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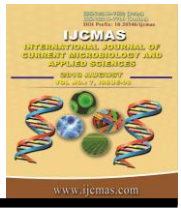

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Solubilization of Phosphorus Containing Mineral by Bacteria from Rhizospheric Region of Walnut (*Juglans regia*)

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A B S T R A C T

Microbial biodiversity in the soil plays a significant role in metabolism of complex molecules and help in plant nutrition. Phosphate solubilizing Bacteria (PSB) increases availability of phosphorus, therefore reducing the application of chemical fertilizers. The present study was aimed to isolate and characterize the selected phosphorus solubilizing bacteria from Rhizospheric regions of Walnut (*Juglans regia*). Walnut rhizospheric region samples were collected from the different sites of Kashmir, India. Each sample was enriched in Pikovskayas's solid medium (pH 7.5 at 30°C for 5 days). Out of 25 isolates, 7 isolates showed highest zone of clearance on Pikovskayas's medium and were morphologically and biochemically characterized. These 7 isolates belong to genus *Bacillus* (PSB8, PSB9, PSB13, 14), *Pseudomonas* (PSB10, PSB11) and *Micrococcus* (PSB12).

Introduction

Numerous soil microflora were reported to solubilize insoluble phosphorus complexes in to solution and make it possible for its use by the plant (Tripura *et al.*, 2005). Phosphorus is the major essential macronutrients for plants growth and development. It plays pivotal in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and some other processes in the plants. It is applied to the soil in the form of chemical fertilizers. Its availability to the plant utilization is limited. As inorganic phosphate, it is immobilized rapidly and becomes unavailable to plants (Akhtar *et al.*, 2010). Among the alternative P sources, the most important is locally available rock phosphate (Khan *et al.*, 2009). Majority of the soils throughout the world are P deficient (Muhammad, 2012). The concentration of Bioavailable P in soil is very low getting to the level of 1.0 mg kg–1 (Goldstein, 1994). Plants absorb P as phosphate anions (HPO4-)

or H_2PO^{4-}) from soil (Rodriguez and Fraga, 1999).

Phosphate anions are highly reactive in the soil and their precipitation is soil pH dependent. In acidic soils phosphate anions get precipitated with free oxides and hydroxides of iron and aluminium, but in alkaline soils calcium is the main element involved in P fixation (I gual *et al.,* 2001). Indian soils are characterized by poor and medium status with respect to available P (Baby, 2002, Li *et al.,* 2003, Ramanathan *et al.,* 2004).

Phosphorus solubilizing microorganisms (PSM) play a vital role in soil P dynamics and availability of phosphate to plants (Richardson, 2001). There are various types of soil microbes which can solubilize this fixed form of P and make available to plants (Illmer *et al.,* 1995; Kucey *et al.,* 1989; Richardson, 2001; White law *et al.,* 1999). Many soil microorganisms particularly those present in rhizosphere of plants, are able to solubilize fixed form of P to soluble form and makes it available to plants (Dave and Patel, 2003; Dubey *et al.,* 1997; Narayanasamy *et al.,* 1981). The principal mechanism of p solubilization is the production of organic acids and phosphatase enzymes which play key role in phosphatase solubilization in the soil (Surange *et al.,* 1995; Duttan and Evans, 1996; Nahas, 1996; Shankar *et al.,* 2013).

Objectives

Isolation of PSB from rhizospheric soils.

Characterization of PSB strains from rhizospheric soils of walnut.

Materials and Methods

It was a laboratory scale study and whole of the work was undertaken in the laboratories of Division of Plant Pathology SKUAST-Kashmir. Different strains of phosphate solubilizing bacteria (PSB) were isolated from walnut rhizospheric soils

Sample preparation

In order to isolate PSB, 10 mixed soil samples were collected from walnut rhizospheric soils, from various sites of Kashmir, India. The samples were collected at a depth of 10-30 cm. All the tools used for soil sampling were surface sterilized using 70% ethanol and soil samples were placed in sterile bags, transported to laboratory, stored at 4 ^{0}C and processed within a week.

Isolation and screening of efficient PSB strains

The isolation of phosphorus solubilizing bacteria (PSB) from rhizospheric soils of walnut was done by following the serial dilution technique. For isolation, soil samples were serially diluted from 10^{-3} to 10^{-6} and inoculated on modified Pikovskayas's agar medium which consists of : glucose 10 g, Magnesium sulphate 0.1g, ferrous sulphate trace, Manganese sulphate trace, Tricalcium phosphate as P source, agar 15 g, distilled water 1L, pH was adjusted to 7 before sterilization, followed by pour plate technique and the 48 h incubation at 30^{0}C, discrete colonies showing halo zones were picked up, sub cultured in Nutrient agar slants and then preserved (Pikovskayas's, 1948).

Characterization of PSB Strains

Morphological characters

Suspension of each purified culture was prepared and poured on plates having solid media by spread plate method. The inoculate plates were incubated at 25^{0}C till the appearance of colonies. Morphological

characters of colonies like size, shape, color and elevation were measured as (Goenadi *et al.,* 2000).

Microscope characters

The isolated strains were heat fixed called as smear. Crystal violet was flooded for one minute and washed gently by tape water. Then the smears were exposed to Gram's iodine for one minute and washed and drained carefully. 95% alcohol was applied for 30 seconds and washed. Finally the smears were washed and drained with 0.25% safranine for 30 seconds and examined under microscope. It focused on shape and size of bacteria. Pink colored bacteria were named as Gram negative while purple colored were named as Gram positive

Estimation of phosphate solubilization efficiency

For testing the Psolubilizing capability of PSB strains, each PSB culture was poured on Pikovskayas's agar plate containing insoluble tricalcium phosphate. The plates having culture were incubated for 4 days at 28^{o}C.Solubilization index was measured by Edi-Premono formula (Edi- Premono *et al.,* 1996).

$$PSI = \frac{Colony\ Diameter + Halo\ Zone\ Diameter}{Colony\ Diameter}$$

Biochemical tests

The most efficient PSB strains were characterized by biochemical tests (Smibert and Krieg, 1994).

Identification of bacterial strain

Different tests like Biochemical, Morphological and Physiological of the selected phosphate solubilizing bacterial isolates were performed for identification, as per methods defined in Bergey's Manual of Determinative Bacteriology (J.G. Holt, *et al.,* 1994). The isolates belong to genus *Bacillus*, *Pseudomonas*, *Micrococcu*s. PSB 8, 9, 13, 14 belong to *Bacillus*, PSB 10, 11 to *Pseudomonas*, and PSB12 to *Micrococcus*.

Statistical analysis

Statistical comparison of different PSB strains for P solubilization efficiency, PSI was undertaken.

Results and Discussion

Isolation, purification and characterization of PSB

Phosphorus is an important limiting factor in agriculture production and microbial activation seems to be an effective way to solve the solidified phosphorus in the soil.

Microorganisms capable of producing a zone of clearance due to the solubilization of inorganic phosphorus (Das, 1989) and were routinely screened in the laboratory by a plate assay method using Pikovskayas's agar medium (pikoviskayas 1948) or tricalcium phosphate medium (Nautiyal, 1999).

The primary screening protocol used for the identification of PSB strains usually depends on the use of tricalcium phosphate as a sole source of P in indicator plates. We decided to use tricalcium phosphate as source of P for screening of PSB isolated from rhizospheric soils of Walnut.

Results indicates that significant clear halo zone formation around bacterial colonies on Pikovskayas's agar with tricalcium phosphate as P source, which was in agreement with reports of (Chung *et al.,* 2005, Barroso and Mahas, 2005) (Fig. 1and 2).

Table.1 Morphological characters of phosphorus solubilizing and mineralizing bacteria

Isolates PSB	Pigmentation	Margin	Gram reaction	Slightly raised	Highly raised	Trans-parent	Opaque	Shape	Spore
PSB8	White	Entire	+	-	+	+	-	Long rods	+
PSB9	Whitish	Entire	+	+	-	-	+	Short rods	+
PSB10	Creamy	Entire	-	+	-	-	+	Short rods	-
PSB11	creamy	Entire	-	+	-	-	+	Short rods	-
PSB12	Creamy	Elevated	+	-	+	-	+	Minute cocci	-
PSB13	White	Entire	+	-	+	+	-	Short rods	+
PSB14	White	Entire	+	-	+	+	-	Short rods	+

Table.2 biochemical characterization of 7 psb isolates

Isolates PSB	C	U	O	Dn	M.R	VP	ST	Ca	A.P	G.P	H_2S	Gel	NaCl	Su	M	G	Cit	k sol.
PSB8	-	+	-	-	+	-	+	-	+	+	-	-	-	-	+	-	+	+
PSB9	-	+	+	+	-	+	-	-	-	-	-	+	-	+	-	+	+	+
PSB10	+	-	+	-	+	-	-	+	+	+	-	-	-	+	+	+	+	+
PSB11	+	-	+	-	-	+	-	+	-	+	+	-	-	-	+	-	-	+
PSB12	+	+	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	+
PSB13	+	-	+	-	+	-	+	+	+	-	-	+	-	+	-	+	+	+
PSB14	+	+	+	-	+	-	+	-	+	-	-	-	-	-	+	+	+	+

C = Catalase test, U = Urea Hydrolysis, O = oxidase, Dn= Denitrification test, MR = Methyl red test, V.P = V.P test, ST = Starch Hydrolysis test, Ca = Casein test, A.P = Acid production, G.P = Gas production, H2S = H_2S production test, Gel = Gelatine hydrolysis, Cit = citrate test, SU = sucrose, M = Mannital, G = glucose, NaCl = 70 % NaCl, K Sol = Potassium solubilization, + = positive, - = negative.

Table.3 Zone of clearance and phosphorus solubilization index of ten isolates

PSB isolates	Diameter of zone of Clearance (cm)	Colony Diameter (cm)	Phosphorus solubilization index (PSI)
PSB8	0.60	0.50	2.20
PSB9	0.40	0.30	2.33
PSB10	0.70	0.40	2.75
PSB11	0.50	0.30	2.67
PSB12	0.60	0.40	2.50
PSB13	0.60	0.30	3.00
PSB14	0.70	0.40	2.75

Fig.1 P Solubilization on PVK medium

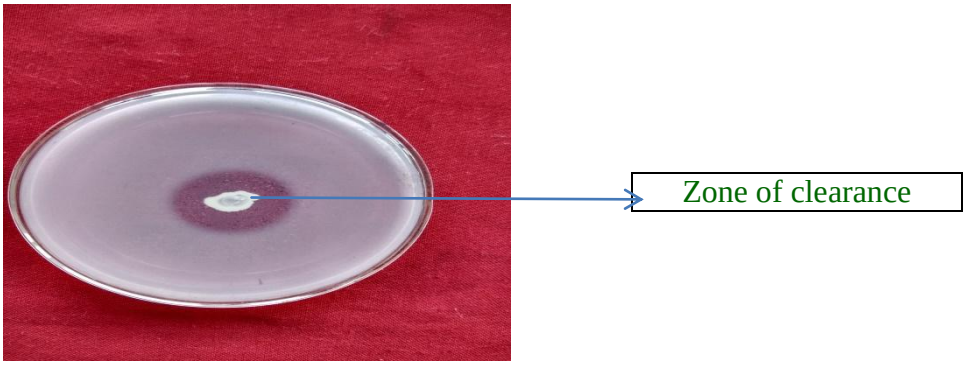
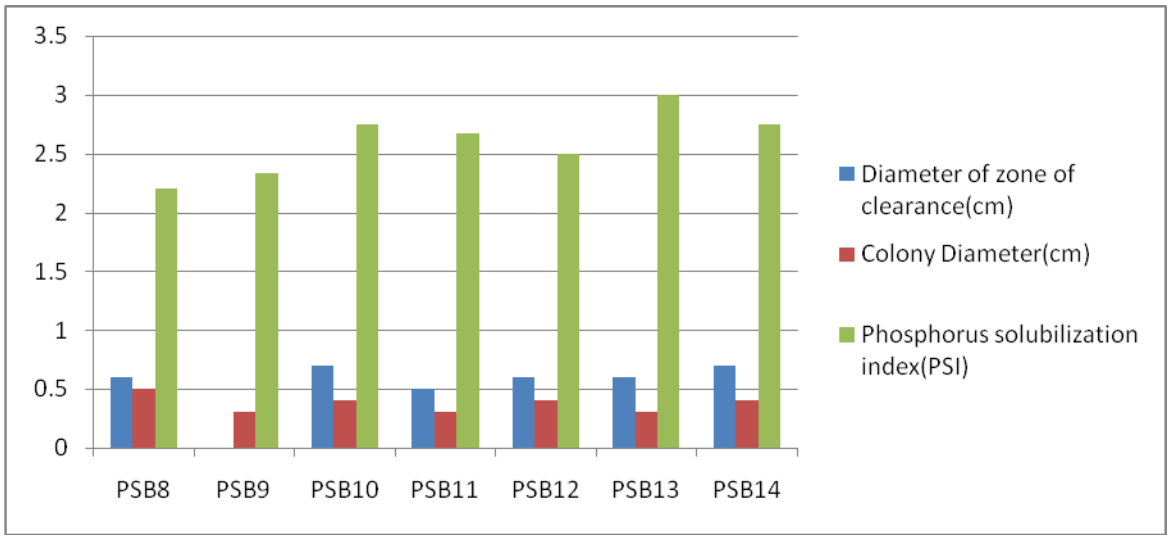


Fig.1a Graphical representation of solubilization index



The isolates were also tested for K solubilization on specific medium. According to morphological and biochemical characterization the microorganisms showing zone of clearance belongs to the bacterial genera, and various biochemical tests were performed (Table 1 to 3). From the various PSB isolates, 7 best strains were selected on the basis of solubilization index for further Characterization. The characterization of PSB was done by the following tests**.**

Gram test

Gram test was performed to check whether PSB strains were gram positive or negative. In this test few strains retained pink color and were categorized as gram negative and few retained purple and are gram positive.

P-solubilization test

For the solubilization test, PSB strain were cultured on Pikovskayas's agar medium and placed in incubator at 28^{o}C for 4 days.

The formation of halo zone around the bacterial colony confirmed them as P solubilizer (Seshadari *et al.,* 2000).

All the 7 strains are best P solubilizers (Table 3)

Effect of PSB on pH of the medium

All the selected 7 PSB strains, showed maximum changes in pH. Change in pH by PSB isolates in broth medium was determined by using pH meter. Maliha *et al.,* (2004) and Chen *et al.,* (2005) reported that PSB strains secrete various organic acids which drop the pH of the mediums resulting in P-solubilization. The drops of pH in PSB broth culture has been reported by several researchers like Rashid *et al.,* (2004) and Panhwar *et al.,* (2009), who found PSB very effective in lowering the pH of the broth medium. Present study results are in line with their findings as they said about PSB's inoculation result in change of pH. The morphological, biochemical and solubilization index is given in tables 1 to 3.

Walnut rhizospheric soil presented a diverse population of PSBs. All the screened isolates PSB8 to PSB14 were more efficient in P solubilization. Therefore these isolates can be used in the production of biofertilizers in order to improve growth of agricultural crops in P deficient soils, constituting an alternative to the application of P fertilizers.

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