

Phosphate Solubilizing Rhizobia Isolated from *Vigna trilobata*

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Abstract Rhizobial strains were isolated from root nodules of *Vigna trilobata* plants raised in soils of different districts of Andhra Pradesh, India. Among the 23 strains of rhizobia isolated, 6 strains were proved to be positive for phosphate solubilization. The strains were identified as *Sinorhizobium* sp. strain MRR101-KC428651, *Agrobacterium tumefaciens* strain MRR102- KC428652, *Rhizobium* sp. strain 103 – JX576499 ; *Sinorhizobium kostiense* strain MRR104- KC428653; *Agrobacterium tumefaciens* strain MRR105- KC428654 and *Rhizobium* sp. strain MRR106- KC428655 after 16S rDNA sequencing. *S. kostiense* strain MRR104 was found to be better than strains of *Agrobacterium tumefaciens* and *Rhizobium* sp. in phosphate solubilization with maximum zone of solubilization (18mm) on standard Pikovskaya's medium and with maximum P₂O₅ liberation of 510 µg/ml in liquid medium. The optimization for maximum phosphate solubilization was done by using different carbon and nitrogen sources. Glucose was preferred as carbon source by all the strains studied, with 10 fold increase in solubilization of phosphorous than other carbon sources. Phosphate solubilization increased with increase in concentration of glucose up to 3% in all the strains studied. Some strains preferred ammonium sulphate and others preferred nitrates as nitrogen source for phosphate solubilization, indicating that strains of *V. trilobata* are adopting two different mechanisms for solubilization. Reduction in pH with increased phosphate solubilization efficiency was also observed among the strains, irrespective of the carbon sources tested. Strain *S. kostiense* MRR104 was proved to be the better strain with maximum liberation of phosphorous along with maximum reduction in pH as 5.13. *Sinorhizobium* strains performed better than that of *Rhizobium* and *Agrobacterium* in solubilization of phosphorous.

Keywords: *Vigna trilobata*, tricalcium phosphate, carbon, nitrogen

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1. Introduction

Phosphorus is one of the essential major macronutrient for plant growth, which has no source in atmosphere as in case of nitrogen [1]. Most of the soils contain phosphorus, mostly in the unavailable form to the plants. Indian soils contain phosphorus mostly in the form of phosphate rock deposits. This contributes cheapest source of phosphorus fertilizer for crop production [2]. Large amount of phosphorous applied to the soil as fertilizer enters in to immobile form through precipitation with metal ions like Al³⁺ and Fe³⁺ in acidic soils and Ca²⁺ in alkaline or normal soils [1]. Phosphate solubilizing bacteria release phosphate from these immobile insoluble forms. Several bacteria and fungi were reported to have the ability to solubilize insoluble phosphorus in soils [2]. Bacteria are more effective phosphate solubilizers than fungi [3]. Phosphate solubilizing bacteria not only includes free living forms but also the symbiotic bacteria like *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium* [2,4]. Symbiotic nitrogen fixing bacteria [5] are advantageous than free living soil microbes in phosphorous solubilization as these bacteria are protected inside the nodule and face little

competition with indigenous rhizosphere microflora. Rhizobia are reported to have high phosphate solubilizing potential in solubilizing both organic and inorganic phosphates and are preferred by virtue of their dual role in nitrogen fixation and P solubilization [6].

Vigna trilobata, commonly called as 'pillipesara', was mainly cultivated as short term pasture and green manure crop in India, Pakistan and Indonesia. So far, the symbiont in the root nodules was reported as rhizobial strain but not characterized completely. The present study was aimed at screening of rhizobial strains from root nodules of *V. trilobata* growing in different areas of Andhra Pradesh, for their potential for phosphate solubilization and selection of an effective strain with high P solubilizing ability. The strains with high phosphate solubilizing efficiency will have high nitrogen fixation ability because nodulation and bacterial growth are affected at low phosphorous levels [7]. The effective rhizobial strain with high phosphate solubilizing ability can be more useful as bioinoculant.

2. Materials and Methods

Rhizobial strains were isolated from root nodules of *V. trilobata* plants raised in earthen pots filled with soils

collected from 23 districts of Andhra Pradesh, and maintained properly in the botanical garden of our University. For the isolation, pink colored healthy root nodules were collected by gently uprooting the plants, 21 days after sowing, surface sterilized with 0.1% mercuric chloride and washed several times with sterile distilled water. Bacterial suspension was prepared by crushing these nodules with sterile glass rod using sterile distilled water. A loopful of suspension was spread on media plates containing selective medium Yeast mannitol agar (YMA) with congo red and incubated at room temperature for three days. After incubation, the white, translucent, convex colonies with high mucilage were isolated and pure cultures were maintained after sub culturing on the same medium. Pure cultures were authenticated as rhizobia by performing the biochemical tests [8] viz., acid production, catelase production, growth on Hofer's alkaline medium, growth on glucose peptone agar medium, ketolactose agar medium. Those strains which are positive to acid production, catelase production and are negative to ketolactose test and growth on glucose peptone agar and on Hofer's alkaline medium were finally tested for nodulation ability on homologous hosts by plant infection tests [9].

The ability of rhizobial strains to solubilize tricalcium phosphate was tested on Pikovskaya's medium [10] containing 0.5% of Tri Calcium Phosphate as insoluble phosphate source. The halo zone formed surrounding the colony revealed phosphate solubilization and was expressed as solubilizing efficiency (SE%) [11].

Solubilization Efficiency (SE%)

$$= \frac{\left(\begin{array}{l} \text{Diameter of solubilization zone} \\ - \text{diameter of the colony} \end{array} \right)}{\text{Diameter of the colony}} \times 100$$

The strains which showed zone of solubilization on pikovskaya's agar plates were further tested for P₂O₅ liberation in liquid medium. For P₂O₅ liberation test, the strains were inoculated (1ml) in to conical flasks containing 100 ml of pikovskaya's broth having initial pH of 7.0. After inoculation, the flasks were incubated at room temperature (28°C) on a rotary shaker (200 rpm) for 7 days. After incubation, 10ml aliquot of each culture was taken and made cell free by centrifugation at 3000 x g for 15 minutes. The final pH of the supernatant was measured and the P₂O₅ liberated was estimated by the method given in [12].

Out of the 23, the six strains which gave positive results on phosphate solubilization were further identified up to species level through 16S rDNA sequencing (Macrogen, South Korea) and sequences were deposited in the gene bank. The strain names with accession numbers are *Sinorhizobium* sp. strain MRR101 – KC428651, *Agrobacterium tumefaciens* strain MRR102- KC428652, *Rhizobium* sp. strain 103 – JX576499; *Sinorhizobium kostiense* strain MRR104- KC428653; *Agrobacterium tumefaciens* strain MRR105- KC428654 and *Rhizobium* sp. strain MRR106- KC428655. These are the first reports of rhizobial strains from *Vigna trilobata*.

Effect of carbon sources on TCP solubilization was studied by using 5 different carbon sources- glucose, mannitol, maltose, sucrose and arabinose. The carbon sources (1%) were sterilized separately and were added

aseptically to the standard Pikovskaya's medium before inoculation. The carbon source of the standard Pikovskaya's medium, glucose, was considered as control and other carbon were added by replacing glucose in the standard Pikovskaya's medium. All the strains were inoculated separately in to the flasks containing media with different carbon sources (1%). After incubation for 72 hours at room temperature on a rotary shaker (200 rpm), the final pH of the supernatant was measured after centrifugation and the P₂O₅ liberated was estimated. Three replicates were maintained for every treatment.

To study the effect of different concentrations of glucose on TCP solubilization, glucose at 5 different concentrations (1, 1.5, 2, 2.5, 3%) were added to the standard Pikovskaya's medium and incubated at room temperature on rotary shaker at 200 rpm after inoculation with all the six strains separately in to each of the conical flasks containing different concentrations of glucose. The glucose concentration of the standard Pikovskaya's medium (1%) was taken as control. Three replicates were maintained for every treatment. After incubation for 72 hours, the final pH of the supernatant was measured after centrifugation and the P₂O₅ liberated was estimated.

To study the effect of different nitrogen sources on TCP solubilization, different inorganic and organic nitrogen sources were added (0.1%) to the standard Pikovskaya's medium by replacing ammonium sulphate in the standard Pikovskaya's medium. Ammonium sulphate of the standard Pikovskaya's medium was considered as control. All the strains were inoculated separately in to the flasks containing media with different nitrogen sources. Three replicates were maintained for every treatment. After incubation for 72 hours at room temperature on a rotary shaker (200 rpm), the final pH of the supernatant was measured after centrifugation and the P₂O₅ liberated was estimated.

Statistical analyses were performed using SPSS software (version 20). Duncan's test was used for multiple range analyses to determine the significant difference between groups of data. The results were considered to be significant at $P < 0.05$. Correlation coefficient was calculated for the data wherever necessary.

3. Results and Discussion

Out of the 23 strains of rhizobia tested, 2 strains each of *Sinorhizobium* sp., *Rhizobium* sp. and *Agrobacterium tumefaciens* produced clear zone of solubilization surrounding the colonies after 3 days of incubation on Pikovskaya's medium. That all the strains of *Rhizobium* could not solubilize phosphate was reported earlier by [6,13,14] indicating that phosphate solubilization is not a wide spread character among rhizobia. The zone of solubilization increased up to 7 days of incubation and decreased thereafter in all the strains tested. Whereas the size of the colony increased up to 3 days of incubation and with no considerable change thereafter, up to 7 days of incubation. Though zone of solubilization showed progressive increase with increase in incubation period, the colony did not show any proportionate increase in growth. The data on colony diameter, zone of solubilization, solubilization efficiency, P₂O₅ liberated along with final pH of the medium for all the 6 strains was given in Table 1.

Table 1. Solubilization of tri calcium phosphate by rhizobial strains isolated from *Vigna trilobata*

Rhizobial strains	Colony diameter (mm)	Zone of solubilization (mm)	Solubilization Efficiency (SE) %	Final pH of the medium	P ₂ O ₅ liberated (µg/ml)
<i>Sinorhizobium</i> sp. MRR101	8	14	75	6.35	250
<i>Agrobacterium tumefaciens</i> MRR102	8	12	50	6.50	253
<i>Rhizobium</i> sp. 103	10	14	40	6.42	250
<i>Sinorhizobium kostiense</i> MRR 104	8	18	125	5.13	510
<i>Agrobacterium tumefaciens</i> MRR 105	7	12	71	6.23	250
<i>Rhizobium</i> sp MRR 106	8	13	62	5.93	410

Correlation coefficient between final pH and P₂O₅ liberated (r = - 0.95) ; between zone of solubilization and P₂O₅ liberated (r = 0.75).

Highest solubilization of phosphorous with zone of 18 mm diameter was recorded in the strain MRR 104 followed by strain MRR 101 and 103 with 14 mm and strains MRR102 and MRR105 with 12 mm. The least zone of solubilization with 8 mm diameter was recorded in the strain MRR 106. Maximum solubilization efficiency of 125% was recorded with the strain MRR104 while it is between 40 – 75% in rest of the strains. In broth medium, strain MRR 104 produced P₂O₅ of 510 µg/ml with the proportionate decrease in the pH to 5.13, followed by strain MRR106 with the P₂O₅ liberation of 410 µg/ml and decrease in pH to 5.93. Statistical analysis also reveals that there is negative correlation between pH of the medium in broth and the P₂O₅ liberated. There is a positive correlation between zone of solubilization on Pikovskaya's agar medium and the P₂O₅ liberated in broth.

In the present study, strains of *Sinorhizobium* species were proved to be better than *Rhizobium* sp. in phosphate solubilization. However, rhizobial strains were reported to be better than *Sinorhizobium* and *Mesorhizobium* in *Sesbania* [6] and *Bradyrhizobium* [14]. This indicates that the rhizobial strains exhibit much variation in phosphate solubilization and is probably related to the host and environmental factors. The strains in the present study were proved to be better with zone of solubilization in the range of 14-18mm diameter in Pikovskaya's medium containing 0.5% TCP, than the *Bradyrhizobium* strains [15] which produces only 9-15 mm diameter zones on AYG medium containing 0.2% TCP. Similarly, the strains in the present study produced soluble phosphorous in the range of 250 - 510 µg / ml while rhizobial strain of *Sesbania sesban* produced only 80.6 µg /ml [14] in AYG-P medium supplemented with 0.2% of TCP. Reduction in pH of the medium during solubilization was commonly observed in

all strains, with maximum reduction up to 5.6 was recorded in strain MRR 104. This decrease in pH is a basic principle in phosphate solubilization and may be related to the production of organic acids [13] and the release of protons[16]. This type of negative correlation between phosphate solubilization and pH by rhizobial strains [14,17] was reported previously.

3.1. Effect of Carbon and Nitrogen Sources on Solubilization of Phosphorus:

Rhizobial strains of *V. trilobata* utilized different carbon and nitrogen sources for the solubilization of phosphorous.

Among the carbon sources tested (Table 2), highest solubilization of phosphorus was recorded when glucose was used as carbon source while sucrose was least preferred by the strains. This clearly shows that glucose (the carbon source in the standard Pikovskaya's medium) supported maximum P₂O₅ liberation by rhizobial strains. This is in conformity with the previous reports [15] in *Bradyrhizobium* sp. from *Cicer arietinum*. Maximum solubilization of phosphorus was observed in strain MRR104 with 510 µg/ml followed by strain MRR106 with 410 µg/ml in glucose containing medium. Among the other carbon sources tested, maltose supported relatively high P₂O₅ liberation in strain MRR104. Previous reports reveal that fructose supported maximum P₂O₅ liberation by *Rhizobium* strain from *V. trilobata*, [17]. In the present study though from the same host none of the strains preferred fructose for maximum solubilization. Similar type of strain difference within the same host species was previously reported [5] in 23 strains of *Rhizobium tropici* and 3 strains of rhizobia from *sesbania sesban* [17].

Table 2. Effect of different carbon sources on solubilization of tricalcium phosphate by rhizobial strains from *V. trilobata*

Carbon Sources (%)	<i>Sinorhizobium</i> sp. MRR101		<i>Agrobacterium tumefaciens</i> MRR102		<i>Rhizobium</i> sp. 103		<i>Sinorhizobium kostiense</i> MRR 104		<i>Agrobacterium tumefaciens</i> MRR 105		<i>Rhizobium</i> sp MRR 106	
	Final pH	P ₂ O ₅ µg/ml	Final pH	P ₂ O ₅ µg/ml	Final pH	P ₂ O ₅ µg/ml	Final pH	P ₂ O ₅ µg/ml	Final pH	P ₂ O ₅ µg/ml	Final pH	P ₂ O ₅ µg/ml
Glucose (control)	6.23	250	6.18	253	6.26	250	5.13	510	6.33	250	5.93	410
Manitol	6.61	200	6.26	250	6.75	120	6.20	250	6.64	140	6.73	120
Maltose	6.73	120	6.71	100	6.68	50	5.89	410	6.73	120	6.83	140
Fructose	6.76	120	6.61	200	6.78	120	6.83	50	6.73	120	6.82	50
Sucrose	6.78	120	6.63	180	6.37	230	6.59	200	6.71	120	6.60	200
Arabinose	6.65	200	6.26	250	6.62	180	6.24	250	6.20	270	6.35	230

The overall model is significant as given by the F-value of 5.559 with p<0.05. The adjusted R-square value is 0.566. The F-value for carbon source, rhizobial strain and interaction are all significant with p<0.05.

Phosphate solubilization was observed in all the strains studied irrespective of the carbon sources used however, with slight difference in the quantity. The effective strain, MRR104, showed 10 fold increase in P₂O₅ liberation than the strains 103 and MRR106. The strain MRR104 liberated an amount of 510 µg/ml of P₂O₅ in glucose medium while strains 103 and MRR106 releases only 50 µg/ml of P₂O₅ in maltose and fructose medium respectively. This 10 fold increase was observed even in the single stain MRR104 when different carbon sources are used, with P₂O₅ liberated as 510 µg/ml and 50 µg/ml in glucose and fructose containing media respectively.

The decrease in pH with increase in phosphate solubilization was observed in the present study, irrespective of the carbon sources tested. This type of negative correlation between pH and phosphate solubilization was earlier reported [18] with respect to rhizobia.

Statistical analysis reveals that there is almost perfect negative correlation (r = - 0.99) between pH and P₂O₅ liberated of strain MRR105 while the remaining strains showed very high to high (r = - 0.81 to - 0.96) negative correlation. Maximum decrease in final pH of the medium was observed when glucose was used as carbon source for all the strains studied. In rest of the carbon sources the decrease was minimal from the initial pH of 7.0. Early reports [18] revealed that the relative phosphate

solubilization efficacy of rhizobial strains on different carbon sources could be due to the organic acid secreted by the strain rather than the total acidity. However, a positive correlation between acid production and phosphorous solubilization by rhizobia was also reported [6] by earlier workers.

The solubilization efficacy increased with increase in concentration of glucose up to 3% in all the strains studied (Table 3). However it was reported at [15] 2% of glucose in *Bradyrhizobium* strain 27A15 isolated from *Cicer arietinum*. In the present study, initially at 1% glucose, all the strains except MRR104 and MRR106, the amount of P₂O₅ liberated was almost same, with increase in concentration of glucose, the P₂O₅ liberation also showed gradual increase in all these strains. After MRR 104, the second highest P₂O₅ liberation was recorded in strain MRR106, indicating that these two strains are better than the rest of the strains in P₂O₅ liberation from tricalcium phosphate. This strain difference was further supported by the observation that decrease in pH of the medium containing 1% glucose was 5.13 and 5.93 in strains MRR104 and MRR106 respectively while it is in the range of 6.11- 6.33 in rest of the strains. That the strains with maximum reduction in pH of the medium can have the greater ability to solubilize phosphates [7] was proved with these two strains which showed highest reduction in pH and consequently maximum P₂O₅ liberation.

Table 3. Effect of different glucose concentrations on solubilization of tricalcium phosphate by rhizobial strains from *V. trilobata*

Glucose Concentration (%)	<i>Sinorhizobium</i> sp. MRR101		<i>Agrobacterium tumefaciens</i> MRR102		<i>Rhizobium</i> sp. 103		<i>Sinorhizobium kostiense</i> MRR 104		<i>Agrobacterium tumefaciens</i> MRR 105		<i>Rhizobium</i> sp MRR 106	
	pH	P ₂ O ₅ µg/ml	pH	P ₂ O ₅ µg/ml	pH	P ₂ O ₅ µg/ml	pH	P ₂ O ₅ µg/ml	pH	P ₂ O ₅ µg/ml	pH	P ₂ O ₅ µg/ml
1.0	6.23	250	6.18	253	6.26	250	5.13	510	6.33	250	5.93	410
1.5	5.85	480	5.30	510	5.76	440	4.74	600	5.78	410	5.71	410
2.0	5.24	510	5.07	570	5.31	500	4.65	670	5.75	450	5.59	420
2.5	5.19	510	4.71	600	5.22	510	4.57	700	5.18	500	5.82	470
3.0	4.53	600	4.58	600	4.59	630	4.24	800	4.58	670	5.14	570

• The overall model is significant as given by the F-value of 19.874 with p<0.05. The adjusted R-square value is 0.854. The F-value for glucose conc., rhizobial strain and interaction are all significant with p<0.05.

Statistical analysis reveals that there is almost perfect positive correlation (r = 0.98) between glucose conc. and P₂O₅ liberated of rhizobial strain MRR104. The remaining

strains showed very high to high positive (r = 0.84 to 0.98) correlation between glucose concentration and P₂O₅ liberated.

Table 4. Effect of different nitrogen sources on solubilization of tricalcium phosphate by rhizobial strains from *V. trilobata*.

Nitrogen Sources (0.1%)	<i>Sinorhizobium</i> sp. MRR101		<i>Agrobacterium tumefaciens</i> MRR102		<i>Rhizobium</i> sp. 103		<i>Sinorhizobium kostiense</i> MRR 104		<i>Agrobacterium tumefaciens</i> MRR 105		<i>Rhizobium</i> sp MRR 106	
	Final pH	P ₂ O ₅ µg/ml	Final pH	P ₂ O ₅ µg/ml	Final pH	P ₂ O ₅ µg/ml	Final pH	P ₂ O ₅ µg/ml	Final pH	P ₂ O ₅ µg/ml	Final pH	P ₂ O ₅ µg/ml
Ammonium sulphate (Control)	6.23	250	6.18	253	6.26	250	5.13	510	6.33	250	5.77	410
Potassium nitrate	6.13	280	6.03	300	6.18	280	5.49	350	6.23	280	5.93	200
Ammonium nitrate	5.49	440	6.25	250	5.52	350	5.61	340	5.47	360	5.98	320
Sodium nitrate	5.75	350	5.42	360	5.26	480	6.28	280	6.61	200	6.62	200
Asparagine	6.43	220	6.67	200	6.70	140	6.83	50	6.77	120	6.67	140

• When each strain was statistically analyzed separately, there is almost perfect negative correlation (r = -0.98) between pH of the final medium and P₂O₅ liberated was observed in strain MRR102. The remaining strains showed very high to high negative correlation (r = - 0.59 to - 0.98) between pH and P₂O₅ liberated.

The rhizobium strains utilized different nitrogen sources and solubilized the phosphate. Among the nitrogen sources tested (Table 4), ammonium sulphate supported maximum phosphorous solubilization (510 and

410 µg/ml) by the strains MRR104 and MRR 106 with concomitant decrease in pH. While in strains MRR101 and MRR105 maximum solubilization (440 and 360 µg/ml) was recorded when ammonium nitrate was used

and strains MRR102 and 103 preferred sodium nitrate for maximum (360 and 480 µg/ml) phosphorous solubilization. Reduction in pH was commonly observed in all the strains when different nitrogen sources are used. This clearly shows that strains of *V. trilobata* adopted two possible mechanisms for solubilization of phosphorus [15] one being, production of inorganic acids by proton exchange mechanism in the presence of NH_4^+ and the other in the absence of ammonium by the production of organic acids. However, potassium nitrate was not preferred by any strain for maximum phosphorous solubilization. This may be due to better growth of the organism and lowering of acid production and low pH when compared to the control as reported for *Aspergillus niger* [19].

In the present study, *Sinorhizobium* strains (MRR101 and MRR104) were proved to be better than that of *Agrobacterium* and *Rhizobium* in production of maximum amount of P_2O_5 . However, an earlier report [7] reveals that *Rhizobium* strains were found to be more effective than *Bradyrhizobium*. From the present study, it is evident that the variation in solubilization was observed between strains of different species and also within the same species. This strain difference can be attributed to the fact that different types of acids are secreted by different strains [19] and it may be an adaptation by the strains, to the soil environment from which they were isolated.

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