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Isolation and Optimization studies of Phosphate solubilizing *bacteria*

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Abstract

The study presents the detailed account of isolation of phosphate solubilizing bacterial strains from the root nodule of *Vicia faba* from different locations of Thanjavur district, Tamilnadu state, India. Phosphorous plays an important role in plant metabolism as most of the soils are deficient in phosphorous. Phosphate solubilizing bacteria, if employed as biofertilizer, would actually enhance the nutrient quality of the soil. Thirteen *Rhizobium* colonies were separated and subjected to confirmation tests and biochemical studies. The effect of different concentrations of glucose as a carbon sources on the *Rhizobium* strains with phosphate solubilization efficiency were calculated and presented.

Keywords: biofertilizer, phosphate solubilizer, *Rhizobium*, root nodule, *Vicia*

INTRODUCTION

Agriculture and soil management activities such as fertilization, tillage and biomass alteration represent great challenge towards food and environment worldwide (McLauchlan 2006, Gordon *et al.*, 2010, Spiertz 2010,). The extensive use of chemical fertilizers in agro systems for enhancing fertility and agronomic yield induce several issues including soils depletion and pollution (Bohlool *et al.*,1992, Peoples and Craswell, 1992, Velthof *et al.*, 2009, Gupta *et al.*,2014). Indeed, these chemical fertilizers are expensive and are known to be immobilized soon after their application in soils and become unavailable to plant for nutrition (Dey,1988).

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The phosphorus based chemical fertilizers derived from phosphate rocks, have several impacts especially on landscape transformation, and water alteration with radioactive compounds and heavy metals. Besides, phosphorus is one of the major macronutrients for biological development and growth along the plant cycle (Spiertz, 2010 and Enrilich, 1982). However, it represents a limiting factor to plant nutrition due to its low soluble forms in soil (varying from 0.001 mg./l^{-1} mg./l^{-1} in deficient soils to 1 mg./l^{-1} in heavily fertilized soils) (Hani, 2012; Shekhar, 1999). Furthermore, phosphorus is involved in different cellular processes including photosynthesis, respiration energy storage and transfer, cell division and early stages of seed formation. Phosphate Solubilizing Rhizobia (PSR) as an biofertilizers, in order to reduce the cost of chemical fertilizers and to decrease soil degradation and pollution was already investigated (Peix *et al.*, 2001, Tagore *et al.*, 2013, Pereira and Castro, 2014). The mechanism behind phosphate solubilization was explained by the ability of some soil bacteria to produce organic acids and chelate oxoacids from carbonic compounds (Dadarwal *et al.*, 1989, Leyval and Barthelin, 1989).

Phosphorus is one of the major plant nutrients limiting plant growth hormones. Most of the essential plant nutrients, including phosphorus, remain in insoluble form in soil (Abd-Alla, 1994; Jones and Darrah, 1994). A large portion of inorganic phosphates applied to soil as fertilizer rapidly get immobilized after application and becomes unavailable to plants (Murphy and Riley, 1962; Yadav and Dadarwal, 1997). The release of insoluble and the forms of phosphorus is an important aspect of increasing soil phosphorus availability. Seed or soil inoculation with phosphate-solubilizing bacteria is known to improve solubilization of soil phosphorus. This paper gives an account of phosphate solubilization bacterial strains isolated from the root nodules of *Vicia faba*.

MATERIALS AND METHODS

Phosphate solubilizing microorganisms routinely are screened by a plate assay method using Pikovskaya (PVK) agar (Pikovskaya, 1948). The test of the relative

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Table 1. Identification of *Rhizobium* isolated from *Vicia faba* root nodules

S. No.	Strain no	G	M	I	MR	VP	C	Gl	S	Ma	L
1	PSRV1	-	+	-	-	-	-	+	-	+	+
2	PSRV2	-	+	-	-	-	-	+	-	+	+
3	PSRV3	-	+	-	-	-	-	+	-	+	+
4	PSRV4	-	+	-	-	-	-	+	-	+	+
5	PSRV5	-	+	+	-	-	-	+	-	+	+
6	PSRV6	-	+	+	-	-	-	+	-	+	+
7	PSRV7	-	+	+	+	+	+	-	-	+	-
8	PSRV8	-	+	-	+	+	-	-	+	-	+
9	PSRV9	+	+	+	-	+	+	+	-	-	+
10	PSRV10	+	-	+	+	-	+	-	+	-	+
11	PSRV11	+	-	+	+	-	+	-	+	-	+
12	PSRV12	+	+	+	-	-	-	+	-	+	-
13	PSRV13	-	+	-	+	-	+	-	-	+	-

G- Gram stain, M- Motility, I-Indole, MR- Methyl red, VP- Voges proskeaur, C- Citrate, Gl- Glucose, S-Sucrose, Ma- Maltose, L-Lactose, + Present, - Absent

efficiency of isolated strains is carried out by selecting the microorganisms which are capable of producing a halo/clear zone on plate due to the production of organic acids into the surrounding medium (Katznelson *et al*., 1962; Hajjam *et al*., 2016). However, the reliability of this halo-based technique is questioned as many isolates which did not produce any visible halo / zone on agar plates could solubilize various types of insoluble inorganic phosphates in liquid medium (Louw and Webley, 1959 and Gupta *et al*., 1994)

Root nodules collection and preservation (Beck *et al.*, 1993)

Root nodules of *Vicia faba* was collected from different locations of Thanjavur district, Tamilnadu state, India. The sampling made by digging nearly 15 cm to either side of the faba bean (*Vicia faba* L.) plant to 25 cm depth. Then, the root nodules of plants were transferred to the laboratory for further nodules preservation. From each plant, thirty nodules were collected and preserved in silica gel tubes at room temperature, in order to keep them dry and to inhibit any growth of fungi or other bacteria.

Isolation, purification and preservation of rhizobial isolates (Vincent 1970)

Fresh nodules were carefully surface sterilized by ethanol 70% for 60 seconds, then transferred and soaked in 3% of calcium hypochlorite or chlorox ($CaCl_2$) solution for 5 to 6 minutes. Nodules were washed immediately by distillated water, five to seven times. Each nodule was covered by a drop of distillated water for further crush and isolation. The nodule

Table 2. Identification and biochemical characterization of phosphate solubilizing strains of *Rhizobium leguminosarum*

Strain no	BTB	GPA	HA	A
PSRV1	-	-	-	+
PSRV2	+	-	-	+
PSRV3	-	-	-	+
PSRV4	+	-	-	+
PSRV5	+	-	-	+
PSRV6	+	-	-	+
PSRV7	+	+	+	+
PSRV8	+	-	+	_
PSRV9	+	+	-	-
PSRV10	-	+	-	+
PSRV11	+	-	+	+
PSRV12	+	+	-	+
PSRV13	-	+	+	-

BTB-Bromothymol blue, GPA- Glucose peptone agar, HA-Hoffer's Alkaline agar, A- Agar

Table 3: Screening of Phosphate solubilization with different strains of *Rhizobium leguminosarum* by plate method

S. No.	Strain no	Zone diameter (mm)
1	PSRV1	5.6
2	PSRV2	5
3	PSRV3	9
4	PSRV4	15
5	PSRV5	13
6	PSRV6	3.4
7	PSRV7	4.3
8	PSRV8	18.4
9	PSRV9	5.7
10	PSRV10	6.9
11	PSRV11	18.3
12	PSRV12	8.4
13	PSRV13	16.5

PSRV1- PSRV13 – Different strains of *Rhizobium leguminosarum*

suspension was streaked out on YEM medium (Yeast extract mannitol) supplemented with Congo red. As a confirmatory test of rhizobia, Gram staining was performed on the isolates and the observation was made by a microscope at 100x magnification using oil immersion. The selected pure cultures recognized by their white and creamy appearance, which is the morphological characteristic of rhizobia, comparing to the other cultures that can absorb Congo red. Finally, pure cultures were preserved in glycerol 50% (v/v)

Table 4. Environmental stress tolerance in pH and temperature of phosphate solubilizing strains of *Rhizobium leguminosarum*.

Isolates	pH					Temperature (°C)			
	5	6	7	8	9	20	30	40	50
PSRV1	+	+	+	+	+	+	+	+	+
PSRV2	-	-	+	+	-	+	+	+	-
PSRV3	-	-	+	+	-	+	+	+	-
PSRV4	-	+	+	+	-	+	+	+	-
PSRV5	-	-	+	+	-	+	+	+	+
PSRV6	-	-	+	+	-	+	+	+	-
PSRV7	+	+	-	-	+	-	+	-	+
PSRV8	+	+	-	-	+	-	+	-	+
PSRV9	-	-	-	+	+	+	-	+	-
PSRV10	+	-	+	-	+	+	-	+	+
PSRV11	+	-	+	-	+	+	-	+	-
PSRV12	+	+	-	+	-	+	-	+	-
PSRV13	+	+	-	+	-	-	+	-	+

(+) growth (-) no growth

Table 5. Effect of different concentrations of glucose as carbon on the phosphate solubilizing strains of *Rhizobium leguminosarum*.

Different Strains	Glucose Concentration			
	2g		4g	
	PSI	PSE %	PSI	PSE %
PSRV1	2.4	60.14	2.5	50
PSRV2	2.2	48	NS	NS
PSRV3	2.3	60.15	2.2	20
PSRV4	2.42	66.65	2.56	56.25
PSRV5	2.52	67.27	2.55	56.4
PSRV6	2.32	32.07	1.73	36.36
PSRV7	2.25	25	NS	NS
PSRV8	2.41	65.45	2.41	66.87
PSRV9	2.25	25	2.25	25
PSRV10	NS	NS	1.47	32.34
PSRV11	2.47	66.23	2.65	62.54
PSRV12	2.38	37.82	2.16	20.34
PSRV13	2.36	36.66	2.43	65.49

NS – No significant solubilization; **PSI –** Phosphate solubilization index; **PSE –** Phosphate solubilization efficiency

and kept in the freezer at (-80°C) for further analysis. Screening of Rhizobial strains was performed.

Bromothymol blue test (BTB) (Beck *et al.*, 1993)

Five ml of BTB (0,016N) added to YEM medium before autoclaving. This indicator turns yellow at pH=6.0 and blue at pH=7.6 and is green between pH 6.0 and 7.6. The selection of isolates in terms of fast/ slow growth and production of acids/alkali based on the appearance of agar plates (yellow color; fast growing rhizobia with production of acids; blue color: slow growing rhizobia with production of alkali).

Effect of pH and temperature

The resistance of phosphate solubilizing rhizobia (PSR) to ranges 5 to 9 pH, temperature (between 20 and 50°C) was examined on agar plates by blotting technique. The reading of agar plates was made after 48 hours of incubation at 28 ±2°C.

Phosphate solubilization ability on agar plates assay (Edi-Premono *et al.*, 1996)

Phosphate solubilizing PSR ability was assessed on Sperber's basal medium (glucose: 10g; yeast extract: 0.5 g; $MgSO_4.7H_2O$: 0.25g; $CaCl_2$:0.1g; agar: 15g) supplemented with 2.5g of tricalcium phosphate $Ca_3(PO_4)_2$ in 1000 ml of distillated water; pH=7.2. The ability of solubilization was visualized by the appearance of a clear zone halo on Sperber's basal plates. The index and the efficiency of solubilization were calculated based on the colony diameter and halo zone diameter of each isolate.

Estimation of phosphate solubilizing ability by PSR in broth culture (Jackson, 1958)

Phosphate solubilizing ability of selected PSR was estimated in Sperber's basal medium broth culture. Each 1ml (1.108 CFU/ml) of selected PSR, according to their significant symbiotic effectiveness of inoculated plants, were transferred into 250 ml Erlenmeyer flask filled with liquid Sperber's basal medium and incubated on a rotator shaker during 8 days at 28±2°C, (200 rev/min). Phosphate solubilizing ability was estimated after 24 hours.

RESULTS AND DISCUSSION

In the current study *Rhizobium leguminosarum* were isolated from *Vicia faba* root nodules from different areas of Thanjavur district, Tamilnadu. From serial dilutions 10^{-3} to 10^{-6} a total of 109 colonies were isolated and identified by biochemical tests. Out of which thirteen strains showed excellent growth from different dilutions, the biochemical characterization of those thirteen strains of *Rhizobium leguminosarum* are represented in Tables 1 and 2.

Screening of phosphate solubilization test were performed from the thirteen strains. The maximum zone diameter was 18.4 mm with PSRV8 strain and minimum zone diameter was 3.4 mm with PSRV6 strain recorded, respectively (Zephania *et al.*,2016). Some of the *Rhizobium* strain had moderate zones of 15.0, 18.4 and 18.3 mm i.e. PSRV4, PSRV8 and PSRV11 strains, respectively (Table-3).

Environmental stress tolerance to PH and temperature of different strains are given in Table-4 and the effects

of different concentrations of glucose as carbon source on *Rhizobium leguminosarum* strains are given in Table-5. The strains with phosphate solubilization were analyzed with reference the phosphate solubilization efficiency. It was observed that phosphate solubilization efficiency of PSRV5 was 67.27% and 56.40% when 2g and 4g of glucose was added respectively. Followed by PSRV4, PSRV11, PSRV3 with 66.65%, 66.23%, 60.15% respectively when treated with 2g of glucose. But in higher concentrations i.e. 4g of glucose the phosphate solubilization efficiency was performed less (Table-5).

CONCLUSION

Thirteen strains of *Rhizobium leguminosarum* was isolated from *Vicia faba* bean root nodules from Thanjavur district, Tamilnadu, India. All isolates were identified by various biochemical tests and confirmed as *Rhizobium leguminosarum*. Some of the isolates were positive for phosphate solubilization activity and some specific strains showed excellent zone diameter, which were analyzed further. The present *Rhizobium leguminosarum* isolates with phosphate solubilization efficiency can be employed as biofertilizer and would have a significant role in achieving sustainable agricultural development.

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