British Microbiology Research Journal
4(9): 996-1006, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Screening of *Rhizobium* Strains Isolated from the Root Nodules of *Vigna mungo* Cultivated in Rice Fallows for Their Phosphate Solubilizing Ability and Enzymatic Activities

T. Satyanandam[1], K. Babu[1], G. Rosaiah[1*] and M. Vijayalakshmi[1]

[1]Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjunanagar, Guntur- 522 510, Andhra Pradesh, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author TS Performed the work and wrote the protocol along with he drafted first manuscript. Author KB Performed the statistical analysis and drafted the paper according to the journal format. Authors GR and MV managed the analyses of the study, managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

Received 15th February 2014
Accepted 28th April 2014
Published 26th May 2014

ABSTRACT

Aims: The present work is aimed to find out the enzymatic activities and phosphate solubilizing efficiency of indigenous rhizobia confined to rice fallows.
Study Design: In this experiment we maintained random block design (RBD).
Place and Duration of Study: This work was carried out in the Department of Botany and Microbiology, Acharya Nagarjuna University between October 2012 and December 2013.
Methodology: In this study, we have isolated 19 *Rhizobium* strains collected from the healthy root nodules of *Vigna mungo* cultivated in rice fallows on yeast extract mannitol agar (YEMA) medium. The strains were confirmed as Rhizobia by using Gram staining, growth on YEMA with congo red, growth in Hofer's alkaline broth, growth on glucose peptone agar, acid production, ketolactose test and nodulating ability was tested on homologous hosts by plant infection tests. Phosphate solubilization ability of the isolated *Rhizobium* strains were carried out Pikovskaya's agar medium.
Results: Eight out of 19 strains tested for phosphate solubilizing ability on Pikovskaya's agar medium containing tri calcium phosphate (TCP) as insoluble phosphate source showed zone of TCP solubilization. The strain VM-2 exhibited maximum solubilization

**Corresponding author: Email: gorrepati_r@yahoo.co.in;*

after 48h of incubation, while least activity was found with VM-11. Effect of different carbon and nitrogen sources on phosphate solubilizing ability of Rhizobial strains was tested and maximum phosphate solubilization (799µg/ml) by VM-2 was observed when glucose and ammonium sulphate were used as carbon and nitrogen sources.
Conclusion: In this study it is concluded that along with symbiotic nitrogen fixtation, some *Rhizobium* species were found to be involved in phosphate solubilization and this ability of phosphate solubilization by the *Rhizobium* strains can be exploited as PGPR.

Keywords: Rhizobium; biochemical analysis; Phosphate solubilization; tri calcium phosphate.

1. INTRODUCTION

Pulse crops have been an important component of agriculture since ancient times. These leguminous plants on symbiotic association with *Rhizobium* form nitrogen fixing root nodules which are agronomically significant as they provide an alternative to the use of energy expensive nitrogenous chemical fertilizer. The black gram (*Vigna mungo* (L.) Hepper) a major source of protein (24%) is one of the important pulse crops of Andhra Pradesh. It also improves the soil fertility by fixing 38kg N/ha/year in soil from atmosphere [1]. It is mainly cultivated in a cereal-pulse cropping system primarly to conserve soil nutrients and utilize the left over soil moisture particularly after rice cultivation.

For effective utilization of land and human resources, the cultivation of rice fallow land is very important. Rice fallow soils are characterized by flooding during rice growing season. At the end of a flooded rice crop, organic and NH_4-N dominate in the soil with negligible amounts of NO_3. Subsequent drying of the soil favours the transmission of aerobic N and finally NO_3 accumulates in soil during the aerobic phase. Recent evidences indicate that large amounts of accumulated soil NO_3 may be lost from rice fallows upon the flooding of aerobic soil for rice production [2]. Cultivation of legumes in rice fallows can conserve soil NO_3 from potential loss and additionally capture atmospheric N through biological nitrogen fixtation (BNF) [3]. The amount of N derived by legumes through BNF depends on the interaction of microbial, plant and environmental determinants. Legume-*Rhizobium* interactions are unique because they supply 80-90% of total nitrogen requirement of legumes. *Rhizobium* has dual beneficial nutritional effect resulting both from N_2 fixtation and P mobilization [4,5].

Phosphorous is one of the most important macro nutritents that plays an important role in plant metabolism [6]. Several plant growth promoting rhizobacteria (PGPR) and rhizosphere bacteria are capable of increasing availability of phosphorous to plants either by mineralization of organic phosphate or by soluibilization of inorganic phosphate by production of acids [7]. Rhizobia are often considered to be one of the most powerful P-solubilizers and these are the first group of bacteria, which are attributed to the ability of PGPR to solubilize insoluble phosphates [8,9]. Effective indigenous *Rhizobium* strains have recently been used to increase the yield of legumes [10]. Hence the present study was carried out to test the effective indigenous *Rhizobium* strains isolated from the root nodules of *Vigna mungo* cultivated in rice fallow soils for enhancing the yield of black gram.

2. MATERIALS AND METHODS

In the present study, 19 *Rhizobium* strains were isolated from the freshly collected healthy root nodules of *Vigna mungo* cultivated in rice fallows on yeast extract mannitol agar (YEMA)

medium. For the pure cultures the *Rhizobium* strains obtained during isolation was sub cultured on a series of YEMA plates. The pure culture of each isolates was maintained on YEMA slants and incubated at 28±2ºC for 48h. After sufficient growth the slants were preserved in the refrigerator at 4ºC [11]. The identity of the isolates as *Rhizobium* was confirmed by tests including Gram staining, growth on YEMA with congo red, growth in Hofer's alkaline broth, growth on glucose peptone agar, acid production, ketolactose test and nodulating ability was tested on homologous hosts by plant infection tests [12]. All these isolates were screened for their ability to produce some enzymes involved in biochemical reactions by following standard methods [13]. In all the tests, by using a pre sterilized inoculation loop the isolates were inoculated into the media or broth.

2.1 Screening of Rhizobial Strains for Enzymatic Activity

The activity of amylase was studied by growing the isolate on Starch Agar Medium (SAM) plates. After 48 h of incubation at room temperature (28±2ºC), the plates were flooded with iodine solution and observed for zones of starch hydrolysis around the colony [13].

Catalase activity was tested by growing the strains on YEMA medium and incubated for 72h at room temperature. After incubation 3% H2O2 was added over the culture. Appearance of effervescence (bubbles) with in 20s indicates positive catalase activity [13].

Nitrate reductase activity was studied by growing the test isolates into 10ml of the YEM broth containing 1% KNO3 and incubated for 72h at room temperature. After incubation, 0.1ml of test reagent was added to the test tubes. Development of red colour within minutes was considered as positive and absence of colour indicates negative [13].

For the *Rhizobium* isolates, Urease activity was tested by growing each isolate in urea broth (10ml) adjusted to pH 6.0. To the broth bromo thymol blue indicator was added and kept for incubation for 48h. After incubation, change in colour of the medium from green to blue indicates that the isolate was positive for urease activity [13].

Citrase activity was studied by streaking the test isolates across the sodium citrate amended YEMA medium plates (Mannitol in YEMA was replaced by 1% sodium citrate) containing bromo thymol blue indicator. After incubation at room temperature for 48h the change in the colour of the medium from green to blue indicates citrase activity [13].

2.2 Screening of Rhizobial Strains for Phosphate Solubilizing Ability

The phosphate solubilizing ability of the isolates was tested on Pikovskaya's agar medium [14] with TCP as insoluble phosphate source. The solubilization efficiency (SE) on agar medium was expressed interms of SE (%) [15,16]. The isolates which formed zone of solubilization on agar medium were further tested in flasks containing 100ml of Pikovskaya's broth having initial pH 7. One ml of the inoculum was inoculated into the broth and the flasks were incubated on rotary shaker (200rpm) at 28±2ºC for 72h. The supernatant was separated from the bacterial cells by centrifugation of flasks at 3000rpm. Later the final pH of the supernatant was measured and the liberated P_2O_5 was estimated by adding 2.5ml of Barton's reagent to a 10ml aliquot of the clear culture supernatant and the volume was made up to 50 ml. After 10 minutes, the resultant yellow colour was read in a calorimeter at 430 nm [17] and the liberated P_2O_5 was estimated by comparing the values with standard curve prepared with K_2HPO_4.

2.3 Effect of Carbon Sources and Nitrogen Sources on Phosphate Solubilization

Effect of different carbon sources (1%) on phosphate solubilization was tested by replacing the glucose with mannitol, fructose, sucrose, maltose and galactose. 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 phosphate solubilization was tested by replacing the ammonium sulphate with sodium nitrate, potassium nitrate and l-asparagine.

2.4 Statistical Analysis

The data were statistically analyzed by correlation coefficient and ANOVA (two way classification technique), using minitab-14.

3. RESULTS AND DISCUSSION

A total of 19 strains were isolated from the root nodules of *Vigna mungo*. The *Rhizobium* colonies on congo red medium appeared as white, round, transparent, elevated with entire margin. They were Gram-negative rods and did not grow on Hofer's medium and glucose-peptone agar. All the strains were negative for the production of 3-ketolactose from lactose and are finally confirmed as Rhizobia by the nodulation test.

3.1 Screening of Rhizobial Strains for Enzymatic Activity

The ability to produce different enzymes to utilize various organic substrates is an important biochemical characterstic feature of *Rhizobium* strains. Out of the 19 strains tested for enzymatic activities, 18 are positive to catalase, nitrate reductase and urease and one is negative to these three tests. Five strains (VM-1, VM-3, VM-7, VM-11 and VM-17) showed all the enzymatic activities tested (Table 1). Some variations were observed among the isolates for the enzymatic activities like amylase and citrase. Variation in enzymatic activities of Rhizobial strains was also reported by earlier workers [18,19].

Out of 79 Rhizobial strains tested, 58 were positive to nitrate reduction while 21 were negative [20]. In the present study 18 out of 19 strains were positive to nitrate reductase activity. Catalase activity of *Rhizobium* strains isolated from *Vigna mungo* was also reported [21]. Catalase, urease, oxidase, nitrate reduction and citrate utilization activities of *Rhizobium* and *Brady Rhizobium* strains isolated from soybean were reported earlier [22,23]. In the present study, 18 out of 19 were positive to catalase activity. Nitrate reductase activity, catalase activity and ammonia production play an important role in nitrogen fixtation metabolism. During nodule formation, urease, amylase and protease play key role [19]. *Rhizobium* strains which produce these enzymes are considered as best for nodulation and nitrogen fixtation.

3.2 Screening of Rhizobial Strains for Phosphate Solubilizing Ability

Among the 19 strains tested, only eight strains (VM-1, VM-2, VM-3, VM-8, VM-9, VM-11, VM-15 and VM-17) produced clear zone around the colonies after 24 h of incubation on Pikovskaya's agar medium, which gradually increased up to 72h (Fig. 1). The solubilization efficiency of *Rhizobium* strains ranged between 16% and 170% (Table 2). *Rhizobium* strain VM-2 showed maximum solubilization efficiency followed by VM-3, VM-1, VM-11, VM-8, VM-

15, VM-17 and VM-9. *Rhizobium* strain VM-2 recorded maximum solubilization (799µg/ml) in liquid medium. A drop in a pH was accompanied with phosphate solubilization.

Table 1. Enzymatic activities of *Rhizobium* strains isolated from the root nodules of *Vigna mungo*

Name of the strain	Enzymatic activity tested				
	Amylase	Catalase	Nitrate Reductase	Urease	Citrase
VM-1	+	+	+	+	+
VM-2	−	+	+	+	−
VM-3	+	+	+	+	+
VM-4	−	+	+	+	+
VM-5	+	+	+	+	−
VM-6	−	+	+	+	−
VM-7	+	+	+	+	+
VM-8	−	+	+	+	−
VM-9	−	+	+	+	+
VM-10	−	+	+	+	−
VM-11	+	+	+	+	+
VM-12	−	+	+	+	+
VM-13	−	+	+	+	+
VM-14	+	+	+	+	−
VM-15	+	+	+	+	−
VM-16	−	+	+	+	+
VM-17	+	+	+	+	+
VM-18	−	+	+	+	−
VM-19	+	−	−	−	−

(+ = positive), (− = negative)

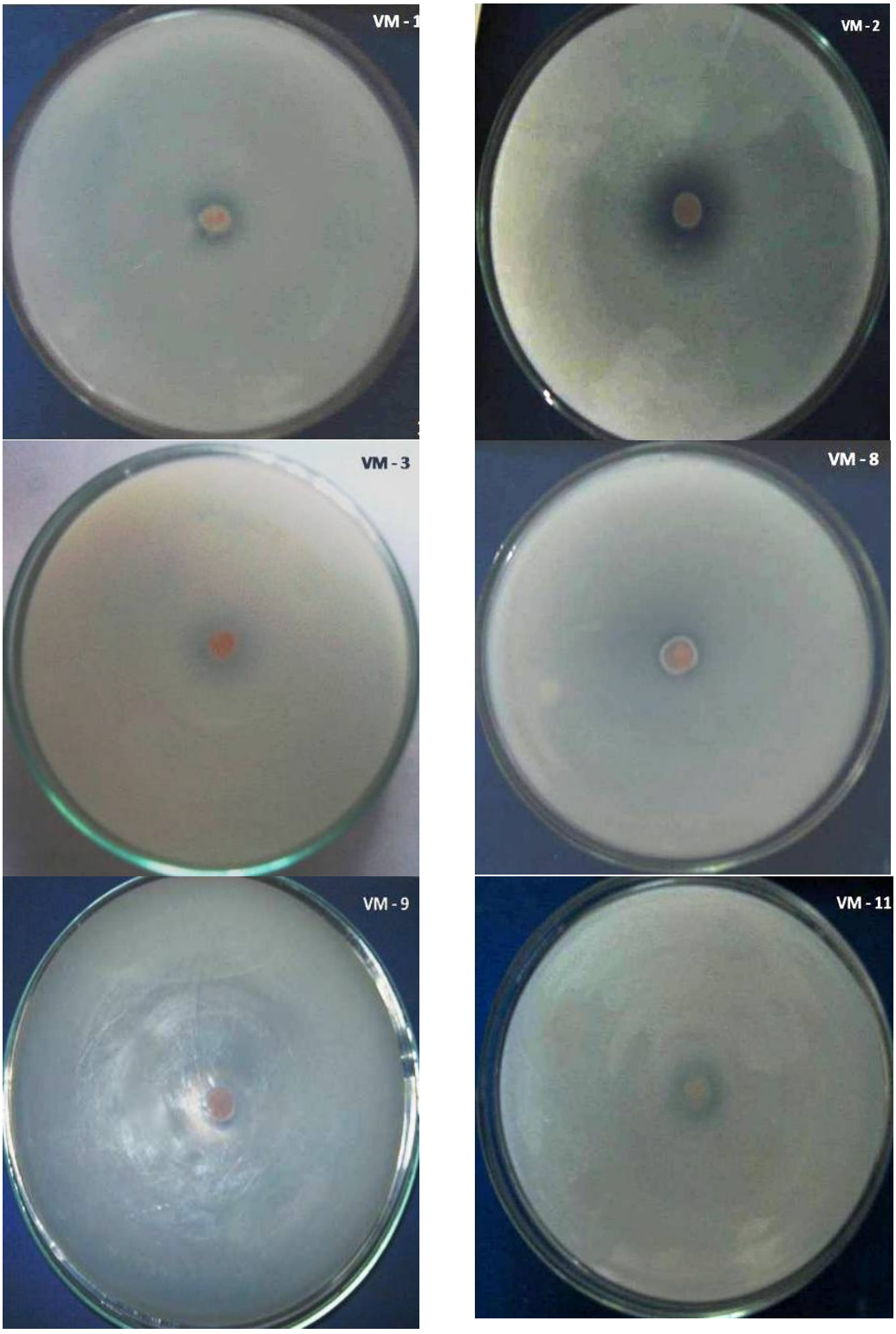
Phosphate solubilizing microorganisms dissolve insoluble phosphates by the production of inorganic or organic acids and/or by the drop of the pH [24,7]. This drop in pH was observed due to production of organic acids up to 48h but after 48h there was sudden increase in pH. This may be due to utilization of organic acids produced during phosphate solubilization by the strains as reported earlier in *Arthrobacter* and *Pseudomonas* [25,26]. The data are statistically analysed using correlation coefficient and it was found that there is a positive correlation between diameter of the zone of solubilization on Pikovskaya's agar medium and liberated P_2O_5 in broth and negative correlation between final pH of medium and liberated P_2O_5.

Effect of different carbon sources (1%) on phosphate solubilization revealed glucose as the best carbon source to support maximum TCP solubilization in *Rhizobium* strain VM-2 (799 µg/ml), followed by VM-3 (666µg/ml), while least solubilization (74µg/ml) was observed in maltose by VM-17 (Table 3). Glucose as the best carbon source for phosphate solubilization was reported earlier for *Bradyrhizobium* from *Cicer arietinum* and *Rhizobium* from *Crotalaria* species [27,28]. The maximum decrease in pH was also found in medium with glucose as compared to media supplemented with other carbon sources. Statistical analysis showed that the effect of different carbon sources on TCP solubilization was significant.

Table 2. Solubilization of tri calcium phosphate by *Rhizobium* strains isolated from *Vigna mungo*

Name of the strain	Incubation time (hrs)	Colony diameter on Pikovskaya's medium (mm)* (C)	Diameter of Zone of solubilization (mm)* (Z)	Phosphate solubilization Efficiency (%)	Final pH of the medium*	P_2O_5 liberated (µg/ml)*
	24	4.0	6.0	50	5.33	416
VM-1	48	4.0	8.0	100	5.21	433
	72	4.0	8.0	100	5.30	433
	24	5.0	13.0	160	4.82	786
VM-2	48	5.0	13.5	170	4.78	799
	72	5.0	13.5	170	4.93	799
	24	4.0	9.0	125	4.96	631
VM-3	48	4.5	9.5	111	5.09	666
	72	4.5	9.5	111	5.19	666
	24	5.5	7.5	36	5.02	366
VM-8	48	6.0	8.0	33	4.94	372
	72	6.0	8.0	33	4.96	372
	24	5.0	5.8	16	5.26	246
VM-9	48	5.0	6.0	20	5.16	261
	72	5.0	6.0	20	5.24	258
	24	4.5	6.5	44	5.17	411
VM-11	48	4.5	7.5	66	5.08	432
	72	4.5	8.5	88	5.11	434
	24	6.0	8.0	33	5.24	337
VM-15	48	6.0	8.0	33	5.18	349
	72	6.0	9.0	50	5.19	353
	24	5.0	6.0	20	5.42	326
VM-17	48	5.0	7.0	40	5.30	346
	72	5.0	8.0	60	5.33	348

*Solubilization efficiency (SE)% =Z-C/C×100, where Z=diameter of solubilization zone, C= colony diameter * Between diameter of zone of solubilization and P_2O_5 liberated (r =0.89) between final pH and P_2O_5 liberated (r= –0.66)*


VM - 1
VM - 2
VM - 3
VM - 8
VM - 9
VM - 11

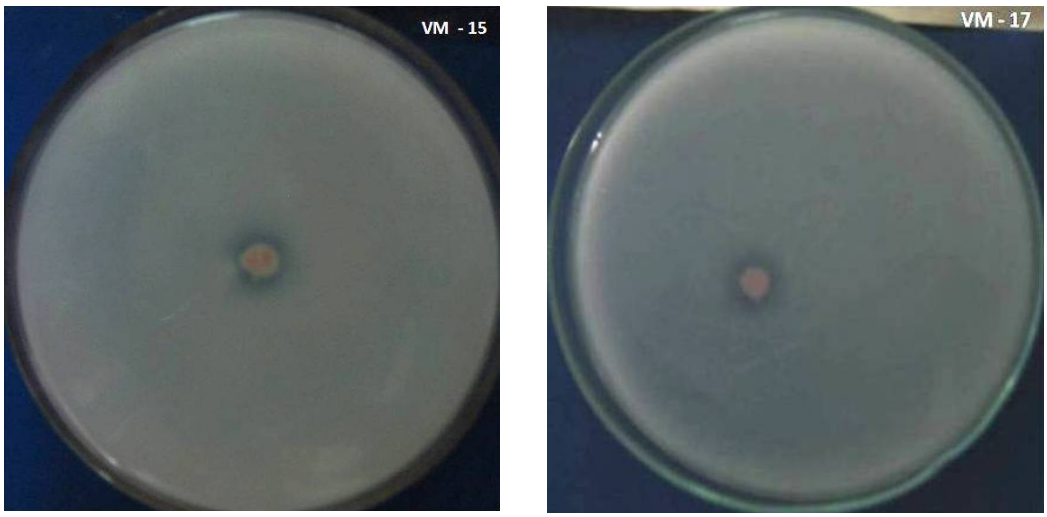


Fig. 1. Phosphte solubilized zones of *Rhizobium* isolates (VM - 1 to VM - 17)

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, 2.0, 2.5, 3.0%) was studied. Phosphate solubilization increased with increase in glucose concentration in *Rhizobium* strains from VM-1, VM-2, VM-3, VM-8 and VM-9 (Table 4).In the case of *Rhizobium* strains VM-11, VM-15and VM-17 maximum solubilization was observed at 2.0% 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* [29]. The variation in the effect of different concentrations of glucose found to be statistically significant.

Effect of different nitrogen sources (0.1%) on phosphate solubilization revealed ammonium sulphate supported maximum solubilization of TCP and maximum decrease in pH than in media with other nitrogen sources (Table 5). Among the eight strains, VM-2 showed maximum solubilization (799µg/ml) inammonium sulphate containing medium, while least solubilization (90µg/ml) was observed in l-aspargine by *Rhizobium* strain VM-15. Further it was observed that the inorganic nitrogen sources supported better solubilization of TCP than organic nitrogen sources. This may be due to the production of inorganic acids by proton exchange mechanism in the presence of NH_4^+ cause accelerated phosphate solubilization [30,27]. Statistical analysis showed that the effect of different nitrogen sources on TCP solubilization was also significant.

Table 3. Effect of different carbon sources on solubilization of tri calcium phosphate by *Rhizobium* strains

*Carbon sources	VM-1		VM-2		VM-3		VM-8		VM-9		VM-11		VM-15		VM-17	
	Final pH	P2O5 liberated (µg/ml)	Final pH	P2O5 liberated (µg/ml)	Final pH	P2O5 liberated (µg/ml)	Final pH	P2O5 liberated (µg/ml)	Final pH	P2O5 liberated (µg/ml)	Final pH	P2O5 liberated (µg/ml)	Final pH	P2O5 liberated (µg/ml)	Final pH	P2O5 Liberated (µg/ml)
Glucose (control)	5.21	433	4.78	799	5.08	666	4.94	372	5.16	261	5.05	432	5.18	359	5.30	348
Mannitol	5.94	326	5.83	423	6.02	315	6.02	261	6.08	267	6.10	148	6.01	310	6.03	226
Fructose	6.25	267	5.93	343	6.12	209	6.25	329	6.14	178	6.03	249	6.23	127	6.13	190
Sucrose	6.08	222	6.13	441	6.24	165	6.11	121	5.83	210	6.08	197	6.22	105	6.13	124
Maltose	5.12	138	5.86	293	5.21	387	5.37	176	5.15	163	5.69	86	5.71	120	5.95	74
Galactose	5.64	172	5.90	316	5.61	348	5.68	284	5.75	124	5.73	111	5.57	151	5.61	111

**significant at 1% (between carbon sources p=0.000)*

Table 4. Effect of different concentrations of glucose on solubilization of tri calcium phosphate by *Rhizobium* strains

Glucose conc. (%)	VM-1		VM-2		VM-3		VM-8		VM-9		VM-11		VM-15		VM-17	
	Final pH	P_2O_5 liberated (µg/ml)	Final pH	P_2O_5 liberated (µg/ml)	Final pH	P_2O_5 liberated (µg/ml)	Final pH	P_2O_5 liberated (µg/ml)	Final pH	P_2O_5 liberated (µg/ml)	Final pH	P_2O_5 liberated (µg/ml)	Final pH	P_2O_5 liberated (µg/ml)	Final pH	P_2O_5 Liberated (µg/ml)
1.0 (control)	5.21	433	4.78	799	5.08	666	4.94	372	5.16	261	5.05	432	5.18	359	5.30	348
1.5	5..11	441	4.67	812	5.0	672	4.86	384	5.13	267	4.93	448	5.01	387	5.03	366
2.0	4.95	453	4.55	824	4.96	684	4.79	390	5.03	278	4.79	454	4.73	398	4.73	390
2.5	4.68	459	4.43	835	4.87	687	4.68	396	4.83	286	5.08	387	4.22	255	4.63	224
3.0	4.15	465	4.31	844	4.76	692	4.59	396	4.89	293	4.91	324	4.56	213	4.84	170

**significant at 1% level (p=0.000)*

Table 5. Effect of different nitrogen sources on solubilization of tri calcium phosphate by *Rhizobium* strains

*Nitrogen sources	VM-1		VM-2		VM-3		VM-8		VM-9		VM-11		VM-15		VM-17	
	Final pH	P_2O_5 liberated (µg/ml)	Final pH	P_2O_5 liberated (µg/ml)	Final pH	P_2O_5 liberated (µg/ml)	Final pH	P_2O_5 liberated (µg/ml)	Final pH	P_2O_5 liberated (µg/ml)	Final pH	P_2O_5 liberated (µg/ml)	Final pH	P_2O_5 liberated (µg/ml)	Final pH	P_2O_5 liberated (µg/ml)
Ammonium sulphate (control)	5.21	433	4.78	799	5.08	666	4.94	372	5.16	261	5.05	432	5.18	359	5.30	348
Sodium nitrate	5.40	248	5.05	532	5.12	355	5.57	244	5.34	203	5.65	321	5.33	171	5.04	113
Potassium nitrate	5.86	321	5.36	544	5.87	248	6.06	221	6.07	202	5.80	210	6.11	299	6.00	167
l-Aspargine	6.14	170	5.71	273	6.03	138	6.01	166	6.08	97	5.90	134	6.04	90	6.27	94

**Significantat 1% (between nitrogen sources p=0.000)*

4. CONCLUSION

It may be concluded that in addition to symbiotic nitrogen fixtation, some species of *Rhizobium* are also involved in phosphate solubilization and this capacity of phosphate solubilization by the *Rhizobium* strains can be exploited as PGPR.

ACKNOWLEDGEMENTS

The authors are highly thankful to the University Grants Commission, New Delhi, India for financial support through a Major research project.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Reddy DKR, Venkateswarlu O, Siva Jyothi GL, Obaiah MC. Genetic parameters and inter-relationship analysis in Black gram [*Vigna mungo*.(L.) Hepper]. Legume Research. 2011;34(2):149-152.
2. Buresh RJ, Woodhead T, Shepherd KD, Flordelis E and Cabangon RC. Nitrate accumulation and loss in a mungbean/lowland rice cropping system. Soil Sci. Soc. Am. J. 1989;53:477–482.
3. George T, Ladha, JK, Buresh RJ, Arrity DP. Managing native and legume-fixed nitrogen in lowland rice-based cropping systems. Plant and soil.1992;141:69-91.
4. Alikhani HA**,** Saleh Rastin N, Antoun H. Phosphate solubilization activity of *Rhizobia* native to iranian soils. Plant and soil. 2006;287:35-41.
5. Peix A, Rivas Boyero AA, Mateos PF. Growth promotion of chickpea and barley by a phosphate solubilizing strain of *Mesorhizobium mediterraneum* under growth chamber conditions. Soil Biol Biochem. 2001;33:103-110.
6. Sashidhar B, Podile AR. Mineral phosphate solubilization by rhizosphere bacteria and scope for manipulation of the direct oxidation pathway involving glucose dehydrogenase. Journal of applied microbiology. 2010;109:1-12.
7. Rodriguez H, Fraga R. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv. 1999;17(4-5):319-339.
8. Halder AK, Mishra AK, Bhattachary AP, Chakrabartty PK. Solubilization of rock phosphates by *Rhizobium* and *Bradyrhizobium*. Journal of General and Applied Microbiology. 1990;36:81-92.
9. Johri BN, Sharma A, Virdi JS. *Rhizobacterial* diversity in India and its influence on soil. Adv Biochem Eng Biotechnol. 2003;4:49-89.
10. Yadav J, Verma JP, Rajak VK, Tiwari KN. Selection of effective indigenous *Rhizobium* strain for seed inoculation of Chickpea (*Cicer aritenium* L.) production. Bacteriology Journal. 2011;1(1):24-30.
11. Vincent JM. A Manual for the practical study of root nodule bacteria. In I.B.P Hand book No.15. Blackwell Scientific Publications. Oxford, England. 1970;73-97.
12. Somasegaran P, Hoben HJ. Methods in legume-*Rhizobium* technology. Nif TAL Project and MIRCEN. University of Hawaii, Maui. 1985;1-52.
13. Cappuccino JC, Sherman N. Microbiology**:** A laboratory manual New York. 1992;125-179.

14. Pikovskaya RI. Mobilization of phosphorous in soil in connection with vital capacity of source microbial species. Microbiologiya. 1948;17:362-370.
15. Sri Ram Kumar V, Kannapiran E. Isolation of total heterotrophic bacteria and phosphate solubilizing bacteria and *In vitro* study of phosphatase activity and production of phytohormones by PSB. Archives of Applied Science Research. 2011;3(5):581-586.
16. Srivastava S, Yadav KS, Kundu BS. Prospects of using phosphate solubilizing *Pseudomonas* as bio fungicide. Indian J Microbiol. 2004;44:91-94.
17. Jackson ML. Soil Chemical Analysis. Prentice Hall of India, New Delhi, India. 1973;134-182.
18. Salve PB, Gangwanae LV. *Rhizobium* from wild legumes and nitrogen fixation in ground nut. In: Biofertilizers Technology Transfers. Associated Publications Co., New Delhi. 1992;95-100.
19. Kumari BS, Ram MR, Mallaiah KV. Studies on nodulation, biochemical analysis and protein profiles of *Rhizobium* isolated from Indigofera species. Malaysian Journal of Microbiology. 2010;6(2):133-139,
20. Graham PH, Parker CA. Diagnostic features in the characterization of the root nodule bacteria of legumes. Plant and soil. 1964;20:383-396.
21. Mahana SK, Garg R, Parvateesam M. Cultural and biochemical characterstics of root nodule bacteria from induced mutants of *Vigna mungo.* Seed Pathology. Print well publications, Jaipur. 2000;417-421.
22. Sadowsky M, Keyser H, Bohlool BB. Biochemical characterization of fast and slow growing *Rhizobia* that nodulate Soybeans. International journal of systematic bacteriology. 1983;33(4):716-722.
23. Kaur H, Sharma P, Kaur N, Gill BS. Phenotypic and biochemical characterization of *Brady Rhizobium* and *Ensifer* spp isolated from soybean rhizosphere. Bioscience Discovery. 2012;3(1):40-46.
24. Sperber JI. Solution of apatite by soil microorganisms producing organic acids. Australian Journal of Agronomy Research. 1958;9:782-787.
25. Chen YP, Rekha, PD, Arun, AB, Shen FT, Young CC. Phosphate solubilizing bacteria from subtropical soil and their tri calcium phosphate solubilizing abilities. Applied Soil Ecology. 2006;34:33-41.
26. Dave A, Patel, HH. Inorganic phosphate solubilizing *Pseudomonas*. Indian J Microbiol. 199;39:161-164.
27. Halder AK, Mishra AK, Bhattachary AP, Chakrabartty PK. Solubilization of inorganic phosphates by Brady *Rhizobium.* Indian J Exp Biol. 1991;29:28-31.
28. Sridevi M, Malliah KV, Yadav NCS. Phosphate solubilization by *Rhizobium* isolates from crotalaria species. Journal of Plant Sciences. 2007;2(6):635- 639.
29. Rodriguez H, Gonzaez T, Goire I, Bashan Y. Gluconic acid production and phosphate solubilization bythe plant growth-promoting bacterium *Azospirillum* spp. Naturwissenschaften 2004;91:552–555.
30. Halder AK, Mishra AK, Chakrabartty PK. Solubilization of phosphatic compounds by *Rhizobium*. Indian J. Micribiol. 1990;30:311-314.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=525&id=8&aid=4702