maximum finer and total root mass as compared to ordinary potting mixture (Srinivasan and Hamza, 2000). The P uptake by the cuttings grown on different media followed their P availability in respective media. The cuttings from potting mixture (soil:sand:-FYM) have recorded significant uptake of P (0.01104 g/plant) than that from soil:TC media (0.00702 g). This higher uptake might be due to higher P availability from FYM source in the potting mixture grown.

Among treatments, highest P availability was recorded in PSB + RP inoculation followed by P B inoculation. Treatment with RP alone :-cc·rded the next best. P availability from rc :k p.iosphate increased when it was co-inoculat:d with PSB. The inoculation of PSB releases more available P from insoluble source like RP than any water soluble source of P (Chhonkar, 1994). More pronounced effect of PSB in the presence of Mussoorie rock phosphate on P release, uptake and growth promotion was observed in mungbean by Singh and Kapoor (1992). Higher P availability in VAM + PSB inoculation over VAM also reveals the definite role of PSB strain PB-21 in P solubilization. The P uptake by the plant was significantly highest in VAM, PSB and combined inoculations over RP and control. Soil:TC mixture has maintained a population of 14×10^4 cfu/g from the initial population of 0.3×10^4 cfu/g at zero hour of inoculation. FYM based mixture has supported upto 8×10^4 cfu/g. The coir compost base supported good multiplication of PSB culture PB-21 for mass production than FYM base (Figures 3 and 4). PSB population was recorded in all the treatments, whereas

Table 3 Effect of potting media on P availability, uptake and growth promotion of black pepper cuttings

Treatment	Shoot length (mm)	Total root length (mm)	Root (< 1 mm dia.) length (mm)	'ry matter /bag)	P uptake (mg/plant)	Avail. P (mg/kg)	PSB ($\times 10^4$ cfu/g)
Soil:Sand:FYM	16.78	371.5	284.0	02	11.04	56.89	8.0
Soil:TC	15.63	646.4	533.8	(.75	7.02	22.81	14.0
CD 5%	NS	22.6	20.9	NS	0.97	1.72	1.27



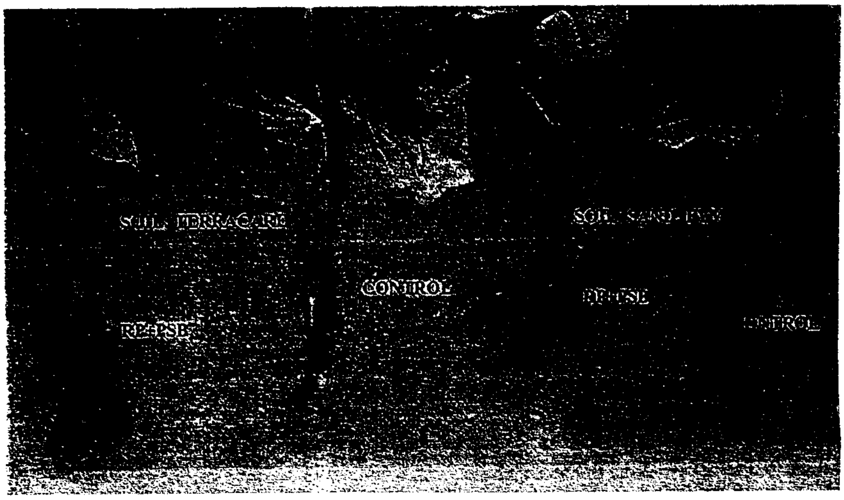


Figure 4. Effect of PSB isolate PB-21 + RP on the growth of black pepper.

it was significantly higher in respective treatments where PSB was added.

Conclusion

In the present study some efficient indigenous phosphate solubilizing bacteria were isolated from bush pepper rhizosphere and their potential worked out. As one of the isolate P1AR$_{6-2}$, identified as *Azospirillum* sp. has a unique characteristic of phosphate solubilization, the same can be used for nitrogen fertilization and phosphate solubilization. PB-21, the efficient PSB strain has efficiently solubilized and released P from insoluble RP and improved the shoot and root growth of the black pepper cuttings either alone or in combination with VAM. The present findings establish the potential for the use of efficient indigenous phosphate solubilizing bacterial isolate as a reliable alternative in low capital agriculture.

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