Phosphate solubilization by *Rhizobium* strains

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Received: 23 March 2007 / Accepted: 24 December 2007

Abstract Forty-six *Rhizobium* isolates from legume root and stem nodules were examined for their phosphate-solubilizing ability on Pikovskaya's agar medium. *Rhizobium* isolates from root nodules of *Cassia absus*, *Vigna trilobata* and three strains from *Sesbania sesban* showed zone of tricalcium phosphate (TCP) solubilization. The isolate from *C. absus* showed maximum solubilization (620 µg/ml) after 12 d of incubation, while the *Rhizobium* sp. strain 26 (from *S. sesban*) showed the least amount (150 µg/ml) of phosphate solubilization. Among the carbon sources tested for their ability to solubilize TCP, maximum solubilization (620 µg/ml) was observed in glucose by *Rhizobium* isolate from *C. absus*. Phosphate solubilization increased with increase in glucose concentration steeply up to 2% and slowly above this concentration in four isolates. Among the nitrogen sources tested, maximum solubilization (620 µg/ml) was observed in ammonium sulphate by *Rhizobium* isolate from *C. absus*.

Keywords Phosphate solubilization · *Rhizobium* · Tricalcium phosphate

Introduction

Availability of phosphate in soil is greatly enhanced through microbial production of metabolites leading to lowering of pH and release of phosphate from organic and inorganic complexes [1]. Phosphorus (P) deficiency in soil can severely limit plant growth productivity, particularly in legumes, where both the plants and their symbiotic bacteria are affected, and this may have a deleterious effect on nodule formation, development and function [2].

Besides, symbiotic nitrogen fixation, a few strains or species of *Rhizobium* are involved in phosphate solubilization also [3]. But studies on phosphate solubilizing ability of *Rhizobium* strains are very limited [4–8]. The main advantage of using rhizobia as a phosphate-solubilizing microorganism will be their beneficial nutritional effect resulting both from phosphate mobilization and nitrogen fixation [9]. In the present study, 46 rhizobia, isolated from root and stem nodules of 20 different legume hosts, were tested for their ability to solubilize tricalcium phosphate (TCP).

Of the 46 isolates, 26 are from *Sesbania sesban* and the remaining 20 isolates include one each from *Abrus precatorius*, *Arachis hypogaea*, *Cajanus cajan*, *Cassia absus*, *Clitoria ternatea*, *Dolichos lablab*, *Indigofera hirsuta*, *I. linnaei*, *I. trita*, *Leucaena leucocephala*, *Macrotyloma uniflorum*, *Pisum sativum*, *Rhynchosia minima*, *Sesbania grandiflora*, *S. procumbens* (root), *S. procumbens* (stem), *Trigonella foenum-graecum*, *Vigna mungo*, *V. radiata* and *V. trilobata*. The selective medium yeast extract mannitol agar (YEMA) with congo red was used for isolation of rhizobia [10] and a pure culture of each isolate was prepared after subculturing on the same medium. Pure cultures were authenticated as rhizobia through biochemical tests [11] and nodulating ability was tested on homologous hosts by plant

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infection tests [10]. A representative isolate from *S. sesban* was identified as *Rhizobium radiobacter* MTCC 8917 (= *Agrobacterium radiobacter*). Since *Agrobacterium* and *Rhizobium* are still treated as separate genera in Bergey's Manual of Systematic Bacteriology [12], we used the term *Rhizobium* sp. with strain numbers as 1–26. The other 20 root nodule isolates were designated by the common term rhizobia with host name in parenthesis. The phosphate-solubilizing ability of the isolates was tested on Pikovskaya's agar medium [13] containing TCP as insoluble phosphate source. The solubilization efficiency (SE) on agar medium was expressed in terms of SE (%) [14]. The isolates, which showed zone of solubilization on agar medium was further tested in flasks containing 100 ml of Pikovskaya's broth having initial pH 7.0. The broth was inoculated with 1 ml of the inoculum and incubated at $28 \pm 2^\circ\text{C}$ on rotary shaker (200 rpm) for 12 d. 10 ml aliquots of each culture were aseptically taken from each flask after 3 d intervals up to 12 d, and the supernatant was separated from bacterial cells by centrifugation (3000 g). The final pH of the supernatant was measured and the liberated P_2O_5 was estimated [15].

Among the 46 *Rhizobium* isolates, only 5 isolates, one each from *C. absus* and *V. trilobata* and three strains from *S. sesban* (*Rhizobium* sp. strain 17, 19 and 26) produced clear zone around the colonies after 3 d of incubation, and

it gradually increased up to 9 d, while the colony diameter is almost similar throughout the incubation period. Solubilization efficiency (SE) of *Rhizobium* isolates on solid media ranged between 33% and 150% (Table 1). Maximum efficiency was observed in *Rhizobium* isolate from *C. absus* (150%) followed by *S. sesban Rhizobium* sp. strain 17 (125%), *Rhizobium* sp. strain 19 (120%), *Rhizobium* sp. strain 26 (100%) and *Rhizobium* isolate from *V. trilobata* (60%). In liquid medium maximum solubilization was recorded with *Rhizobium* isolate from *C. absus* (620 $\mu\text{g/ml}$) followed by *Rhizobium* sp. strain 19 (391 $\mu\text{g/ml}$), and least in *Rhizobium* sp. strain 26 (156 $\mu\text{g/ml}$) from *S. sesban*. It was also observed that there is no correlation between phosphate solubilization efficiency on solid and liquid medium as also noticed earlier [14]. A fall in pH accompanied phosphate solubilization, due to production of organic acids was observed up to 9 d, but there was sudden increase in pH after 9 d. This could be due to utilization of organic acids by the strains as was reported in *Pseudomonas* [16]. The data were statistically analyzed using correlation coefficient and it was found that there is positive correlation between zone of solubilization on Pikovskaya's agar medium and liberated P_2O_5 in broth, and negative correlation between liberated P_2O_5 and final pH of the medium in broth.

Table 1 Solubilization of tricalcium phosphate by *Rhizobium* isolates

<i>Rhizobium</i> isolates	Incubation time (d)	Colony diameter on Pikovskaya's medium (mm)	Colony diameter of on YEMA (mm)	Diameter of zone of solubilization (mm)*	Solubilization efficiency (%)	Final pH of the medium*	P_2O_5 -liberated ($\mu\text{g/ml}$)*
<i>Rhizobium</i> isolate (<i>Cassia absus</i>)	3	4.0	3.5	6.2	55	6.02	600
	6	4.0	3.9	9.0	125	5.82	612
	9	4.0	4.2	10.0	150	5.02	620
	12	4.0	4.2	10.0	150	5.22	620
<i>Rhizobium</i> isolate (<i>Vigna trilobata</i>)	3	3.0	3.2	4.0	33	5.62	342
	6	3.0	3.5	4.5	50	5.60	344
	9	3.0	3.9	4.8	60	4.05	352
	12	3.0	3.9	4.8	60	5.44	352
<i>Rhizobium</i> sp. strain 17 (<i>Sesbania sesban</i>)	3	2.0	4.2	4.0	100	6.99	324
	6	2.0	4.5	4.2	110	6.93	329
	9	2.0	4.5	4.5	125	4.98	381
	12	2.0	4.5	4.5	125	5.09	380
<i>Rhizobium</i> sp. strain 19 (<i>S. sesban</i>)	3	2.0	3.9	4.2	110	6.84	363
	6	2.0	3.9	4.2	110	6.62	369
	9	2.0	4.1	4.4	120	5.25	390
	12	2.0	4.2	4.4	120	6.34	391
<i>Rhizobium</i> sp. strain 26 (<i>S. sesban</i>)	3	2.5	3.5	4.0	60	6.02	93
	6	2.5	3.8	4.2	68	6.02	99
	9	2.5	3.9	4.2	68	6.00	155
	12	2.5	4.1	4.2	68	6.00	156

Solubilization efficiency (SE) % = $Z - C / C \times 100$, where Z = diameter of solubilization zone, C = colony diameter

*Between diameter of zone of solubilization and P_2O_5 liberated ($r = 0.84$); between final pH and P_2O_5 liberated ($r = -0.47$).

Effect of different carbon sources (1%) on phosphate-solubilizing activity was studied by replacing the glucose with 14 carbon compounds sterilized separately and added aseptically to the flasks containing 100 ml of the Pikovskaya's medium before inoculation. The medium was incubated on a rotary shaker (200 rpm) at room temperature ($28 \pm 2^\circ\text{C}$) for 72 h. Among the 14 carbon sources tested, glucose supported maximum TCP solubilization in *Rhizobium* isolate from *C. absus* (620 $\mu\text{g/ml}$), followed by *Rhizobium* sp. strain 19 (390 $\mu\text{g/ml}$), *Rhizobium* sp. strain 17 (381 $\mu\text{g/ml}$) and *Rhizobium* sp. strain 26 (155 $\mu\text{g/ml}$) (Table 2). While the isolate from *V. trilobata* showed maximum solubilization (466 $\mu\text{g/ml}$) in fructose. That glucose was the best carbon source for phosphate solubilization was also reported earlier in *Bradyrhizobium* species isolated from *Cicer arietinum* [5]. The 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 quantity of P_2O_5 liberated were observed. These results indicated that mannitol was not a good carbon source for phosphate solubilization studies in rhizobia, and therefore all tests were performed with Pikovskaya's medium containing glucose as carbon source. The relative efficiency of *Rhizobium* isolates on different carbon sources could be due to the organic acids secreted by the isolates rather than the total acidity [16]. The data on effect of carbon sources on TCP solubilization by *Rhizobium* strains were statistically analyzed using analysis of variance (two-way classification technique) and it was found

that variations due to both carbon sources and *Rhizobium* strains were found to be significant.

As the glucose at 1% concentration (as in Pikovskaya's medium) supported maximum solubilization of TCP, effect of different concentrations of glucose (1, 1.5, 2, 2.5, 3 %) was studied. Phosphate solubilization increased with increase in glucose concentration in *Rhizobium* isolates from *C. absus*, *V. trilobata* and *Rhizobium* sp. strain 17 and 19 from *S. sesban* (Table 3). It was also observed that increase in glucose concentration from 2% to 3% did not show much change in TCP solubilization. In the case of *Rhizobium* sp. strain 26, maximum solubilization was observed at 1.5% glucose concentration and above this concentration TCP solubilization decreased. This could be due to the auto-consumption of soluble phosphate by the growing bacterial population as reported in *Azospirillum brasilense* [17]. The variation in the effect of different concentrations of glucose is found to be statistically significant.

Effect of different nitrogen sources (0.1 %) on TCP solubilization was also studied by replacing ammonium sulphate with five different nitrogen sources in flasks containing 100 ml of the Pikovskaya's medium and incubated for 72 h at room temperature ($28 \pm 2^\circ\text{C}$) in a rotary shaker (200 rpm). Among the nitrogen source tested, ammonium sulphate reduced the pH of the medium and supported maximum solubilization of TCP (Table 4). However, nitrates were also effective. If the production of inorganic acids by proton exchange mechanism in the presence of NH_4^+ causes accelerated phosphate solubilization, the second mechanism operative

Table 2 Effect of different carbon sources on solubilization of tricalcium phosphate by *Rhizobium* isolates

Carbon source (1 %)*	<i>Rhizobium</i> isolates*									
	<i>Rhizobium</i> isolate (<i>C. absus</i>)		<i>Rhizobium</i> isolate (<i>V. trilobata</i>)		<i>Rhizobium</i> sp. strain 17 (<i>S. sesban</i>)		<i>Rhizobium</i> sp. strain 19 (<i>S. sesban</i>)		<i>Rhizobium</i> sp. strain 26 (<i>S. sesban</i>)	
	Final pH	P_2O_5 ($\mu\text{g/ml}$)	Final pH	P_2O_5 ($\mu\text{g/ml}$)	Final pH	P_2O_5 ($\mu\text{g/ml}$)	Final pH	P_2O_5 ($\mu\text{g/ml}$)	Final pH	P_2O_5 ($\mu\text{g/ml}$)
Glucose (control)	5.02	620	4.05	352	4.98	381	5.25	390	5.02	155
Mannitol	6.24	320	6.03	37	6.09	56	6.02	44	5.09	30
Galactose	6.02	92	6.09	74	6.06	184	6.06	170	6.09	124
Fructose	4.82	152	6.80	466	6.68	102	6.90	98	5.08	76
Rhamnose	6.01	360	6.03	351	6.44	99	6.55	101	6.91	30
Arabinose	6.55	176	5.09	303	5.20	106	5.22	103	5.45	45
Trehalose	4.81	166	5.46	86	5.99	111	6.02	104	6.00	98
Inositol	6.22	160	6.20	23	6.69	29	6.55	35	6.19	49
Raffinose	6.54	400	6.62	26	6.80	76	6.89	80	6.72	74
Sucrose	6.09	100	5.40	43	6.66	72	6.46	69	6.50	72
Maltose	5.82	76	5.96	66	5.09	43	5.10	49	5.12	55
Mannose	5.09	200	6.01	81	5.21	23	6.02	29	6.09	20
Dulcitol	6.09	22	6.54	29	5.99	31	6.39	34	6.04	30
Ribose	6.48	72	5.03	49	6.66	46	5.89	54	5.99	53
Xylose	6.89	120	5.09	122	6.91	11	6.84	20	6.89	49

*Significant at 1% (F-calculated = 6.419, F-tabulated = 3.674)

Table 3 Effect of different concentrations of glucose on TCP solubilization by *Rhizobium* isolates

Glucose concentration* (%)	<i>Rhizobium</i> isolates*									
	<i>Rhizobium</i> isolate (<i>C. absus</i>)		<i>Rhizobium</i> isolate (<i>V. trilobata</i>)		<i>Rhizobium</i> sp. strain 17 (<i>S. sesban</i>)		<i>Rhizobium</i> sp. strain 19 (<i>S. sesban</i>)		<i>Rhizobium</i> sp. strain 26 (<i>S. sesban</i>)	
	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)
1.0 (Control)	6.0	280	6.10	210	5.98	210	5.99	200	6.01	214
1.5	6.0	288	6.00	243	5.99	216	6.00	210	6.01	224
2.0	5.01	620	4.05	352	4.98	381	5.25	390	5.01	150
2.5	5.09	622	4.09	356	4.99	389	5.05	394	5.00	154
3.0	4.98	628	4.04	358	4.90	392	5.09	396	5.04	155

*Significant at 1% (F-calculated = 9.414, F-tabulated = 4.772)

Table 4 Effect of different nitrogen sources on solubilization of tricalcium phosphate by *Rhizobium* isolates

Nitrogen source* (0.1 %)	<i>Rhizobium</i> isolates*									
	<i>Rhizobium</i> isolate (<i>C. absus</i>)		<i>Rhizobium</i> isolate (<i>V. trilobata</i>)		<i>Rhizobium</i> sp. strain 17 (<i>S. sesban</i>)		<i>Rhizobium</i> sp. strain 19 (<i>S. sesban</i>)		<i>Rhizobium</i> sp. strain 26 (<i>S. sesban</i>)	
	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)	5.02	620	4.05	352	4.98	381	5.25	390	5.02	155
Sodium nitrate	5.92	410	4.98	196	5.01	160	5.93	186	5.91	120
Potassium nitrate	5.65	369	5.95	193	5.95	136	6.08	146	6.91	104
Calcium nitrate	5.94	364	5.65	184	5.05	124	6.09	129	6.24	96
Asparagine	5.74	84	5.94	81	6.09	73	6.30	44	6.42	39
Urea	5.94	43	5.25	36	6.81	20	6.25	14	6.90	12

*Significant at 1% (F-calculated = 10.265, F-tabulated = 2.866)

in the absence of NH_4^+ also affects phosphate solubilization efficiently [5]. Of all the isolates, *Rhizobium* isolate from *C. absus* showed maximum solubilization (620 µg/ml) in ammonium sulphate containing medium, while least solubilization (12 µg/ml) was observed in urea by *Rhizobium* sp. strain 26. Further it was observed that 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 the presence of NH_4^+ cause accelerated phosphate solubilization [5]. Statistical analysis showed that the effect of different nitrogen sources on TCP solubilization was also significant. From this study it is clear that phosphate solubilization is not a widespread character among rhizobia, and not all the *Rhizobium* strains, even from a single host (*S. sesban*), exhibit phosphate solubilization. Hence, it is necessary to study the phosphate-solubilizing activity of a large number of *Rhizobium* strains from a single host. Furthermore, the *Rhizobium* strains showing phosphate solubilization could be used for understanding the mechanism of phosphate acquisition in host plants from which they were isolated.

Acknowledgements We thank Dr. G. V. S. R. Anjaneyulu, Department of Statistics, A.N.U for statistical analysis.

We greatly acknowledge Andhra Pradesh Council of Science and Technology (APCOST) for Young Scientist Fellowship (YSF) to MS. We sincerely acknowledge the help of Institute of Microbial Technology (IMTECH), Chandigarh for the identification of culture.

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