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***In vitro* Solubilization of Tricalcium Phosphate by Phosphate Solubilizing Fungi Isolated from Crop Rhizosphere**

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Fifteen most efficient phosphate solubilizing fungi (PSF) isolated from different crop rhizosphere were studied for biochemical characteristics. PSF were grown *in vitro* for fifteen days on Pikovskaya's medium and following analyses i.e. solubilization index, pH change, phosphorus (P) solubilized, P immobilized and organic acids produced were carried out under *in vitro* conditions. P solubilization index of these isolates ranged from 1.15-3.75. Drop in pH of the medium ranged from 7 to 3.20 with the continuous growth of these isolates for eighteen days. Study showed that more P was immobilized (0.2-0.46%) than solubilized (0.088-0.22%). *Aspergillus sulfureous* and *Penicillium bilaii* were found to be more active fungi in conversion of insoluble P to soluble P. Tartaric acid and gluconic acid were two common organic acids produced by PSF. Correlation studies suggested that besides organic acids, there might be other factors such as microbial respiration, shaking or stagnant condition of culture growth, carbon source used for culture growth, that are also involved in the process of inorganic phosphorus solubilization.

Key words : Phosphate solubilization fungi (PSF), organic acid, solubilization index

Phosphorus is the second most important plant nutrient after nitrogen (Donahue *et al.*, 1990). It exists in nature in a variety of organic and inorganic forms. P availability is low in soils because of its fixation as insoluble phosphates of iron, aluminium and calcium. Since deficiency of P is the most important chemical factor restricting plant growth, chemical phosphatic fertilizers are widely used to achieve optimum yields. Soluble forms of P fertilizer used are easily precipitated as insoluble forms, which leads to their excessive and repeated application to crop. Phosphorus supply through biological means is a viable alternative. Phosphate solubilizing bacteria (PSB), phosphate solubilizing fungi (PSF) and actinomycetes have been reported to be active in conversion of insoluble phosphate to soluble primary and secondary orthophosphate ions (Chabot *et al.*, 1993; Pal, 1998). Although mechanism of phosphate solubilization is still not fully understood, several mechanisms have been implicated for the process. The production of organic acids seems to be the main mechanism (Illmer *et al.*, 1995). In broth studies many workers have shown that phosphate solubilization is due to organic acid

production (Gupta *et al.*, 1994; Illmer & Schinner, 1992, 1995). Omar (1998) tested 36 fungal species on agar plates and found that *Aspergillus niger* and *Penicillium citrinum* caused a marked drop in pH of liquid culture media and solubilized considerable amounts of phosphate. Nautiyal (1999) stated that organic acid production is an important mechanism in 'P' solubilization but not the sole mechanism. Chabot *et al.* (1993) found that PSM constitute 26-46% of microbial population of four Quebec soils studied. They isolated 10 bacteria and three fungi capable of phosphate solubilization on the basis of large clear zone on solid media containing different insoluble phosphates. Khalil (1995) reported that phosphate availability from rock phosphate was increased in soil from 0.67 ppm in control to 17.78 ppm with PSM inoculation in 20 days. In an effort to develop a PSF inoculum for improving P uptake by wheat crop, numerous PSF were isolated from the rhizosphere of many commonly grown crops. Biochemical characteristics of the most efficient 15 isolates were studied.

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MATERIALS AND METHODS

Solubilization index (SI).

0.1 ml of each PSF culture preserved in sterile distilled water was placed on Pikovskaya's agar (PA) (Pikovskaya, 1948) plates [containing insoluble tricalcium phosphate 2.5 g, glucose 13 g, (NH₄)SO₄ 0.5 g, NaCl 0.2 g, MgSO₄·7H₂O 0.1 g, KCl 0.2 g, Yeast Extract 0.5 g, MnSO₄ trace, FeSO₄·7H₂O trace, Agar 15 g, pH adjusted to 7.2 and dissolved in 1000 mL distilled water] and incubated for fifteen days. Solubilization Index was measured using following formula (Edi-Premono *et al.*, 1996).

$$SI = \frac{\text{Colony diameter} + \text{halozone diameter}}{\text{Colony diameter}}$$

pH change

1 ml of three day old culture in sterile distilled water (containing about 1×10^4 CFU) was added to sterile 100 mL Pikovskaya's broth (PB) medium in 250 ml conical flask and shaken intermitantly upto twenty-one days. Sterile uninoculated medium served as control. Initial pH and change in pH was noted at the interval of three days up to three weeks by digital pH meter.

'P' Analysis in broth

Phosphorus solubilized

The clear filtrate after measurement of pH was transferred to 100 ml volumetric flask and the volume was made up with distilled water. Solubilization of tricalcium phosphate in terms of water soluble phosphorus was estimated periodically by chlorostannous reduced molybdophosphoric acid blue colour method as described by Jackson (1958) by taking known quantity of filtrate.

Test for available P

Broth phosphorus was determined by ascorbic acid method (Watanabe and Olsen, 1965). One ml of broth sample extract was taken in 50 ml conical flask and 9 mL of distilled water + 2.5 ml of freshly prepared colour reagent [12 g ammonium molybdate {(NH₄)₆Mo₇·4H₂O} + 250 mL distilled water and 0.2908 mg antimony potassium tartrate {K(SbO)C₄H₄O₆·1/2 H₂O} in 1000 mL of 5N H₂SO₄ (148 mL conc. H₂SO₄ L⁻¹)]. Both the solutions were mixed and the volume was raised upto 2 lt. 140 ml of this mixture was added to 0.74 g ascorbic acid and stirred gently. The optical density of the blue colour developed after 15 min. was measured at 880 nm

by spectrophotometer and the concentration of available P (ppm) was calculated.

Phosphorus immobilized

Pellets of centrifuged cultures after decanting supernatant were taken in a 50 ml calibrated pyrex Kjeldahl flask. Ten ml of perchloric acid and nitric acid mixture (1:2) was added to the pellet. Samples were digested on a micro Kjeldahl digestion rack using gentle heat until clear solution was obtained. The volume of sample was reduced to 2-3 ml. When effervescence stopped, volume of digest was made 100 ml and stored in plastic bottles.

Estimation of P

Immobilized P was estimated by "Vanadomolybdo phosphoric acid colour method" (Jackson, 1958). To 5 ml plant digest, 5 ml colour reagent (50 gL⁻¹ ammonium molybdate + 2.5 gL⁻¹ ammonium vanadate + 20 mL conc. HNO₃ + 250 ml⁻¹ HNO₃) was added. Same procedure was repeated with P standards of 5, 10, 15, 20, 25 and 30 ppm. After 30 min, optical density of yellow colour was noted with spectrophotometer at 430 nm and concentration of percent immobilized P was calculated.

Organic acids determination

Organic acids produced by PSF in PB medium were analyzed by paper chromatography. The PSF were grown in 100 ml PB medium in 250 ml sterilized conical flask for 15 days in B.O.D. at 28 ± 2°C. The broths were filtered through filter paper under vacuum Buchner funnel. The filtrate was concentrated at 90°C on water bath and evaporated up to 3 ml and then poured in 5 ml volumetric flask and the volume was made up with distilled water. The type and amount of organic acids produced by fungal culture were identified by paper chromatography in 20 cm × 5 cm paper stripe (Whatman No 1).

Two parallel lines were drawn with lead pencil at a distance of 2 cm from both ends longitudinally. There after the concentrated culture sample as well as standard solution of organic acids were applied with the help of capillary tube on a horizontal line from one end of the paper, and were dried at room temperature. After drying of spots, the chromatographic papers were put in the cylindrical developing chamber (containing, mixture of n- butanol: formic acid: water: 5:1:4, v/v) for 10 - 12 hours. The chamber was made air tight by ground glass cover. After the developments of spots in chromatographic papers, they were allowed to dry and

these dried papers were sprayed with bromophenol blue reagent (0.05 % solution in ethanol) with a chromatographic sprayer and the areas of yellow coloured spots in sky blue background were immediately marked with lead pencil. The distances travelled by the advancing front in papers were noted for finding out the retention factor (Rf values).

$$\text{Rf values} = \frac{\text{The distances moved by solute (organic acid)}}{\text{The distance moved by solvent front}}$$

The Rf values were calculated for different organic acids and were compared with those of standard Rf values of organic acids (fumaric acid, lactic acid, succinic acid, malonic acid, malic acid, oxalic acid, citric acid, tartaric acid and gluconic acid) and simultaneously organic acids produced by fungal culture were identified through Rf values.

Quantification of organic acids

For quantification, the yellow coloured spots were stripped out from the paper with the help of scissors and were kept in petriplate. About 3 ml of n- butanol was spread over the stripped part of chromatograph in order to dissolve the compounds of yellow spots in n- butanol. When the strip paper became colourless, the solvent was poured into 5 ml of volumetric flask and final volume was made 5 ml with n- butanol. The intensity of light yellow coloured spots of the entire sample was measured at 426 nm wavelength, where it gave the maximum absorbance (%). As most of the fungal isolates produced tartaric acid, so the standard was prepared with this acid. The standard curve was plotted with the reaction product of tartaric acid and bromophenol blue of different strengths of tartaric acid viz. 2.5, 5.0, 10, 15, 20, and 25 mg L⁻¹ dissolved in butanol. The percentage absorbance of sample was measured and its actual amount produced was calculated from standard curve.

Statistical analysis

Simple correlation was run to determine correlation coefficients. The results were depicted diagrammatically.

RESULTS AND DISCUSSION

Solubilization index (SI).

Solubilization index based on colony diameter and halozone for each PSF isolate is presented in Table 1. Results showed that among PSF, F₁₇ was the most efficient phosphate solubilizer on PA plates with SI = 3.75 followed by F₁₀. Studies on agar plates revealed

that phosphate solubilizing fungi formed clear zones by solubilizing suspended tricalcium phosphate. Measurements ranged from 4.07- 8.84 cm for fungi. Generally, halozone increased with increase in colony diameter. Fluctuations in solubilization index were observed during the fifteen days observation period. In most of the cases it gradually increased (F₇, F₁₁, F₁₃, F₁₄ and F₁₆) while in few cases it first decreased and then increased (F₃, F₆ and F₂₂ & F₂₃) and in certain cases (F₂₅ and F₃₀) no systematic trend was observed. These results are in accordance to Chabot *et al.* (1993) and Nahas (1996). Similar results were found by Kucey (1983, 1987), Edi- Premono *et al.* (1996) and Kumar and Narula (1999). The solubilization index was determined for fifteen days only because of overgrowth of fungus. Most of PSF strains lost their ability to form halozone on Pikovskaya Agar medium on repeated subculturing. This result is in accordance to Kucey and Leggett (1989), and Illmer and Schinner (1992).

pH

Most phosphate solubilizing fungi studied lowered the pH of PB medium compared to uninoculated sterile PB (control) incubated for twenty-one days under conditions as inoculated (Table 2). All fungal isolates were found to be active in lowering of the pH. Drop in pH by PSF ranged from 5.98 to 3.20 at the end of incubation period. Few PSF isolates (F₇, and F₂₂) although showed larger halozones but, did not lower the pH accordingly. Fluctuations in pH drop were also noted. pH studies showed a drop of pH from 7.01 (control) to 3.2 (F₁₄, F₂₀). Similar results were observed by Bar – Yosef *et al.* (1999), and Khalil and Sultan (2000).

Immobilized and solubilized P

P solubilization and immobilization data indicate that fungal isolates solubilized appreciable amount of phosphorus, which in turn immobilized, at the same rate. The percentage of P solubilized and P immobilized were calculated at every incubation period, but only the solubilized and immobilized P percentage at 15 days of incubation, which was the highest among entire incubation period, was represented (Fig. 1). Results indicated that more P was immobilized than solubilized. These results are in contrast to the findings of previous studies (Kucey, 1983; Nahas, 1996). This contradiction may be due to the fact that fungi immobilize more P because they form larger colonies and their biomass is greater than bacteria (Illmer & Schinner, 1995). Thus, it may be concluded that F₂₅ was the most efficient strain among all PSF,

because it immobilized the lowest amount of phosphorus (69.48%) in comparison to its solubilized amount of phosphorus (65.24), where as F₁₃ was the least efficient contributor of phosphorus for solubilization of tricalcium phosphate because it immobilized the highest amount of phosphorus (64.63%) in comparison to the solubilized amount of phosphorus. Solubilized P by all studied PSF strains was higher than uninoculated control. Values of solubilized P ranged from 54.67- 66.55% and the values for P immobilized ranged from 64.63 – 72.36%. Illmer and Schinner (1995) noted that P contents of PSM biomass varied from 0.4 - 0.8%. Johri *et al.* (1999) found 0.036 - 0.44% P solubilized by PSM. This difference might be due to different strains used by them.

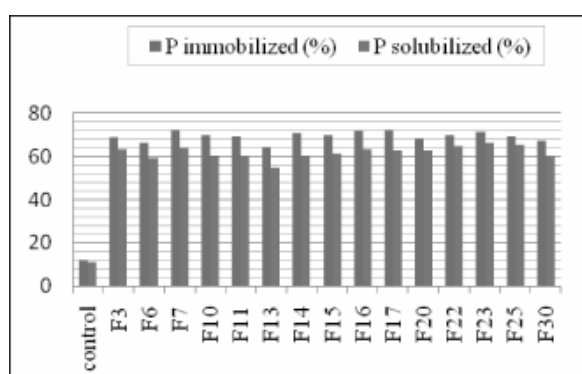


Figure 1: Percent P immobilized and solubilized by selected PSF isolated from different rhizosphere

Organic acids

Organic acids produced by PSF isolated from different commonly grown crop rhizosphere have been presented in Table 3. Results indicated that the highest amount of total organic acid produced was 8.918 mgL⁻¹ by strain F₂₅, followed by 8.612 mgL⁻¹ (F₃) and 8.068 mgL⁻¹ (F₇) while, the lowest was 2.272 mgL⁻¹ in F₆. Most common acids produced were tartaric and gluconic acids while lactic acid was produced by only one strain (F₁₂). F₁₆ produced the highest concentration of tartaric acid (7.489 mgL⁻¹). Among all isolated fungi, *Aspergillus flavus* (F₆) produced three types of organic acids viz. tartaric, gluconic and malonic (1.751, 0.265 & 0.2605 mgL⁻¹ respectively), while the total amount of organic acids produced were the lowest (2.272 mg L⁻¹) with this isolate. Studies related to the production of organic acids have shown that tartaric and gluconic acids were the two major organic acids commonly produced by PSF. Production of oxalic acid as major organic acid from different species of *Aspergillus*, an unknown species of *Penicillium* and *Sclerotium rolfisii* has been reported

by Banik and Dey (1983), Gupta *et al.* (1994), and Illmer and Schinner (1995). Contrary to their findings, the tartaric acid was first common organic acid produced by PSF except F₂₂ and gluconic acid was second. This is similar to the findings of Illmer and Schinner (1995) indicating production of gluconic acid from *A. niger*. However, it is contradictory to the reports by Illmer and Schinner (1992) and Whitelaw *et al.* (1999) that gluconic acid was not produced by PSM strains. Venkateswarlu *et al.* (1994) detected lactic acid from *A. niger*, while none of the 10 isolates of *Aspergillus* produced lactic acid in the study whereas *Penicillium digitatum* (F₂₂) produced lactic acid in little amount (0.941 mg L⁻¹). Contradiction or similarities may be explained on the basis that type and quantity of acid produced depends on the PSF strain, media composition, growth conditions and several other factors. Cunningham and Kuyack (1992) reported that citric acid was the major organic acid produced by *P. bilji* when nitrogen source was nitrate but it was not detected when ammonium was used. PSF used glucose as carbon source to produce organic acids as found by Nautial (1999). They stated that more organic acids were produced by microorganisms when glucose was used as carbon source.

Correlation coefficients

Correlation coefficient values showed significant ($p < 0.05$) correlation between P solubilized, P immobilized and solubilization index (Table 4). The result is similar to observation made by Illmer and Schinner (1995). But P solubilized was non-significantly correlated with colony diameter ($r = 0.106$) and colony+halozone diameter ($r = 0.18$). Significant ($p < .05$) and negative correlation was found between pH and P solubilized, P immobilized, solubilization index and halozone produced. Similar correlation is reported by Kumar and Narula (1999) and Whitelaw (2000). Least correlation was found with P solubilized and colony + halozone diameter ($r = 0.106$). Non-significant positive correlation was found between P solubilized and halozone produced. The result is similar to that of Kucey (1983) study but opposite to the findings of Edi- Premono *et al.* (1996) and, Kumar and Narula (1999). This is because most PSF show fluctuations in their behaviour. Production of halozone on solid medium is important but it is not necessary that PSF with larger halozones will solubilize more phosphorus. Johri *et al.* (1999) suggested that isolation of efficient PSF based on formation of a visible zone on agar plates was not a reliable technique. Some PSM lost their ability to produce halozone on sub culturing (Kucey, 1983). Highest positive

Table 1. Solubilization index of selected strains of phosphate solubilizing fungi

Strains	Crop rhizosphere	Solubilization index through fifteen days				
		Day 3	Day 6	Day 9	Day 12	Day 15
Control	0.00	0.00	0.00	0.00	0.00	
<i>Aspergillus awamori</i> (F ₃)	Potato	1.21	1.27	1.20	1.35	1.44
<i>Aspergillus flavus</i> (F ₆)	Sugarcane	1.45	1.40	1.30	1.36	1.47
<i>Aspergillus fomitopsis</i> (F ₇)	Wheat	1.55	1.80	1.98	2.17	2.28
<i>Aspergillus niger</i> (F ₁₀)	Radish	3.30	3.13	3.45	3.67	3.27
<i>Aspergillus niger</i> (F ₁₁)	Lobia	1.45	1.46	1.47	1.49	1.65
<i>Aspergillus sulfureous</i> (F ₁₃)	Rajmash	2.34	2.34	2.63	2.70	2.71
<i>Aspergillus sulfureous</i> (F ₁₄)	Lobia	2.24	2.25	2.33	2.62	2.81
<i>Aspergillus sulfureous</i> (F ₁₅)	Parwal	2.44	2.50	2.31	2.11	2.21
<i>Aspergillus sulfureous</i> (F ₁₆)	Wheat	1.25	1.50	1.84	1.93	2.22
<i>Aspergillus sulfureous</i> (F ₁₇)	Pea	2.30	3.75	2.80	2.59	2.22
<i>Penicillium bilaii</i> (F ₂₀)	Parwal	1.21	1.15	1.86	1.80	1.76
<i>Penicillium digitatum</i> (F ₂₂)	Okra	3.02	2.24	1.62	1.62	2.99
<i>Penicillium digitatum</i> (F ₂₃)	Rajmash	3.05	2.29	1.65	1.66	2.81
<i>Penicillium fuscum</i> (F ₂₅)	Maize	1.92	2.21	2.08	1.54	2.05
<i>Rhizopus stolonifer</i> (F ₃₀)*	Sugarcane	1.89	2.20	1.63	-	-

*Solubilization index for this fungus was determined for nine days only because of fungal over growth.

Table 2. Drop in pH by selected strains of phosphate solubilizing fungi in Pikovskaya's broth

Strains	pH Drop through twenty-one days						
	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21
Control 7.01	7.01	7.01	7.01	7.01	7.01	7.01	
<i>Aspergillus awamori</i> (F ₃)	6.91	4.10	4.02	3.84	3.89	3.76	4.04
<i>Aspergillus flavus</i> (F ₆)	6.99	4.82	3.95	3.68	3.52	3.45	3.43
<i>Aspergillus fomitopsis</i> (F ₇)	6.93	5.19	4.74	4.55	4.62	4.60	4.30
<i>Aspergillus niger</i> (F ₁₀)	6.99	3.89	3.58	3.38	3.30	3.29	3.33
<i>Aspergillus niger</i> (F ₁₁)	6.94	3.55	3.44	3.25	3.25	3.23	3.33
<i>Aspergillus sulfureous</i> (F ₁₃)	6.99	3.83	3.73	3.61	3.49	3.39	3.43
<i>Aspergillus sulfureous</i> (F ₁₄)	6.99	4.37	4.58	4.29	3.60	3.33	3.20
<i>Aspergillus sulfureous</i> (F ₁₅)	6.99	4.50	4.44	4.39	4.29	4.25	4.33
<i>Aspergillus sulfureous</i> (F ₁₆)	6.99	5.06	3.65	3.54	3.40	3.45	3.40
<i>Aspergillus sulfureous</i> (F ₁₇)	6.97	4.38	3.76	3.72	3.71	3.70	3.25
<i>Penicillium bilaii</i> (F ₂₀)	6.99	6.83	5.58	4.91	4.59	3.91	3.20
<i>Penicillium digitatum</i> (F ₂₂)	6.99	6.93	6.40	5.50	4.34	4.32	4.00
<i>Penicillium digitatum</i> (F ₂₃)	6.98	7.03	5.08	4.70	3.40	3.33	3.30
<i>Penicillium fuscum</i> (F ₂₅)	6.95	6.92	6.35	5.49	4.33	4.30	4.28
<i>Rhizopus stolonifer</i> (F ₃₀)	6.96	6.92	6.85	6.45	6.33	6.02	5.98

Table 3. Low molecular weight organic acids produced by phosphate solubilizing fungus isolated from different crop rhizosphere

PSF	Total organic acids (mg L ⁻¹)	Tartaric acid	Citric acid	Gluconic acid	Malonic acid	Fumaric acid	Lactic acid
F ₃	8.612	5.98	-	-	-	2.632	-
F ₆	2.272	1.751	-	0.2605	0.2605	-	-
F ₇	8.068	2.195	-	5.873	-	-	-
F ₁₀	4.544	1.576	-	2.968	-	-	-
F ₁₁	4.408	4.408	-	-	-	-	-
F ₁₃	6.272	4.089	-	2.183	-	-	-
F ₁₄	4.238	2.978	-	-	-	1.26	-
F ₁₅	7.932	5.985	-	-	-	1.947	-
F ₁₆	9.360	7.489	-	1.871	-	-	-
F ₁₇	7.258	6.981	-	-	0.277	-	-
F ₂₀	7.364	6.914	-	0.450	-	-	-
F ₂₂	2.816	-	1.875	-	-	-	0.941
F ₂₃	5.768	4.565	-	-	-	1.203	-
F ₂₅	8.918	6.983	1.935	-	-	-	-
F ₃₀	3.36	3.03	-	0.33	-	-	-
Control	-	-	-	-	-	-	-

Table 4. Correlation coefficients of different biochemical characters of PSF isolated from different crop rhizosphere

Parameter	P Immobilized (%)	Solubilization index	Colony diameter (cm)	Halozone (cm)	pH	Organic acid (mg/L)
P Solubilized (%)	0.67**	0.552*	0.106	0.18	-0.57*	0.44
P Immobilized (%)		0.574*	0.67**	0.472	-0.69**	0.37
Solubilization Index			0.058**	0.485	-0.79**	0.825**
Colony diameter(cm)				0.515*	-0.302	0.11
Halozone (cm)					-0.625**	0.468
pH						-0.49

* significant (p<0.05); ** highly significant (p<0.01)

correlation was obtained with solubilization index and organic acids produced ($r = 0.825$). This is due to the fact that perhaps organic acids are the main factors responsible for P solubilization. A nonsignificant positive correlation was observed between P solubilized and organic acids produced by PSF suggesting that organic acids may play important role in solubilization of tricalcium phosphate and rock phosphate but are not the only possible mechanism for P solubilization (Illmer &

Schinner, 1992). pH and organic acids were also found to be non significantly correlated with each other. Microbial respiration may be involved for drop in pH e.g (Illmer & Schinner, 1992).

Thus, it may be concluded that PSF showed variation in their biochemical characteristics. Organic acid production was not the only possible reason for phosphorus solubilization but also many other factors like

microbial respiration, shaking or stagnant condition of culture growth, carbon source used for culture growth are also involved in this processes. Present study also showed that F₂₂ and F₂₅ are the most efficient strains on the basis of their P solubilizing activity.

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