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## Phosphate Solubilization Potential of Fluorescent *Pseudomonas* spp. Isolated from Diverse Agro-Ecosystems of India

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### ABSTRACT

Phosphorus is one of the major nutrients limiting plant growth. The solubilization of P in the rhizosphere is the most common mode of action implicated in PGPR that increase nutrient availability to host plants. About 75 isolates of *Pseudomonas* spp. were obtained by plating the rhizosphere soil samples from different agro-ecosystem locations of India. All the isolates were initially screened for tri calcium phosphate (TCP) solubilization under *in vitro* conditions on Pikovskaya's agar medium and followed by in liquid medium. A total of 10 promising isolates were further checked for their ability to solubilize rock phosphate in liquid medium. Results showed that the amount of P solubilized by different isolates ranged between 2.11 and 15.19 mg L<sup>-1</sup>. Highest P solubilization was observed with strain P17 (15.19 mg L<sup>-1</sup>) that showed spent medium pH of 3.36. The fall in pH corresponded with the solubilization of insoluble P. Maximum rock phosphate solubilization of 12.9 mg L<sup>-1</sup> was observed with P29 followed by P17 which solubilized 12 mg L<sup>-1</sup>. The P29 which showed highest rock phosphate solubilization recorded lowest pH of 3.7. From this study it is evident that, most of the strains solubilizing rock phosphate were isolated from semi-arid, deep alfisols of Deccan Plateau agro-ecological sub region of India suggesting that these strains are potential phosphate solubilizers. And strains like P17 and P29 could be viable, cost-effective alternatives of chemical fertilizers for management of plant nutrition.

**Key words:** Plant nutrition, *Pseudomonas* sp.-P17, rock phosphate, TCP solubilization

### INTRODUCTION

Phosphorus is one of the major nutrients limiting plant growth. Most of the soils worldwide are P deficient (Batjes, 1997). The use of rock phosphate as a phosphate fertilizer and its solubilization by microbes (Kang *et al.*, 2002), through the production of organic acids (Rashid *et al.*, 2004; Khan *et al.*, 2007), has become a valid alternative to chemical fertilizers. Several studies have shown that Phosphate Solubilizing Microorganisms (PSM) solubilized the fixed P in the soil resulting in higher crop yields (Zaidi, 1999; Gull *et al.*, 2004). Phosphate solubilizing bacteria are ubiquitous (Gyaneshwar *et al.*, 2002) and *Bacillus*, *Enterobacter*, *Erwinia* and *Pseudomonas* spp. are among the most potent strains. The solubilization of P in the rhizosphere is the most common mode of action implicated in PGPR that increase nutrient availability to host plants (Richardson, 2001). The PSMs solubilize the unavailable forms of inorganic-P like tricalcium, iron, aluminium

and rock phosphates into soluble forms by release of succinic, citric, maleic, fumaric, glyoxalic and gluconic acids (Kucey *et al.*, 1989; Khan *et al.*, 2007). Under controlled growth conditions, various studies have demonstrated enhanced growth and P nutrition of plants inoculated with PSM (Rodriguez and Fraga, 1999; Gyaneshwar *et al.*, 2002).

Phosphorus is an essential element for plant development and growth making up about 0.2% of plant dry weight. Plants acquire P from soil solution as phosphate anions. However, phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Al}^{3+}$ , depending on the particular properties of a soil. In these forms, P is highly insoluble and unavailable to plants. As the results, the amount available to plants is usually a small proportion of total amount. Most agricultural soils contain large reserves of total P, commonly in the range of 200-5,000 mg P  $\text{kg}^{-1}$  with an average of 600 mg P  $\text{kg}^{-1}$  and a part of P accumulates depend on regular application of chemical fertilizers or sludge from wastewater treatment (Gyaneshwar *et al.*, 2002; De-Bashan and Bashan, 2004).

To meet food grains requirement of 300 Mt by 2025, 45 Mt of  $\text{N}+\text{P}_2\text{O}_5+\text{K}_2\text{O}$  is estimated to be required per annum. Among these P fertilizer requirement would be around 9 MT. There has been a gradual increase in chemical fertilizer consumption for crop production in India. About 0.25 MT of P fertilizers in the form of NPK in 1966-67 to 6.5 MT of P fertilizers in the year 2012 (Prasad, 2012). Organic farming is becoming a major tool for sustaining the soil quality degraded by intensive use of synthetic chemicals for increasing crop production and therefore, use of bio-agents as biofertilizers or biopesticides is an integral part of organic farming (Srivastava *et al.*, 2007).

Even though, a lot has been reported on use of microorganisms in agriculture, much of soil microbial diversity is to be explored for the benefit of the small and marginal farming community. Therefore, in this study an attempt was made to isolate and characterize the phosphate solubilization potential of different fluorescent *Pseudomonas* spp. from diverse agro-ecosystems of India.

## MATERIALS AND METHODS

**Bacterial cultures:** Seventy five soil samples of different crops representing 31 locations from 13 states of India were used for isolation of fluorescent *Pseudomonas* spp. (Kumar *et al.*, 2012) using King's B medium (King *et al.*, 1954).

**Screening for (TCP) solubilization:** All the 75 *Pseudomonas* isolates were initially qualitatively screened for TCP solubilization on solid media employing Pikovskaya's medium (Pikovskaya, 1948) amended with tri-calcium phosphate with following composition (g  $\text{L}^{-1}$ ): Yeast extract-0.5; Dextrose 10.0;  $\text{Ca}_3(\text{PO}_4)_2$  5.0;  $(\text{NH}_4)_2\text{SO}_4$  0.5;  $\text{MgSO}_4$  0.1; KCl 0.2;  $\text{MnSO}_4$  and  $\text{FeSO}_4$  traces; Agar-agar 20.0; pH-7.2. Petridishes containing medium were spot-inoculated with 10  $\mu\text{L}$  of overnight grown bacterial cultures and incubated at 28°C. The solubilization zone size was measured after 7 and 14 days of inoculation.

**Quantitative estimation of TCP solubilization:** For quantification of phosphate solubilization, Olsen and Sommers (1982) method was adapted. The method is briefly described here. Pikovskaya's broth medium was prepared with 1 mg  $\text{mL}^{-1}$  of tri-calcium phosphate (or rock phosphate) as known

insoluble phosphorus source. About 50 mL of medium was transferred to 150 mL conical flask. Medium was sterilized and inoculated with 100  $\mu$ L of overnight-grown fresh bacterial cultures. The flasks were incubated in an incubator shaker at 28°C for 10 days. Each treatment had 3 replicates and an un-inoculated control. After incubation, the broth was centrifuged and pH of the supernatant was recorded. Five milliliter of sulphomolybdic acid containing ascorbic acid was added to 1 mL of supernatant. Final volume of the solution was made up to 50 mL. The absorbance of blue colour of the reaction mixture was measured at 660 nm and amount of soluble phosphorus was determined from the standard curve of  $\text{KH}_2\text{PO}_4$ .

**Quantitative estimation of rock phosphate solubilization:** About 10 isolates that showed promising TCP solubilization in liquid medium were further evaluated for their ability to solubilize rock phosphate by a similar method as described previously.

## RESULTS

**Isolation of fluorescent *Pseudomonas* spp.:** In total, 75 fluorescent *Pseudomonas* spp. were isolated from 75 soil samples obtained from 35 different locations representing 13 states, across India (Table 1).

**TCP solubilization on solid medium:** All the isolates were qualitatively characterized for phosphate solubilization on solid medium amended with Tri-Calcium Phosphate (TCP). After 14 days of incubation, 8 strains viz., P10, P11, P17, P24, P25, P26, P38 and P42 effectively solubilized insoluble P and produced >10 mm solubilization zone and hence were considered as

Table 1: Details of crop, locations/states surveyed and fluorescent *Pseudomonas* isolates obtained from various rainfed agro-ecosystems of India

Type of crop	No. of isolates	No. of locations	No. of states
Greengram	05	05	03
Blackgram	03	03	02
Redgram	11	10	05
Horsegram	02	02	02
Sorghum	09	07	03
Chickpea	04	04	03
Fingermillet	02	02	01
Foxtailmillet	01	01	01
Pearlmillet	02	02	02
Sesamum	02	02	02
Maize	03	03	02
Castor	02	02	02
Groundnut	04	04	02
Cotton	05	04	02
Safflower	02	02	02
Oats	01	01	01
Rainfed rice	01	01	01
Rice	01	01	01
Composite soil	15	15	09

efficient phosphate solubilizers. Thirty eight isolates showed poor phosphate solubilization and produced zones of <5 mm diameter (Table 2 and Fig. 1). Nine isolates did not produce any zone of solubilization even though they could grow on Pikovskaya's agar medium.

**Estimation of TCP solubilization:** Phosphate solubilization was quantified for all isolates. The amount of P solubilized by different isolates ranged between 2.11 and 15.19 mg L<sup>-1</sup> (Table 3). Lowest P solubilization was observed with P68 (2.11 mg L<sup>-1</sup>) and the highest with P17 (15.19 mg L<sup>-1</sup>). Similarly, other isolates which showed efficient solubilization of TCP were P42, P18,

Table 2: Qualitative screening for tri-calcium phosphate solubilization by *Pseudomonas* isolates on Pikovskaya's agar medium after 7 and 14 days of incubation

Isolate	Clearing zone (mm)		Isolate	Clearing zone (mm)	
	7 days	14 days		7 days	14 days
P1	-	2	P37	1	3
P2	-	3	P38	10	12
P3	-	2	P39	1.5	3
P5	6	7	P41	-	3
P6	2	7	P42	10	15
P7	1	2	P43	6	8
P8	8	9	P44	-	2
P9	1	2	P45	3	4
P10	10	14	P46	2	3
P11	6	12	P48	-	1
P12	-	3	P49	2	3
P13	3	3.5	P50	2	3
P14	7	8	P51	3	4
P15	5	6	P52	3	4
P16	3	5	P53	4	5
P17	9	14	P54	1	2
P18	5	8	P55	5	6
P19	6	7	P56	-	2
P20	3	7	P57	2	3
P21	8.5	9	P58	3	4
P22	6	7	P59	2	3
P23	2	3	P60	2	3
P24	10	12	P62	2	3
P25	9	13	P63	4	5
P26	8.5	10	P64	3	4
P27	6.5	8.5	P65	2	3
P28	7	7.5	P66	3	4
P29	4	6	P67	3	4
P30	-	1	P68	2	3
P31	-	1	P69	2	4
P32	-	4	P70	2	3
P33	5	7	P74	5	8
P35	-	3	P75	2	2

:- No activity

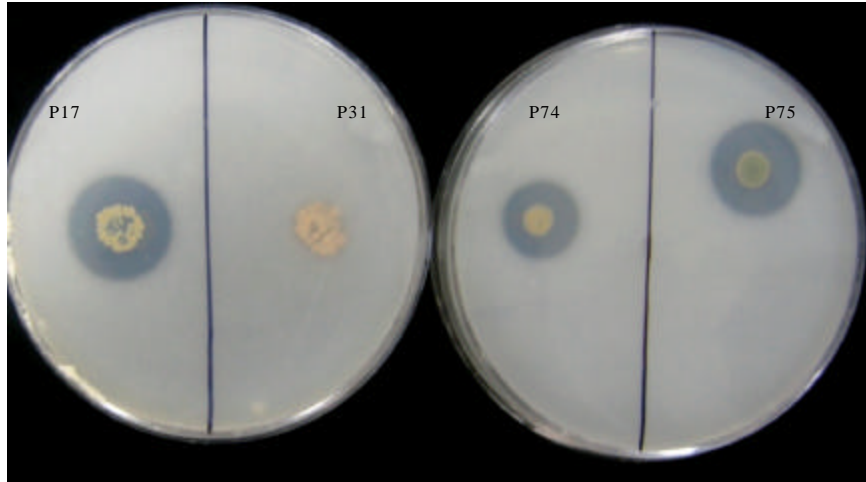


Fig. 1: Solubilization of tri-calcium phosphate on Pikovskaya's agar medium by *Pseudomonas* isolates after 7 days

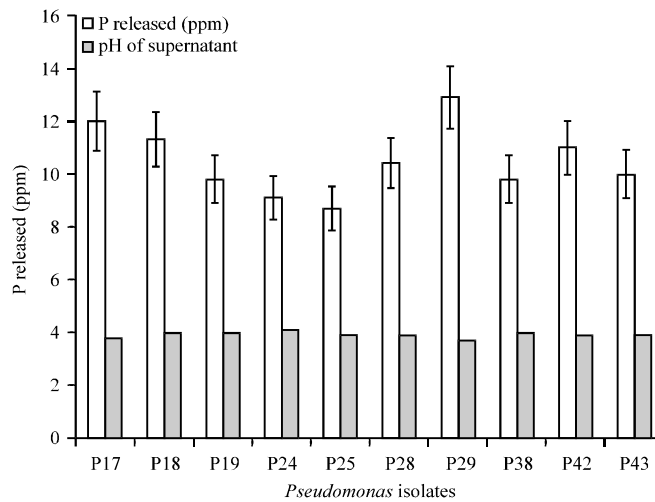


Fig. 2: Solubilization of rock phosphate by selected isolates of *Pseudomonas* spp.

P28, P19, P38, P24, P29, P43 and P25 releasing 14.2, 12.9, 12.3, 12.0, 12.0, 11.0, 11.0, 10.8 and 10.2 mg L<sup>-1</sup>, respectively (Table 3). The pH value of the cell-free culture broth was the lowest for P18 (3.30) followed by P17 (3.36) and the highest pH was recorded in P56 (6.21). The fall in pH corresponded with the solubilization of insoluble P (Table 3). The isolates showing higher solubilization of tri-calcium phosphate were selected further to evaluate their ability to solubilize rock phosphate.

**Rock phosphate solubilization:** Maximum rock phosphate solubilization of 12.9 mg L<sup>-1</sup> was observed with P29 followed by P17 which solubilized 12 mg L<sup>-1</sup> which was on par with P29. Interestingly, P17 which showed highest TCP solubilization could not solubilize rock phosphate as efficiently as P29 (Fig. 2). The P18 released 11.3 mg L<sup>-1</sup> of P, whereas, solubilization by other

Table 3: Quantitative estimation of tri-calcium phosphate solubilization by *Pseudomonas* isolates in liquid medium (after 10 days incubation)

Isolates	Available P in culture filtrate (mg L <sup>-1</sup> ±SD)	pH of culture filtrate	Isolates	Available P in culture filtrate (mg L <sup>-1</sup> ±SD)	pH of culture filtrate
P1	6.37±0.58	4.82	P34	9.06±0.83	4.28
P2	5.31±0.48	4.65	P35	5.94±0.54	4.86
P3	5.02±0.46	4.18	P37	8.60±0.79	5.32
P4	5.37±0.49	4.51	P38	11.90±1.09	3.75
P5	5.22±0.48	4.58	P39	4.43±0.40	4.80
P6	4.72±0.43	4.52	P40	6.17±0.56	4.55
P7	5.00±0.46	4.50	P41	3.92±0.36	6.31
P8	5.14±0.47	4.53	P42	14.20±1.30	3.58
P9	5.35±0.49	4.57	P43	10.84±0.99	4.42
P10	5.17±0.47	5.00	P44	3.03±0.27	5.69
P11	2.94±0.27	5.20	P45	3.51±0.32	5.21
P12	3.64±0.33	5.00	P46	5.21±0.48	6.34
P13	3.23±0.29	5.10	P47	2.74±0.25	6.64
P14	2.96±0.27	5.10	P48	3.67±0.33	6.12
P15	8.75±0.80	3.90	P49	2.91±0.26	6.10
P16	4.03±0.37	5.00	P50	3.93±0.36	6.17
P17	15.19±1.40	3.36	P51	4.02±0.37	6.15
P18	12.88±1.18	3.30	P52	3.47±0.31	6.21
P19	11.97±1.10	3.40	P53	4.32±0.39	6.07
P20	9.61±0.88	4.50	P54	4.54±0.41	6.15
P21	9.92±0.91	3.40	P55	4.18±0.38	6.17
P22	9.05±0.83	3.80	P56	4.07±0.37	6.21
P23	2.81±0.25	5.90	P58	3.55±0.32	6.10
P24	11.06±1.01	3.40	P59	6.03±0.55	5.55
P25	10.18±0.93	5.83	P60	7.27±0.67	5.10
P26	5.83±0.53	6.14	P62	3.32±0.30	5.60
P27	5.79±0.53	6.29	P63	3.26±0.30	5.79
P28	12.27±1.13	4.14	P66	4.48±0.41	4.28
P29	11.00±1.00	4.12	P67	6.72±0.61	3.82
P30	8.20±0.75	4.27	P68	2.11±0.19	5.20
P31	5.53±0.50	4.87	P69	2.15±0.19	4.46
P32	5.70±0.52	5.01	P70	5.34±0.49	4.15
P33	6.12±0.56	5.21	P74	3.15±0.29	4.44

Values represented in the columns are means of triplicates and significant at p<0.05

isolates ranged between 8.7 and 11 mg L<sup>-1</sup>. The P25 solubilized very less RP with the release of 8.7 mg L<sup>-1</sup>. The pH of the cell-free culture filtrates of the isolates ranged between 3.7-4.1. The P29 which showed highest rock phosphate solubilization recorded lowest pH of 3.7 and P24 isolate which was the second best isolate culture filtrate showed a pH of 4.1 (Fig. 2).

## DISCUSSION

**Isolation of *Pseudomonas* spp.:** Microorganisms bring about a number of transformations of the chemical elements in soil, including the oxidation or reduction of inorganic phosphorus (P) compounds. In this process, P that is in excess to the required amount is released into the soil environment. The efficiency of the process depends, amongst other, on the population of the

organisms and environmental factors. *Pseudomonas* spp. were isolated from soil samples collected from various agro-ecosystems of India. Eighteen different crop production systems rhizosphere and bulk soil samples were used for the isolation of *Pseudomonas* spp. (Table 1). Constituents of plant root exudates decide the type and density of bacterial communities in a given crop production system (Curl and Truelove, 1986). This diversity in turn may also lead to variability in their morpho-physiological traits. In the present study also, species of *Pseudomonas* were isolated from different crop production systems of diverse agro-ecological regions. Van Overbeek *et al.* (1997) reported that survival strategies of the organisms depend on the physiological adaptation to nutrient-limited conditions and/or other physico-chemical conditions, efficient utilization of root-released compounds or specific interactions with plants.

**Phosphate solubilization:** Screening of TCP solubilization on solid media revealed that some of the strains could not solubilize TCP and therefore, no zone of solubilization was observed (Table 2). But when tested for solubilization in liquid media for 10 days such isolates showed release of soluble P into the medium. This could be due to the meagre availability of nutrients on solid media and vice-versa in liquid media. However, the degree of phosphate solubilization varied with the type of organisms involved. It was reported that di-calcium phosphate could be solubilized more readily than tri-calcium phosphate by some bacteria (Sujatha *et al.*, 2004). All the 75 isolates of *Pseudomonas* were tested for their ability to solubilize Tri-Calcium Phosphate (TCP) *in vitro*. Among which P17 solubilized highest TCP with the release of 15.19 mg L<sup>-1</sup> and the pH of the culture filtrate was lowest (3.36) (Table 3). Ten selected isolates were checked for their Rock Phosphate (RP) solubilization ability (Fig. 2). It was interesting to note that strain P17 which showed highest TCP solubilization did not show higher RP solubilization where as P29 was the best among the tested isolates for RP solubilization which solubilized less TCP than that of P17. This investigation is co-inciding with the observations of Gupta *et al.* (2007). The reason for higher RP solubilization by P29 may be due to higher acids production in the medium than P17. So, utilization of such strains will be useful to combat the nutrient deficiencies in soils devoid of phosphorous because most of the Indian soils are P deficient.

In the present study, the solubilization of TCP could be due to the production of various organic acids (Table 3) into the medium which can also be explained by the following facts. Phosphorous solubilization correlated with drop in pH of the medium among all the isolates. Inorganic P is solubilized by the action of organic and inorganic acids secreted by PSB in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe, Ca) and decrease the pH in basic soils (Deubel *et al.*, 2000; Stevenson, 2005). Organic acid production by microbial strains has been reported to be a major mechanism of solubilization (Nguyen *et al.*, 1992; Fasim *et al.*, 2002). Organic acid producing phytate solubilizing bacteria and their effect on pigeonpea growth is recently documented (Patel *et al.*, 2010). Even though, in the present study isolates were not evaluated for their ability to solubilize phytic acid, it is presumed that the secretion of organic acids as evidenced by a fall in pH in the TCP/RP containing medium could also solubilize phytates in the soil. The PGPR with phosphate solubilizing ability isolated from composts were demonstrated to have plant growth promoting effect on maize (Hameeda *et al.*, 2006).

The potential of a given rhizobacteria to promote nutrient uptake and plant growth could be due to synergistic action of the growth promoting traits than individual effect (Glick *et al.*, 1999). The solubilization of TCP was possible by simple acidification of the medium and acidity contributed to its solubilization by the release of carboxylic acids (Puente *et al.*, 2004; Rodriguez *et al.*, 2006).



Application of phosphate solubilizing bacteria to improve plant growth by solubilizing sparingly soluble inorganic phosphates in soil was demonstrated by Rodriguez and Fraga (1999). The importance of microbial strains solubilizing insoluble Zn and P sources *in vitro* for management of nutrient requirements of crops has been demonstrated by Suseelendra *et al.* (2012). Diverse factors (salts, pH and temperature) can affect the capacity of phosphobacteria to solubilize P (White *et al.*, 1997; Johri *et al.*, 1999).

## CONCLUSION

In conclusion, we isolated about 75 isolates of *Pseudomonas* spp. from diverse agro-ecosystems of India and evaluated their phosphate solubilization ability *in vitro*. Most of the soils are rich in total P. However, its availability to plants is very limited, as P in soils tend to change its form and becomes unavailable. Recurrent use of inorganic phosphate fertilizers to alleviate deficiency in soils is not only cost-effective but also may lead to excessive deposition over time. Strains of *Pseudomonas*, proven to solubilize P, offer best possible nutrient re-cycling mechanism at a low input cost where expensive inorganic fertilizers are becoming unaffordable by small and marginal farmers. Further, in our study it became evident that, most of the strains solubilizing rock phosphate were isolated from semi-arid, deep alfisols of Deccan Plateau agro-ecological sub region of India suggesting that these strains are potential phosphate solubilizers. Strains like *Pseudomonas*-P17 and P29 could be viable cost-effective alternatives to chemical fertilizers for management of plant nutrition.

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