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Isolation and Characterization of Phosphate Solubilising Bacteria Isolated from Different Vegetable Crops

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Phosphate solubilizing bacteria (PSB) were isolated from the rhizosphere of different vegetable crops. A total of 18 isolates were selected and evaluated for P solubilization on solid as well as liquid medium. All the isolates showed large variations in P-solubilization, and they also produced indole-3-acetic acid (IAA) and phosphatase. On the basis of the 19 biochemical tests, these isolates were tentatively placed under three genera-*Klebsiella, Pseudomonas and Enterobacter.* All the selected PSB were able to establish well in the rhizosphere of wheat irrespective of the host. The shoot biomass was improved with seed bacterization by PSB. The plant growth promotion by PSB was not directly proportional with the solubilization of inorganic phosphate. The isolate that showed the maximum increase in shoot biomass did not possess the maximum P-solubilization ability; rather it produced the maximum IAA.

Key words: Phosphate solubilization, Plant-growth-promoting bacteria, Phosphatases, Rhizosphere, Seed bacterization.

Phosphorus is one the most essential elements for plant growth after nitrogen. However, the availability of this nutrient for plants is limited by different chemical reactions especially in arid and semi-arid soils[4]. The mobility of this element is very slow in the soil and can not respond to its rapid uptake by plants. This causes the creation and development of phosphorus depleted zones near the contact area of roots and soil in rhizosphere. Mineral phosphate solubilizing bacteria are considered among the most effective plant assistants to supply phosphorus at a favorable level. The principal mechanism for mineral phosphate solubilization is the production of organic acids and acid phosphatases play a major role in the mineralization of organic phosphorus in soil[14]. Chelating substances and inorganic acids such as sulphideric, nitric, and carbonic acid are considered as other mechanisms for phosphate solubilization[5]. Among the soil bacteria communities, *Achromobacter, Aerobacter, Alkaligenes, Bacillus, Serratia, Pseudomonas* and *Xanthomonas* are the important genera. In addition, certain fungi have also been shown to solubilize insoluble phosphate. These organisms have been in use as biofertilizers since 1962[20] and are gaining importance in the recent years due to their role in maintaining soil nutrient status and structure. Very little information is available on community structure of PSB. Moreover, community structure changes with host, irrigation, fertilizer application and climate[2]. Also, a continued exploration of the natural biodiversity of soil microorganisms, and the optimization and manipulation of microbial interactions in the rhizosphere of crops represents a prerequisite step to develop more efficient

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microbial inoculants. In order to assess the magnitude of changes in bacterial community, it is necessary to gain knowledge on bacterial diversity so that the endogenous PSB with ability to solubilise phosphate can be identified and developed as biofertilisers for various crops. The present study was undertaken to assess the distribution pattern of PSB in rhizosohere soils of different field crops.

MATERIALS AND METHODS

Enumeration, isolation and identification of PSB

PSB were isolated from rhizosphere of different crops such as ladyfinger, tomato, brinjal and cauliflower grown in Faridabad district. Intact root systems from all these host plants were collected and shaken gently to remove the soil closely adhering to the plant roots. These soils were air dried and used for the isolation of PSB. Suspensions from all the rhizospheres were diluted upto 10^{-4} with three replications, and plated with six replicate plates on Pikovskayas (PVK) medium[11]. The colonies distinguished by producing halo zones were identified and subcultured. As the plate assay is not considered a reliable method in determining a strain as phosphate solubiliser[7], the pure cultures were further screened in liquid medium containing tricalcium phosphate as insoluble P source at a concentration of 5 g/l 10^8 cfu/ml of different isolates were inoculated in 50 ml liquid PVK medium. Uninoculated flasks were used as control. The flasks were incubated at 30°C for 7 days and centrifuged at 15,000 r/min. The supernatant was passed through a 0.45uM Millipore filter and the inorganic phosphate content of the culture filtrate was determined by the molybdenum blue method[6].

Estimation of growth regulators produced by PSB

To measure the amount of IAA produced, 1.5 ml bacterial broth culture was centrifuged at 12,000 rpm for 5 minutes. To one milliliter of the supernatant 2 ml Salkowaski reagent was added. Salkowski reagent consisted of a mixture of 15 ml 0.5 M $FeCl_3$, 500 ml distilled water, and 300 ml of concentrated H_2S04. After 30 minutes, the mixture was read in UV-spectrophotometer at 530 nm absorbance. The amount of IAA produced per milliliter culture was estimated using a standard curve.

Determination of phosphatase activity

Phosphatase activity was determined in response to phosphorous enrichment using β-glycerophosphate as the phosphorous source. Culture filtrates were centrifuged and phosphatase activity was estimated following the procedure of Tabatabai and Bremner[18]. The phosphatase activity was calculated by referring to a standard graph prepared with *p*-nitrophenol. Enzyme activity was expressed as µg of *p*-nitrophenol released/mg cell protein in24h.

Biochemical Characterisation

All the strains were phenotypically characterized based on their differential utilization of different substrates.The reactions tested were indole formation, methyl red, Voges proskauer, nitrate reduction, urease hydrolysis,catalase activity, oxidase activity, H_2S production, gelatin hydrolysis, Lysine decarboxylase, arginine dihydrolase and Utilisation of arabinose, inositol,lactose, sorbitol,sucrose, and citrate.

Crop response and establishment of PSB in the rhizosphere of chickpea, mustard and wheat under pot culture conditions.

The PSB strains were checked for crop response and their establishment in the rhizosphere in wheat under pot culture conditions. Seed inoculation with the selected PSB strains was done by soaking the seeds of wheat (Raj 3765) in culture broth containing approx. 10^8 cells/ml for 30 min. Five The experiment was conducted with the following treatments: control (uninoculated), Single super phosphate, SSP (30kg P_2O_5/ha) and SSP along with all the PSB isolates. The plants were uprooted at 30 days after sowing (DAS) and dried in oven at 80ºC and shoot weight was determined. The rhizosphere samples from each treatment were serially diluted and appropriate dilutions (10^{-4}-10^{-6}) were plated on PVK medium plates .PSB were counted after incubating the plates at 30±2ºC for 2 days and the results expressed as average of three plates (cfu/g).

RESULTS AND DISCUSSION

Isolation of PSB from the rhizosphere of different food crops

A total of four samples were collected from the rhizosphere of different field crops and population density was determined. The difference

in the population density was not so significant (Table 1). This may be due to the similar soil types as similar soil types tend to select similar communities[3] .Also, the PSB count was lower than the total bacterial count in all the samples. Kundu *et al.*[9] also reported that the PSB count was about 10 to 100 times lower than the total bacterial count in the samples collected from rhizosphere of chickpea, mustard and wheat.

Table 1. Isolation of PSB from rhizosphere of different crops grown at Faridabad

Crops	Bacterial Counts(10^5 cells/g)		Types	Isolate Designation
	Total	PSB		
Ladyfinger	270	67	7	L1, L2, L3, L4, L5, L6, L7
Brinjal	346	37	2	B1, B2
Tomato	129	33	2	T1, T2
Cauliflower	100	15	1	C1

All the strains were able to solubilize inorganic contents in the medium (Table 2). Phosphate solubilizing efficiency ranged between 32.3-118.2 on solid medium .The isolate L4 from ladyfinger showed maximum P solubilization followed by L7 from ladyfinger and B2 from brinjal.The least P solubilization was shown by T3 from tomato. In PVK liquid medium, a decrease in pH and increase in soluble P content was recorded with all the PSB isolates suggesting the microbial production of organic acids.. Maximum P solubilization was observed with L7 while T3 showed the least. The P solubilization by various isolates on the solid medium and the broth did not show much correlation as also reported earlier[13]. Phosphatase activity was highest in the strain L7 isolated followed by L4 and L6 (Table 2). The enzyme activity was least in T3. However, a positive correlation was observed between phosphate solubilizing capacity and phosphatase activity, the results being in conformity with those reported earlier.

All the strains produced IAA. Maximum IAA was produced by B3 followed by L2 and T1.

Table 2. Phosphatase activity, Solubilization of Tricalcium phosphate in plate and broth assays and IAA production by PSB isolates from rhizosphere of different vegetable crops

Isolate No.	PSE (%)	pH	Phosphatase activity	P-solubilzation (µg/ml)	IAA((µg/ml)
L1	59.8	6.70	34	130.95	12.4
L2	80	5.75	38	150	23.6
L3	63.3	6.11	38	146.43	20.1
L4	118.2	5.50	83	142.86	ND
L5	85.3	6.47	41	150	8.9
L6	57.3	5.64	64	178.57	6.4
L7	112.5	6.01	86	222.6	4.9
B1	82.4	4.89	27	130	6.9
B2	93.3	4.88	60	137.7	ND
B3	81.6	6.50	57	175	29.9
B4	59.7	5.66	56	178.6	8.7
B5	66.3	5.85	42	156.3	3.5
T1	49.4	5.67	40	142	17.1
T2	58.7	3.0	41	146	2.9
T3	32.3	5.78	21	126.4	3.9
T4	5O.8	5.90	39	155	9.1
C1	59.9	5.45.	43	154	12
C2	60.5	4.65	52	166	5.6

IAA may also play an important role in plant growth by PSB. Plant growth promotion by PSB strains might be due to the production of phytoharmones rather than their action to release available phosphorus[12].

Biochemical characterization of PSB isolates

Identification of PSB isolates was carried out according to Bergeys manual of Systematic bacteriology. Gram staining revealed a fairly homogeneous population of Gram negative rods. A total of 17 biochemical tests were performed and on the basis of these tests, the isolates were placed into three genera *Pseudomonas, Klebsiella*, and *Enterobacter* (Table 3). However no host specificity was noticed for PSB suggesting a predominantly homogeneous population among these PSB isolated from different crops. The soil chemical properties (pH, organic C and organic N) and soil management practices such as crop rotation, fertilizer, organic manures and pesticide application are known to affect the soil microbial communities[10, 17] Earlier, Chiarini *et al*.,[1] observed that the greatest effect on density and community structure was exerted by soil types, whereas no significant difference of cultivar was observed. Gelsomino *et al*.[3] also reported that similar soil types select similar type of microbial communities. In the present study the soil samples were collected from same locations having similar soil chemical properties. These soils had similar cropping system and similar management practices were used. These factors did not affect the distribution of PSB.

Table 3. Biochemical Characterization of PSB isolated from the rhizosphere of different vegetable crops

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
L1	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	*Klebsiella*
L2	+	+	+	-	-	+	-	-	+	+	-	+	+	+	+	+	+	+	*Pseudomonas*
L3	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	*Klebsiella*
L4	+	+	+	-	-	+	-	-	+	+	-	+	+	+	+	+	+	+	*Pseudomonas*
L5	+	-	+	+	+	+	-	+	-	+	-	-	+	+	-	+	-	+	*Enterobacter*
L6	+	-	+	+	+	+	-	+	-	+	-	-	+	+	-	+	-	+	*Enterobacter*
L7	+	+	+	-	-	+	-	-	+	+	-	+	+	+	+	+	+	+	*Pseudomonas*
B1	+	+	+	-	-	+	-	-	+	+	-	+	+	+	+	+	+	+	*Pseudomonas*
B2	+	-	+	+	+	+	-	+	-	+	-	-	+	+	-	+	-	+	*Enterobacter*
B3	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	+	+	-	*Pseudomonas*
B4	+	+	+	-	-	+	-	-	+	+	-	+	+	+	+	+	+	+	*Pseudomonas*
B5	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	+	+	-	*Pseudomonas*
T1	+	-	+	+	+	+	-	+	-	+	-	-	+	+	-	+	-	+	*Enterobacter*
T2	+	+	+	-	-	+	-	_	+	+	-	+	+	+	+	+	+	+	*Pseudomonas*
T3	+	+	+	-	-	+	-	_	+	+	-	+	+	+	+	+	+	+	*Pseudomonas*
T4	+	+	+	-	-	+	-	_	+	+	-	+	+	+	+	+	+	+	*Pseudomonas*
C1	+	+	+	-	-	+	-	_	+	+	-	+	+	+	+	+	+	+	*Pseudomonas*
C2	+	+	+	-	-	+	-	-	+	+	-	+	+	+	+	+	+	+	*Pseudomonas*

A, Arabinose utilization; B, Inositol utilization; C, Lactose utilization; D, Sorbitol utilization; E,Sucrose utilization; F, Citrate utilization; G, Methyl red; H,Voges Proskauer; I,Arginine decarboxylase; J,Gelatin hydrolysis; K,H_2S production; L,Indole production; M, Lysine decarboxylase; N, ornithine decarboxylase; O, Urease; P, Catalase; Q,Oxidase; R, Motility; S, Tentative identification.+, Positive;-, Negative

Establishment of PSB in wheat plants under pot house conditions

The establishment of all the strains and their effect on the total bacterial and PSB population in the wheat rhizosphere was determined at 30 DAS. The total bacterial count with the inoculation of the selected PSB varied from 46-89 x 10^6 cfu/g at 30 DAS (Table 4). It was observed that the application of SSP did not affect much the total bacterial population at 30 DAS. However, seed bacterization enhanced the total number of bacteria, highest being observed with the inoculation of B5 (89 x 10^6 cfu/g) and T1 (84 x 10^6 cfu/g). A small gain was observed in PSB count

with the application of SSP at 30 DAS as compared to control. However, the PSB count was increased 2-3 fold upon inoculation with various PSB strains.

The effect of the PSB isolates on growth of wheat was studied under pot house condition in a sandy P deficient soil. Application of SSP did not show significant increase in the shoot biomass of chickpea at 30 DAS. However, inoculation with PSB resulted in significant gain in the shoot biomass (Table 5).

Table 4. Effect of PSB on the number of bacteria in wheat rhizosphere under pot culture conditions at 30 days after sowing (DAS)

Treatment	Total Bacteria ($X10^6$ cfu/g)	PSB ($X10^6$ cfu/g)
Control	47	16
30 kg SSP	59	20
3o kg SSP +L1	68	36
30 kg SSP +L2	58	37
30 kg SSP +L3	88	50
30 kg SSP +L4	65	38
30 kg SSP +L5	79	48
30 kg SSP +L6	46	37
30 kg SSP +L7	69	50
30 kg SSP +B1	73	41
30 kg SSP +B2	58	31
30 kg SSP +B3	67	35
30 kg SSP +B4	74	35
30 kg SSP +B5	89	46
30 kg SSP +T1	84	56
30 kg SSP +T2	79	45
30 kg SSP +T3	59	32
30 kg SSP +T4	66	34
30 kg SSP +C1	69	32
30 kg SSP +C2	76	42

Table 5. Effect of inoculation of PSB on the shoot weight of wheat under pot culture conditions at 30 days after sowing (DAS)

Treatment	Shoot weight	% Increase over control
Control	0.16	
30 kg SSP	0.17	6.25
30 kg SSP +L1	0.19	18.75
30 kg SSP +L2	0.23	43.75
30 kg SSP +L3	0.24	50
30 kg SSP +L4	0.25	56.25
30 kg SSP +L5	0.23	43.75
30 kg SSP +L6	0.31	93.75
30 kg SSP +L7	0.29	81.25
30 kg SSP +B1	0.33	106.25
30 kg SSP +B3	0.49	206.25
30 kg SSP +B4	0.26	62.5
30 kg SSP +B5	0.27	68.75
30 kg SSP +T1	0.34	112.5
30 kg SSP +T2	0.22	37.5
30 kg SSP +T3	0.28	75
30 kg SSP +T4	0.2	25
30 kg SSP +C1	0.3	87.5
30 kg SSP +C2	0.27	68.75

The maximum increase in dried shoot weight (226.25% over the control) was observed with the inoculation of B3 at 30 DAS. This isolate also produced the maximum IAA suggesting that the plant growth promotion by PSB is not only due to the solubilization of inorganic P; it might be due to the production of phytoharmones. The field and pot trials of PSM with or without phosphatic fertilizers showed increase in P-uptake, N-uptake and dry matter production[8]. Increase in the yield of various crops by inoculation with P-solubilizing organisms has been reported[16, 19, 15].

CONCLUSION

The present investigation revealed that the PSB isolates from the rhizosphere of vegetable crops apart from phosphate solubilization also possessed the innate potential of plant growth promoting traits thus making them as promising inoculants for crops. All the selected PSB strains were able to establish well in the rhizosphere of wheat irrespective of the location or the host from which it was isolated indicating that they are not host specific and can effectively be used as bioinoculants. Knowledge generated on the genetics of P- solubilization will help to develop them as successful bioinoculants in sustainable and organic agriculture.

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