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Analyzing the Efficacy of Phosphate Solubilizing Microorganisms by Enrichment Culture Techniques

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Abstract

Phosphate solubilizing microorganisms (PSMs) were isolated from rhizoplane, rhizosphere and non-rhizosphere of different leguminous plants. To isolate efficient phosphate solubilizers the rhizosphere and non-rhizosphere soil samples were enriched with different phosphate sources like tricalcium and rock phosphate. PSMs were detected in all the regions, but their number gradually decreased from rhizosphere, rhizoplane and non-rhizosphere soil. When compared to fungal population, bacterial population was more in number. *Tephrosia purpurea* recorded the highest bacterial population of 30.15 × 10^6 cfu/g, 50.51 × 10^6 cfu/g and 21.10 × 10^6 cfu/g in the rhizoplane, rhizosphere and non-rhizosphere regions respectively. In enrichment culture technique, highest phosphate solubilizing bacterial population was recorded in the rhizosphere soil of *Clitoria ternatea* (23 × 10^3 cfu/g) in tricalcium phosphate containing Pikovskaya's (PVK) medium. In a plate assay method solubilization zone diameter produced by microorganisms was varied from 0.2 cm to 1.0 cm. The phosphate solubilization ability of the isolated microorganisms in a liquid PVK medium varied from 11.85 mg to 61.96 mg P_2O_5. The medium turned acidic during the incubation period. The pH varied among the organisms from the initial 6.5 to the final 3.2 during 15 days of incubation. Citric acid, fumaric acid, gluconic acid, glutaric acid, glyoxalic acid, ketobutyric acid, ketoglutaric acid, malic acid, malonic acid, succinic acid and tartaric acid are produced by the isolated PSMs. Seed or soil inoculation with phosphate solubilizing bacteria (PSB) is known to improve solubilization of fixed and applied phosphates in soil bring about higher crop yield. The PSM are effective as biofertilizers in enhancing crop yields in phosphate deficient soils. They are environmentally friendly and supply phosphate to plants in a sustainable manner.

Keywords: Phosphate solubilization; Rhizosphere soil; Enrichment culture; Insoluble phosphate sources

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Introduction

Phosphorus (P) is a one of the major plant growth-limiting nutrients although it is abundant in soils in both inorganic and organic forms. The nitrogen (N) fixing legume plants usually require more P than the plants depending upon mineral N fertilizer. Nodule establishment and functioning are important sinks for P and nodules have usually the highest P content in the plant. Therefore, P deficiency condition results in reduced biological nitrogen fixation (BNF) potential and P fertilization will usually result in enhanced nodule number and mass as well as greater BNF potential. Soil P transformation are primarily mediated by microbial activity, which in turn, is influenced by a combination of factors including plant species, soil type and environmental conditions. Soil microorganisms play a significant role in mobilizing P for plants by bringing about changes in pH in rhizosphere soil and also by producing chelating substances, which lead to solubilization of phosphates [1]. These microbes are known as phosphate solubilizers consisting predominately of fungal, bacterial and *Actinomycetes* species collectively called the phosphate solubilizing microorganisms (PSM) [2]. Soil microorganisms, specifically bacteria and fungi, growing in the root region of plants play an important role in the supply of P in rhizosphere region. There is a preferential stimulation of gram

negative, non-sporulating rod shaped bacteria. *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Agrobacterium*, *Cellulomonas*, *Flavobacterium*, *Mycobacterium* and *Micrococcus* are mostly present in the rhizosphere region. Microbial inoculants are produced on the basis of selection of beneficial soil microorganisms which have the highest efficiency to enhance plant growth by providing nutrients in a readily absorbable form. Application of microbial inoculants provided an abundant population of active and effective microorganisms to the root activity zone which increases plant ability to uptake more nutrients [3]. The objective of the present study is to isolate phosphate-solubilizing microorganisms from the soil samples of leguminous plants to screen and identify them for phosphate solubilizing efficacy.

Materials and Methods

Isolation of phosphate solubilizing microorganisms

Bacteria, fungi and phosphate solubilizing microorganisms were isolated from three different sites i.e., rhizoplane, rhizosphere and non-rhizosphere [4] of leguminous plants from in and around Gandhigram Rural Institute, Gandhigram, Dindigul. Serial dilutions of the root homogenate of rhizoplane, soil samples of rhizosphere and non-rhizosphere were individually plated on Nutrient Agar (NA), Rose Bengal Agar (RBA) and Pikovskaya Agar Medium (PVK) as described by Gaur [5]. Total bacterial, fungal and phosphate solubilizing microorganisms were enumerated and the RS ratio [6] between the rhizosphere and non-rhizosphere soil samples was calculated by using the formula

$$R/S\ ratio = \frac{No\ of\ bacteria/g\ of\ rhizosphere\ soil(R)}{No\ of\ bacteria/g\ of\ non-rhizosphere\ soil(S)}$$

Enrichment culture technique

For isolation of insoluble phosphate solubilizing microorganisms, the rhizosphere and non-rhizosphere soil samples were enriched by the method adopted by Tardieux-roche and Barjac [7] and Bardiya and Gaur [8]. The colonies exhibiting zones of phosphate solubiliation were transferred to agar slants on the PVK medium containing 0.5 percent $CaCO_2$ and allowed to grow at room temperature for 3 days. Total bacterial, fungal and phosphate solubilizing microorganisms were enumerated.

Screening of phosphate solubilizing microorganism in solid medium

The ability of the isolated microorganisms to solubilize insoluble phosphate was calculated by solubilization efficiency (E) [9]. Those strains showed solubilization zone were presumed to be phosphate solubilizers and they were further tested for solubilization of phosphates quantitatively.

Screening of phosphate solubilizing microorganism in liquid medium

100 ml aliquots of PVK broth was transferred into 250 ml conical flasks and sterilized by autoclaving before the addition of phosphate source. After 15 days of incubation period the filtrate was used to measure the pH and soluble P. The soluble P present in the supernatant was estimated using the Jasco Spectrophotometer V-530 by chlorostannous reduced molybdophosphoric acid blue method [10]. The pH of the supernatant was measured using an Elico L1 120 pH meter. The organic acids produced by selected PS bacterial cultures were detected by thin layer paper chromatography [5].

Results and Discussion

The isolation of microbes like bacteria, fungi and PSM from rhizoplane, rhizosphere and non-rhizosphere soil samples of various leguminous plants are given in **Table 1.** The total number of bacterial and fungal colonies varied in the three different regions; PSM were detected in all the regions, but their number gradually decreased from rhizosphere, rhizoplane and non-rhizosphere soil. When compared to fungal population, bacterial population was greater in number. *Tephrosia purpurea* recorded the highest total bacterial population of 50.51 × 10^6 cfu/g in the rhizosphere region. The lowest total bacterial population of 2.11 × 10^6 cfu/g was noticed in the non-rhizosphere region of *Lablab purpureus*. The capacity of bacterial isolates to solubilize phosphate depends upon the zone of their origin. The large number of phosphate solubilizing bacteria (PSB) observed in the rhizosphere region might be due to the favorable influence of root exudates, containing amino acids, organic acids, sugars, growth promoting substances [11], land use, physico chemical properties, organic matter, available Phosphorus and organic carbon content [12]. Soils poor in organic matter are known to be low in microbial activities except in the rhizosphere of growing plants [4]. Similar findings on occurrence of higher numbers of PSM in the rhizosphere region have been recorded by Swaby and Sperber [13] in legumes, Tomashevska and Manzon [14] and Majumdar [15] in sugarcane rhizosphere, Mehta and Bhide [16], Johri et al. [4], Wahid and Wahid and Mehana [17] and Hwangbo et al. [18] in grass rhizosphere and Rao and Charyalu [19] in foxtail millet. The R:S ratio is the comparison microbial population of rhizosphere and non-rhizosphere regions of a plant and to find out the degree or extent of plant roots exudates effect on soil microorganisms. The R:S ratio of bacterial population was higher in *Cicer arietinum*. The R/S ratio of microbes was calculated by dividing the microbial population in the rhizosphere area by microbial population in the non-rhizosphere area.

Enrichment in PVK liquid medium

Rhizosphere and non-rhizosphere soil samples of *Clitoria ternatea* and *Vigna unguiculata* were enriched with different phosphate sources in PVK liquid medium and their effects were given in **Table 2.** Highest phosphate solubilizing bacterial population was recorded in the rhizosphere soil of *Clitoria ternatea* (23 × 10^3 cfu/g) in PVK medium containing tricalcium phosphate (TCP). Lowest population growth was observed in PVK medium containing $FePO_4$ (9.60 × 10^3 cfu/g) in the rhizosphere soil of *V. unguiculata*. Highest phosphate solubilizing fungal population was observed in rhizosphere soil of *V. unguiculata* (25.33 × 10^3 cfu/g) containing $FePO_4$. $AlPO_4$ inhibited the growth of phosphate solubilizing fungal colonies (Plate 1). This enrichment culture technique enhances the growth of phosphate solubilizing microorganisms.

Table 1 Isolation of phosphate solubilizing microorganisms from rhizoplane, rhizosphere and non-rhizosphere soil of various leguminous plants in and around Gandhigram. Mean value of two replicates *RS Ratio between Rhizosphere and non-rhizosphere soil samples.

Leguminous Plants	Rhizoplane soil				Rhizosphere soil				Non-Rhizosphere soil				*RS Ratio**			
	Bacteria (1×10^6 cfu/g)	Fungi (1×10^4 cfu/g)	PSM (1×10^3cfu/g)		Bacteria (1×10^6 cfu/g)	Fungi (1×10^4cfu/g)	PSM (1×10^3 cfu/g)		Bacteria (1×10^6 cfu/g)	Fungi (1×10^4 cfu/g)	PSM ($\times10^3$ cfu/g)		Bacteria (1×10^6 cfu/g)	Fungi 1×10^4 cfu/g)	*PSM* (1×10^3 cfu/g)	
			Bacteria	Fungi			Bacteria	Fungi			Bacteria	Fungi			Bacteria	Fungi
Arachis hypogaea	15.19	15.68	-	1.8	19.53	19.58	1.4	-	09.00	10.20	-	-	2.2	1.9	-	-
Caesalpinia pulcherrima	26.23	14.71	1.4	1.5	40.74	18.61	1.8	2.3	17.61	9.19	-	1.3	2.3	2.0	-	1.8
Cicer arietinum	09.10	11.97	-	-	29.72	18.92	-	1.2	05.42	06.78	-	-	5.5	2.8	-	-
Clitoria ternatea	09.16	13.46	2.3	1.2	14.25	18.12	4.3	2.6	04.70	05.38	1.1	1.8	3.0	3.4	3.9	1.4
Lab labpurpureus	04.53	11.18	1.1	-	08.01	15.43	2.2	-	02.11	05.24	-	-	3.8	2.9	-	-
Gliricidia maculata	09.28	12.92	2.4	2.2	18.26	16.40	3.5	3.5	06.56	06.47	1.8	1.5	2.8	2.5	1.9	2.3
Vigna mungo	18.76	12.54	2.8	-	25.81	17.76	2.1	1.4	11.02	07.80	1.9	-	2.3	2.3	1.1	-
Sesbania grandiflora	15.56	15.00	-	1.9	25.91	20.21	2.2	2.1	10.62	11.63	-	1.7	2.4	1.7	-	1.2
Tephrosia purpurea	30.15	10.86	2.8	1.4	50.51	13.00	3.1	22	21.10	06.28	-	-	2.4	2.1	-	-
Vigna unguiculata	28.24	14.21	2.6	1.0	34.87	18.16	3.0	2.0	19.06	09.52	1.1	-	1.8	1.9	2.7	-

Screening in solid medium

Phosphate solubilizing bacterial (11 Nos) and fungal (14 Nos) strains isolated from different soil samples having the ability to solubilize TCP in solid PVK medium are presented in **Table 3.**

Bacillus sp. 1 to 4 were able to solubilize TCP and produce solubilization zone range from 0.8 cm to 1.0 cm but isolates belonging to *Fusarium* sp., *Mucor* sp., *Penicillium* sp. (2), *Aspergillus ochraceus*, *A. sydawi*, *A. terreus*, *A. versicolor* and *Trichoderma viride* were able to produce only 0.2 cm to 0.4 cm of solubilization zone. Solubilization efficiency (E) varied from 13.04 percent to 85.71 percent on 7 days of incubation period only. The colony diameter found to vary from 0.7 cm to 2.9 cm. The growth of the fungal colonies, as in diameter was found to be more when compared to bacterial colonies. Solubilization of insoluble phosphate depends on the type of acids secreted by the organism into the medium. The inhibition zone size varied (cm) in the plate assay may be due to the diffusion rate of different organic acid secreted by the organisms [4].

Screening in liquid medium

After 15 days of incubation, isolates showed good zone of solubilization on solid PVK medium. Phosphate solubilization efficiency of the isolates was confirmed by quantitative analysis of available phosphorus in the PVK liquid medium **(Table 4).** All the isolated microorganisms solubilized TCP though they varied in their ability and the growth period for highest phosphate solubilization (PS) activity. Among the 25 isolates, 13 isolates viz., 7 *Bacillus* spp., 4 *Aspergillus* spp., 1 *Pseudomonas* sp. and 1 *Penicillium* sp. showed highest PS activity. The phosphate solubilization ability of the isolated microorganisms varied from 11.85 mg to 61.96 mg P_2O_5. The *Proteus* sp. showed comparatively lesser PS activity. During 15 days of incubation, the medium changed acidic and the pH of the organisms varied from the initial value of 6.7 to 3.2. Production of organic acid by the isolated organisms in the liquid medium coupled with the decrease of the pH value of the medium. The results obtained showed that the solubilization of insoluble phosphates depends on a decrease in pH and acid production, confirming the observations of Kucey et al. [20]. A correlation between final pH and soluble P level have been reported by Arora and Gaur [21], Venkateswarlu et al. [22], Thomas [23], Narsian et al. [24] and Dave and Patel [25].

Organic acid secretion during phosphate solubilisation

Production of organic acid by the selected bacterial sp. in the

Table 2 Enumeration of phosphate solubilizing microorganisms from rhizosphere and non-rhizosphere soil by enrichment culture technique. Mean value of three replicates.

Soil sample	Phosphate sources	Microbial Population			
		Total Microbial population		Phosphate solubilizers	
		Bacteria (1×10^6cfu/g)	Fungi (1×10^6cfu/g)	Bacteria (1×10^3cfu/g)	Fungi (1×10^3cfu/g)
Rhizosphere soil of *Cternatea*	TCP	TNTC	35 ± 5	23 ± 5.12	18 ± 2
	RP	TNTC	31 ± 2	15 ± 8.06	11 ± 1
	$AlPO_4$	88 ± 4	ND	14 ± 7.23	ND
	$FePO_4$	53 ± 13	39 ± 5	11 ± 5.09	13 ± 1
Non rhizosphere soil of *C. ternatea*	TCP	56 ± 5	35 ± 2	16 ± 2.91	20 ± 1
	RP	47 ± 3	12 ± .9	10 ± 2.04	6 ± 0.4
	$AlPO_4$	34 ± 2	ND	11 ± 1.90	ND
	$FePO_4$	39 ± 3	9 ± 0.5	12 ± 2.11	5 ± 0.3
Rhizosphere soil of *V. unguiculata*	TCP	TNTC	48 ± 3	18 ± 5.14	25 ± 3
	RP	TNTC	26 ± 2	13 ± 8.62	12 ± 1
	$AlPO_4$	95 ± 8	ND	12 ± 7.23	ND
	$FePO_4$	73 ± 3	19 ± 1	9 ± 3.23	21 ± 1
Non rhizosphere soil of *V. unguiculata*	TCP	51 ± 4	23 ± 4	16 ± 2.99	15 ± 2
	RP	40 ± 3	13 ± 2	13 ± 2.10	9 ± 0.8
	$AlPO_4$	32 ± 2	ND	12 ± 1.66	ND
	$FePO_4$	35 ± 3	12 ± 1	10 ± 1.75	11 ± 0.5

Mean value of three replicates

Table 3 Screening of microorganisms for tricalcium phosphate solubilization and evaluation of their solubilization efficiency (E) by plate assay method.

Organism	Culture diameter (cm)	Solubilization zone (cm)	Solubilization activity	Solubilization efficiency (E) (%)
Bacillus sp. (B1)	1.3	0.8	+++	61.54
Bacillus sp. (B2)	1.9	0.9	++++	47.37
Bacillus sp. (B3)	1.8	0.9	++++	50.00
Bacillus sp. (B4)	1.9	1.0	++++	52.63
Bacillus sp. (B5)	1.8	0.5	++	27.78
Bacillus sp. (B6)	1.3	0.5	++	38.46
Bacillus sp. (B7)	1.2	0.5	++	41.67
Proteous sp.	0.7	0.6	++	85.71
Pseudomonas sp.(P1)	0.9	0.4	+	44.44
Pseudomonas sp.(P2)	0.7	0.5	++	71.43
Azospirillum sp.	2.2	0.7	+++	31.82
Chaetomium globosum	1.7	0.3	+	17.65
Fusarium sp.	1.4	0.3	+	21.43
Mucor sp.	1.3	0.2	+	15.38
Penicillium sp.(PI1)	1.6	0.5	++	31.25
Penicillium sp.(PI2)	1.6	0.4	+	25.00
Aspergillus flavus	1.9	0.5	++	26.32
A. niger	2.9	0.7	+++	24.14
A. ochraceus	1.8	0.4	+	22.22
A. sydawi	1.5	0.3	+	20.00
A. terreus	2.4	0.4	+	16.67
A. versicolor	2.3	0.3	+	13.04

A. awamori	1.8	0.6	++	33.33
Aspergillus sp.	2.7	0.6	++	22.22
Trichoderma viride	1.6	0.4	+	25.00

Table 4 Screening of microorganisms for TCP solubilization in a broth assay after 15 days.

Organism	Maximum P solubilized as P_2O_5 (mg)	Solubilization of total P in the medium (%)	Final pH
Control	3 ± 0.2	1.60	6.5 ± 0.4
Bacillus sp. (B1)	56 ± 3	24.99	4.6 ± 0.3
Bacillus sp. (B2)	48 ± 2	21.69	4.7 ± 0.2
Bacillus sp. (B3)	61 ± 5	27.51	4.3 ± 0.3
Bacillus sp. (B4)	46 ± 3	20.73	4.3 ± 0.2
Bacillus sp. (B5)	61 ± 5	27.22	4.9 ± 0.4
Bacillus sp. (B6)	51 ± 4	23.08	4.9 ± 0.3
Bacillus sp. (B7)	46 ± 3	20.64	4.2 ± 0.2
Proteous sp.	11 ± 0.9	5.26	4.8 ± 0.3
Pseudomonas sp.(P1)	44 ± 2	19.68	4.9 ± 0.4
Pseudomonas sp.(P2)	37 ± 2	16.60	5.3 ± 0.4
Azospirillum sp.	26 ± 2	11.91	5.6 ± 0.3
Chaetomium globosum	18 ± 1	8.28	3.5 ± 0.2
Fusarium sp.	30 ± 2	13.74	4.3 ± 0.2
Mucor sp.	19 ± 1	8.63	5.2 ± 0.4
Penicillium sp.(PI1)	54 ± 4	24.12	3.9 ± 0.2
Penicillium sp.(PI2)	27 ± 1	12.11	3.6 ± 0.2
Aspergillus flavus	50 ± 4	22.63	4.3 ± 0.3
A. niger	59 ± 4	26.45	3.2 ± 0.2
A. ochraceus	57 ± 3	25.70	4.6 ± 0.3
A. sydawi	47 ± 3	21.07	3.8 ± 0.2
A. terreus	39 ± 2	17.50	3.6 ± 0.3
A. versicolor	36 ± 2	16.39	4.2 ± 0.3
A. awamori	37 ± 3	16.46	4.5 ± 0.2
Aspergillus sp.	32 ± 2	14.56	4.3 ± 0.3
Trichoderma viride	37 ± 3	16.60	5.1 ± 0.4

Table 5 Identification of organic acids produced by the phosphate solubilizers in PVK medium containing TCP after 15 days of incubation.

Phosphate solubilizing bacterial isolates	Organic acids										
	Citric acid	Glutaric acid	Glyoxalic acid	ketoglutaric acid	ketobutyric acid	Malic acid	Malonic acid	Succinic acid	Fumaric acid	Tartaric acid	Gluconic acid
Bacillus sp. (B1)	√	-	√	-	-	√	-	√	√	√	√
Bacillus sp. (B2)	√	-	-	√	-	-	√	√	√		√
Bacillus sp. (B3)	√	-	-	√	√	-	-	√	√		√
Bacillus sp. (B4)	√	√	-	-	-	√	-	√	√	√	√
Bacillus sp. (B5)	√	-	√	-	-	-	-	√	√	-	√
Bacillus sp. (B6)	√	√	-	-	-	√	-	√	√	-	√
Bacillus sp. (B7)	√	√	-	-	-	-	√	√	√	√	√
Proteous sp.	√	-	-	-	-	-	-	√	√		√
Pseudomonas sp.(P1)	√	-	√	-	√	√	-	√	√	√	√
Pseudomonas sp.(P2)	√	-	-	-	√	-	-	√	√	√	√
Azospirillum sp.	√	-	-	-	-	-	-	√	√	-	√

PVK liquid medium after 10-15 days of incubation was coupled with the decrease of the pH value of the medium. The type of the microorganism and concentration of organic acid produced by them varied with respect to TCP.

Acid production during phosphate solubilization appears to be an event of common occurrence. Citric acid, fumaric acid, gluconic acid, glutaric acid, glyoxalic acid, ketobutyric acid, ketoglutaric acid, malic acid, malonic acid, succinic acid and tartaric acid are commonly produced by the selected phosphate solubilizing bacteria **(Table 5).**

Solubilization of insoluble phosphate by microorganisms is mainly by production of organic acids and chelating substances [1,26-31]. The synthesis of organic acids by PSB has been well documented [13,32-35]. The synergistic effect of the microorganisms would permit a better response because of greater diversity of the acids secreted. The concentration of organic acids secreted by the microorganisms varied with the phosphate source employed [36].

Conclusion

The soil is one of the most dynamic sites of biological interaction in nature and it is the site in which the nutrition of agricultural crops occurs. Rhizosphere is the region that surrounds plant roots, where materials released from the roots and the metabolic activities of the root change the characteristics of the soil. Phosphate solubilizing microorganisms (PSMs) were isolated from rhizoplane, rhizosphere and non-rhizosphere of different leguminous plants. Seed or soil inoculated phosphate solubilizing bacteria (PSB) is known to improve solubilization of fixed and applied phosphates in soil and it increase the crop yield. The PSM are effective as biofertilizers in enhancing crop yields in phosphate deficient soils. The supply of phosphate to the plants by this environmentally friendly way is a sustainable manner.

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