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## Phosphate Solubilization by *Rhizobium* Isolates from *Crotalaria* Species

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**Abstract:** *Rhizobium* species isolated from *Crotalaria* species (*C. juncea*, *C. laburnifolia*, *C. retusa* and *C. verrucosa*) were tested for their ability to solubilize tricalcium phosphate (TCP). Among these four species, *Rhizobium* sp. isolated from *Crotalaria retusa* and *C. verrucosa* showed zone of TCP solubilization on Pikovskaya's agar medium. Highest solubilization index (2.70) was observed in *Rhizobium* sp. from *C. retusa*. Effect of different carbon and nitrogen sources was also studied. Among the carbon sources, glucose was found to be best carbon source for TCP solubilization. Presence of highest concentration (3%) of glucose enhanced TCP solubilization. Among the nitrogen sources, ammonium sulphate was the best promoter for the process. Addition of low levels ( $0.5 \mu\text{g mL}^{-1}$ ) of EDTA increased TCP solubilization.

**Key words:** Phosphate solubilization, *Rhizobium* sp., *Crotalaria* sp., *Rhizobium*-legume symbiosis

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## INTRODUCTION

Plant Growth Promoting Rhizobacteria (PGPR) are involved with host plants in mutual interaction. They promote plant growth by the production of phytohormones, solubilization of insoluble phosphates and biocontrol of host plant diseases or improvement of plant nutritional status (Deshwal *et al.*, 2003). Rhizobia are the first group of bacteria, which are attributed to the ability of PGPR to solubilize insoluble phosphates (Johri *et al.*, 2003). So far only a few reports on phosphate solubilizing ability of *Rhizobium* strains (Halder *et al.*, 1990, 1991; Halder and Chakrabarty, 1993; Rivas *et al.*, 2006; Daimon *et al.*, 2006).

*Crotalaria* is one of the largest genera of Fabaceae with more than 500 species commonly occurring in diverse climatological situations (Allen and Allen, 1981). Some *Crotalaria* species are of great agronomic interest, since they are used as green manure to improve soil fertility or control nematode populations in infested soils (Sy *et al.*, 2001). Because of its importance the present study was taken up to investigate phosphate solubilizing ability of *Rhizobium* isolates from commonly occurring *Crotalaria* species (*C. juncea* L., *C. laburnifolia* L., *C. retusa* L. and *C. verrucosa* L.) and the ability of these isolates to solubilize tricalcium phosphate (TCP) in the presence of different carbon sources, nitrogen sources and cell affecting agents was also studied.

## MATERIALS AND METHODS

### Organism and Phosphate Solubilization

*Rhizobium* species isolated from root nodules of *C. juncea*, *C. laburnifolia*, *C. retusa* and *C. verrucosa* on Yeast Extract Mannitol Agar (YEMA) medium. The identification of the isolates as

*Rhizobium* was confirmed by plant infection test (Vincent, 1970). The phosphate solubilizing ability of the *Rhizobium* species was tested on Pikovskaya's agar (Pikovskaya, 1948) containing tricalcium phosphate (TCP) as insoluble phosphate source. The formed halo zone surrounding the colony revealed phosphate solubilization and was expressed in solubilization index (Arun and Sridhar, 2005). The *Rhizobium* species, which formed zone of solubilization on agar medium, was further tested in Pikovskaya's broth having initial pH 7.0.

After incubation the final pH and phosphorus (P) content of the medium were also estimated in culture supernatant (Subba Rao, 1993).

#### Effect of Carbon and Nitrogen Sources

Effect of different carbon sources (1%) on phosphate solubilization was tested by replacing glucose with 14 carbon compounds (mannitol, galactose, fructose, rhamnose, arabinose, trehalose, inositol, raffinose, sucrose, maltose, mannose, dulcitol, ribose and xylose) sterilized separately and added aseptically to the medium before sterilization. The effect of different concentrations (1.0, 1.5, 2.0, 2.5 and 3.0%) of most effective carbon source was also studied. Effect of different nitrogen sources (0.1%) on TCP solubilization was studied by replacing ammonium sulphate with five different nitrogen sources (sodium nitrate, potassium nitrate, calcium nitrate, asparagine and urea).

#### Effect of EDTA

The chelating effect of EDTA on TCP solubilization was tested by adding different concentrations (0.5, 1.0, 2.0, 2.5 and 3.0  $\mu\text{g mL}^{-1}$ ) of EDTA to the medium.

#### Statistical Analysis

The data were statistically analyzed by correlation coefficient and ANOVA (two way classification technique), using Statistica software.

## RESULTS AND DISCUSSION

The *Rhizobium* species isolated from *C. juncea*, *C. laburnifolia*, *C. retusa* and *C. verrucosa* were fast growers as they produced acid in YEM broth and colony diameter was greater than 2.0 mm. Among these species, the *Rhizobium* species from *C. retusa* and *C. verrucosa* formed clear zones around the colonies on Pikovskaya's agar medium after 3 days of incubation and it gradually increased up to 9 days. The colony diameter is almost similar throughout the incubation period. Solubilization Index (SI) of these species on solid media ranged between 2.4 and 2.7 (Table 1). Maximum SI was observed in *Rhizobium* sp. from *C. retusa* (2.7). In liquid medium maximum solubilization was also

Table 1: Solubilization of tricalcium phosphate by *Rhizobium* species from *Crotalaria retusa* and *C. verrucosa*

<i>Rhizobium</i> sp.	Incubation time (day)	Colony diameter (mm)	Zone of solubilization (mm)*	Solubilization Index (SI)	Final pH of medium*	P-liberated* ( $\mu\text{g mL}^{-1}$ )
<i>Crotalaria retusa</i>	3	5.0	8.2	2.64	4.25	824
	6	5.0	8.4	2.68	4.20	832
	9	5.0	8.5	2.70	4.32	840
	12	5.0	8.5	2.70	5.20	840
<i>Crotalaria verrucosa</i>	3	4.2	6.2	2.40	6.00	612
	6	4.2	6.3	2.50	5.90	619
	9	4.2	6.3	2.50	5.80	632
	12	4.2	6.4	2.50	5.70	632

Solubilization Index (SI) = Diameter of the colony+Halo zone/Diameter of the colony; \*: Correlation coefficient (r); Between pH and P liberated = -1; Between zone of solubilization and P liberated = 1

recorded in this species ( $840 \mu\text{g mL}^{-1}$ ). A fall in pH accompanied phosphate solubilization, due to production of organic acids was observed up to 9 days, but there is sudden increase in pH after 9 days. This may be due to utilization of organic acids produced during phosphate solubilization by the strains as reported earlier in *Pseudomonas* (Dave and Patel, 1999). The data were statistically analyzed using correlation coefficient and it was found that there is positive correlation between zone of solubilization on agar medium and liberated P in broth and negative correlation between liberated P and final pH of the medium.

Effect of different carbon sources (1%) on phosphate solubilizing activity by the *Rhizobium* sp. revealed that the maximum solubilization occurred in glucose containing medium (Table 2). That the glucose was the best carbon source for phosphate solubilization was also reported earlier in *Bradyrhizobium* sp. isolated from *Cicer arietinum* (Halder *et al.*, 1991). Maximum decrease in pH was recorded in glucose containing medium. In other carbon sources little decrease in pH and no correlation between acidic pH and P liberated were observed. The relative efficiency of *Rhizobium* spp. on different carbon sources could be due to the organic acids secreted by the isolates rather than the total acidity (Dave and Patel, 1999). The data on effect of carbon sources on TCP solubilization by *Rhizobium* spp. were statistically analyzed using analysis of variance (two way classification technique) and it was found that variations due to both carbon sources and *Rhizobium* spp. were found to be significant and the *Rhizobium* spp. differ significantly with different carbon sources.

As the glucose at 1% concentration (as in Pikovskaya's medium) supported maximum solubilization of TCP, effect of different concentrations of glucose was studied. *Rhizobium* sp. from *C. retusa* and *C. verrucosa* showed maximum solubilization at 3.0% concentration of glucose (Table 3). Linear increase in TCP solubilization was observed as the concentration of glucose increased. This may be due to greater acidity produced at 3.0% concentration of glucose, which is an important factor in phosphate solubilization. The variation in the effect of different concentrations of glucose is found to be statistically significant.

Table 2: Effect of different carbon sources on TCP solubilization

Carbon source (1%)*	<i>Rhizobium</i> sp. ( <i>C. retusa</i> )		<i>Rhizobium</i> sp. ( <i>C. verrucosa</i> )	
	Final pH	P-liberated ( $\mu\text{g mL}^{-1}$ )	Final pH	P-liberated ( $\mu\text{g mL}^{-1}$ )
Glucose (control)	5.20	840	5.70	632
Mannitol	6.20	225	6.10	121
Galactose	5.82	125	6.28	119
Fructose	5.90	76	6.20	92
Rhamnose	6.82	30	6.12	98
Arabinose	6.91	98	6.00	102
Trehalose	6.20	49	6.03	74
Inositol	6.80	72	6.25	45
Raffinose	6.40	55	6.69	29
Sucrose	6.20	30	6.80	76
Maltose	5.90	20	6.25	48
Mannose	5.80	49	6.90	78
Dulcitol	6.00	45	6.39	34
Ribose	6.22	30	6.09	48
Xylose	6.89	35	6.09	44

\*: Significant at 1%

Table 3: Effect of different concentrations of glucose on TCP solubilization

Glucose concentration (%)*	<i>Rhizobium</i> sp. ( <i>C. retusa</i> )		<i>Rhizobium</i> sp. ( <i>C. verrucosa</i> )	
	Final pH	P-liberated ( $\mu\text{g mL}^{-1}$ )	Final pH	P-liberated ( $\mu\text{g mL}^{-1}$ )
1.0	5.2	840	5.70	632
1.5	5.0	852	5.62	645
2.0	4.9	894	5.50	648
2.5	4.2	920	5.40	720
3.0	4.0	980	5.30	784

\*: Significant at 1%

Table 4: Effect of nitrogen sources on TCP solubilization

Nitrogen sources*	<i>Rhizobium</i> sp. ( <i>C. retusa</i> )		<i>Rhizobium</i> sp. ( <i>C. verrucosa</i> )	
	Final pH	P-liberated ( $\mu\text{g mL}^{-1}$ )	Final pH	P-liberated ( $\mu\text{g mL}^{-1}$ )
Ammonium sulphate	5.20	840	5.70	632
Sodium nitrate	5.92	410	6.80	612
Potassium nitrate	5.65	402	6.80	610
Calcium nitrate	5.85	395	6.20	540
Asparagine	5.74	300	6.10	480
Urea	5.94	295	6.68	295

\*: Significant at 1%

Table 5: Effect of EDTA on TCP solubilization

Concentration of EDTA ( $\mu\text{g mL}^{-1}$ )*	<i>Rhizobium</i> sp. ( <i>C. retusa</i> )		<i>Rhizobium</i> sp. ( <i>C. verrucosa</i> )	
	Final pH	P-liberated ( $\mu\text{g mL}^{-1}$ )	Final pH	P-liberated ( $\mu\text{g mL}^{-1}$ )
Control	5.2	840	5.7	632
0.5	4.8	892	5.2	649
1.0	5.7	840	5.9	612
2.0	5.9	812	6.0	592
2.5	6.2	628	6.2	540
3.0	6.2	621	6.8	511

\*: Significant at 1%

Effect of different nitrogen sources on TCP solubilization revealed that maximum solubilization of TCP occurred in ammonium sulphate containing medium (Table 4) and reduced the pH of the medium. Further it was observed that the inorganic nitrogen sources supported better solubilization of TCP than organic nitrogen sources. This could be due to the production of inorganic acids by proton exchange mechanism in presence of  $\text{NH}_4^+$  cause accelerated phosphate solubilization (Halder *et al.*, 1991). Statistical analysis showed that the effect of different nitrogen sources on TCP solubilization was also found to be significant.

The effect of different concentrations of EDTA (0.5, 1.0, 2.0, 2.5 and 3.0  $\mu\text{g mL}^{-1}$ ) revealed that the addition of 0.5  $\mu\text{g mL}^{-1}$  EDTA to the standard medium results in significant increase of TCP solubilization, but increasing concentration of EDTA had little effect on TCP solubilization (Table 5). This could be due to the chelation of  $\text{Ca}^{2+}$  produced during TCP solubilization as reported in *Bradyrhizobium* sp. (Halder *et al.*, 1991). The effect of EDTA on TCP solubilization was found to be statistically significant.

Only a few reports on phosphate solubilization have been reported earlier (Halder *et al.*, 1990; 1991; Halder and Chakrabarty, 1993; Rivas *et al.*, 2006; Daimon *et al.*, 2006) and studies were confined to only limited number of hosts and a single species of *Rhizobium* from a single host. From this it may concluded that, in addition to symbiotic nitrogen fixation, some species of *Rhizobium* can also involved in phosphate solubilization. Furthermore, the capacity of phosphate solubilization by the *Rhizobium* species can be exploited as PGPR.

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