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ISOLATION AND CHARACTERIZATION OF BACTERIAL ISOLATES FOR PHOSPHATE SOLUBILIZATION AND OTHER PLANT GROWTH PROMOTING ACTIVITIES FROM APPLE SOIL OF HIMACHAL PRADESH

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ABSTRACT

Phosphate solubilizing bacteria (PSB) are being used as biofertilizer since 1950s. Release of phosphate by PSB from insoluble and fixed forms is an important aspect regarding phosphorus availability in soils. A total of 20 phosphate solubilizing bacteria were isolated from rhizospheric soil samples of both normal and replant sites of apple orchard. On the basis of morphological and biochemical characterization, these bacteria were placed under the genus *Pseudomonas* especially fluorescent group. Out of 20 isolates, only 11 isolates were shown good potential for phosphate solubilization on Pivkovskaya's (PVK) medium and other plant growth promoting activities. Maximum phosphate solubilization (42mm dia.) on PVK agar was shown by MJN-9 isolate and in broth medium (35μg/mL) by isolate MJN-6. All the isolates were then screened for other plant growth promoting activities like production of ammonia, HCN, siderophore, proleolytic and antifungal activities. All the isolates were able to produce ammonia, siderophore and proleolytic activities both qualitatively and quantitatively. Only 36% isolates were able to produce HCN production and 63% showed antifungal activity against root rot causing fungi (*Dematophora necatrix*). Out of eleven phosphate solubilizing *Pseudomonas* species isolates, four were more promising for the management of replant problem of apple.

INTRODUCTION

Phosphorus (P) is one of the major plant nutrient required by both plants and microorganism. Its major physiological role being, in certain essential steps, the accumulation and release of energy during cellular metabolism. Most agricultural soil contains large reserves of total P, commonly in the range of 200-500 mg /kg with an average of 600 mg/kg and a part of P accumulates depends on regular application of chemical fertilizers (Fernandez *et al.,* 2007). Both P fixation and precipitation occur in soil because of the large reactivity of phosphate ions with numerous soil constituents. Soil microorganisms play a key role in soil P dynamics and subsequent availability of phosphate to plants. Inorganic forms of P are solubilized by a group of heterotrophic microorganisms excreting organic acids that dissolve phosphatic minerals and/or chelate cationic partners of the P ions *i.e.* PO_4^{3-} directly, releasing P into solution. Interest has focused on the inoculation of PSB into the soil so as to increase the availability of native fixed P and to reduce the use of fertilizers (Illmer and Schinner, 1992). Phosphate solubilizing bacteria (PSB) are being used as biofertilizer since 1950s Release of phosphate by PSB from insoluble and fixed forms is an import aspect regarding P availability in soils. There are strong evidences that soil bacteria are capable of transforming soil P to the forms available to plant. Microbial biomass assimilates soluble P and prevents it from adsorption or fixation (Khan and Joergesen, 2009). Microbial community influences soil fertility through soil processes viz. decomposition, mineralization, and storage / release of nutrients. These bacteria enhance the P availability to plants by mineralizing organic P in soil and by solubilizing precipitated phosphates. These bacteria in the presence of labile carbon serve as a sink for P by rapidly immobilizing it even in low P soils. PSB are the group of common PGPR in rhizosphere. Secretion of organic acids and phosphatase to solubilize insoluble phosphate to soluble forms are common in this group (Kim *et al.,* 1998). Although several PSB occur in soil their numbers are not adequate to compete with each other bacteria commonly established in the rhizosphere (Glick *et al*., 1995). Application of PGPR was beneficial showing higher nutrient content in soil (Das and Singh, 2014). So there is a need to screen effective PSB with plant growth promoting activities for further management of replant problem of apple.

MATERIALS AND METHODS

Collection of soil samples

Soil samples were collected around few to 10 cm apart from root and 1 foot deep from plants rhozosphere and transferred under aseptic conditions to laboratory for further process.

Isolation, enumeration and identification

The media employed for the isolation of *Pseudomonas* sp. were nutrient agar (NA) and selective King's B medium supplemented with 3 antibiotics *i.e.*, Ampicillin (40μg/ml), Cycloheximide (100μg/mL) and Streptomycin (30μg/ml). Plates were incubated at 28 ± 2ºC for 24 - 48 h. Twenty isolates were classified on the basis of colony characteristics such as size, color, shape, texture and type of fluorescent pigment production. The most predominant *Pseudomonas* sp. isolates showing greenish/yellowish fluorescent pigments at 302 nm wavelength in BIO-RAD gel doc system XR were assumed to be fluorescent colonies.

Then they were characterized on the basis of morphological and biochemical tests as per their genera as prescribed in Bergey's Manual of Systematic Bacteriology (Palleroni, 1984). The pure culture of these selected strains were maintained on the nutrient agar slants at 4ºC and were sub-cultured periodically on the same media at 28 ± 2ºC. They all were also maintained and preserved in 20% glycerol at -20ºC for further research.

Phosphate Solubilization

For phosphate solubilizing activity, both qualitative and quantitative methods were followed. Test organisms were grown in PVK broth supplemented with 5.0 g/L tri-calcium phosphate (TCP) for 72 hours at 28 ± 2ºC under shake conditions (100 rpm). Supernatant were prepared by centrifugation of culture at 12,000 rpm for 20 min. and was stored at 4ºC. By qualitative method, 100 ml of this supernatant was applied to 10mm wells of PVK (Pikovskaya, 1948) agar plates. Then these plates were incubated at 28 ± 2°C. Phosphate solubilization was expressed in terms of mm diameter of yellow coloured zone produced around the well after 24 to 48 hours. Quantitatively, estimation of phosphorous (Olsen *et al.,* 1954) was done from 72h old cell free supernatant of test organisms grown in PVK broth supplemented with 5.0 g/L tri-calcium phosphate (TCP) and solubilization of phosphorous was calculated from standard curve of KH_2PO_4 (10-100 μg/mL) in the form of μg/mL of Pi released.

Production of other plant growth promoting activities

All the *Pseudomonas* sp. isolates were screened for other plant growth promoting activities like Ammonia and HCN production, siderophore and protease activities and antifungal activity against root rot causing fungi (*Dematophora necatrix*).

HCN Production

Production of hydrogen cyanide (HCN) was checked according to Bakker and Schippers, (1987). All the *Pseudomonas* sp. was streaked on prepoured plates of King's medium B amended with glycine (1.4 g/l). Whatman No.1 filter paper strips were soaked in 0.5 per cent picric acid in 2 per cent sodium carbonate and were placed in the lid of each petriplates and then Petriplates were sealed with parafilm and were incubated at 28 + 2°C for 1-4 days. Uninoculated control was kept for comparison of results. Plates observed for change of color of filter paper from yellow (-) to dark brown (+ + +) to orange brown (+ + + +).

Assay for Ammonia Production

For the detection of ammonia production, the method of Lata & Saxena (2003) was used. Phosphate solubilizing bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown culture were inoculated into 5 ml peptone water in each tube and incubated for 96 hour at 28 ± 2ºC. After incubation, Nessler's reagent (0.5 ml) was added to each tube. Development of brown to yellow colour was a positive test for ammonia production. . Presence of very light brown color (+) indicates small amount of ammonia production and light brown (+ +) to orange brown color (+ + + +) indicates large amount of ammonia production.

Siderophore production

PSB isolates were tested for siderophore production both by qualitative and quantitative methods (Schwyn and Neilands, 1987). For qualitative estimation, CAS agar plates were prepared and 100 ml supernatant of *Pseudomonas* sp. were applied on these plates. Development of yellow orange halo around the well was described as positive for siderophore production. By quantitative detection, siderophore production in liquid medium of Chrome-azurol-S (CAS) was carried out and change in the color of reaction mixture was observed from dark blue to orange or pink.

Protease activity

Protease activity (Casein degradation) was determined from clear zone in skimmed milk agar. Skimmed milk agar plates were prepared by using 1 per cent skimmed milk in nutrient agar medium and 100 ml supernatant was applied on to these plates. Development of halo zone around the well was considered as positive for protease activity.

Antifungal activity

Antifungal activity of each test isolate of *Pseudomonas* sp. was checked by standard well/bit plate assay method (Vincent *et al.*, 1947). Fresh culture bit of root rot causing fungi (*Dematophora necatrix*) in apple were cut with the help of sterile well borer and placed on the one side of prepoured malt extract agar (MEA) plates with the help of inoculating needle. On the other side of plates, 10mm well was cut with the help of sterile cork borer. 100 μl of 72 h old cell free culture supernatant of each test bacterial isolates was added to each well (10mm). Plates were incubated at 28 + 2°C for 5 -7 days and observed for inhibition zone produced around the well. For control culture bit of indicator fungi kept in the centre of MEA plate and incubated at 28 ± 2°C for 5-7-days. Antifungal activity expressed in terms of mm diameter of clear zone around the bit/well and also expressed in terms of per cent inhibition of fungal mycelium as calculating from equation:

$$\frac{C-Z}{X}\times 100 = \%\text{inhibition}$$

C : growth of mycelium in control

Z : growth of mycelium in treatment

RESULTS AND DISCUSSION

Having screened soil samples from the rhizosphere of apple, the twenty bacterial isolates were isolated. Out of twenty, eleven were found to be fluorescent, with transparent to translucent colonies (irregular to circular colonies with entire edge and flat elevation on nutrient agar plate), pigmented (greenish and yellowish pigmentation on King's B medium plates), Gram's

Table 1: Morphological characteristics of *Pseudomonas* like bacterial isolates isolated from rhizospheric soil of normal and replant site of apple

*Pseudomonas** Isolate	Colony morphology				Pigment/Fluorescence
	Shape	Elevation	Edge	Opacity	
MJN-2	Irregular	Raised	Entire	Transparent	Yellowish
MJN-3	Circular	Raised	Entire	Transparent	Yellowish
MJN-4	Circular	Flat	Entire	Transparent	Yellowish
MJN-5	Circular	Raised	Entire	Transparent	Yellowish
MJN-6	Circular	Raised	Entire	Transparent	Yellowish
MJN-7	Circular	Flat	Entire	Transparent	Yellowish
MJN-9	Irregular	Raised	Entire	Transparent	Greenish
MJN-10	Circular	Flat	Entire	Transparent	Yellowish
MJR-1	Circular	Raised	Entire	Transparent	Brown
MJR-8	Circular	Raised	Entire	Transparent	Yellowish
MJR-11	circular	Raised	Entire	Transparent	Greyish

*All isolates were found to be gram negative, rods/circular and non spore producer.

Table 2: Physiological and biochemical characteristics of selected isolates of fluorescent *Pseudomonas* isolates

Isolate	Characteristics Growth (on King'B agar)											
	Gram's staining	Tween 80 hydrolysis	Metabolism (oxidative)	Lecithinate	Denitrifi -Cation	Oxidase	Catalase	Gelatin Liquification	4°C	25°C	37°C	41°C
MJN-2	-	-	+	-		+	+	-	+	+	+	-
MJN-3	-	-	+	-	+	+	+	+	+	+	+	-
MJN-4	-	-	+	-	+	+	+	+	+	+	+	-
MJN-5	-	-	+	-	-	+	+	-	+	+	+	-
MJN-6	-	-	+	-	-	+	+	-	+	+	+	-
MJN-7	-	-	+	-	+	+	+	+	+	+	+	-
MJN-9	-	-	+	-	+	+	+	+	-	+	+	+
MJN-10	-	-	+	-	+	+	+	+	+	+	+	-
MJR-1	-	-	+	-	+	+	+	+	-	+	+	+
MJR-8	-	-	+	-	+	+	+	-	+	+	+	-
MJR-11	-	-	+	-	+	+	+	-	+	+	+	-

(+) indicates positivity of test; (-) indicates negativity of test

Table 3: Plant Growth Promoting Activities from normal and replant soil of apple rhizosphere

Strains	Phosphate mm dia.	Solubilization μg of available Pi	HCN	Ammonia	Siderophore mm dia.	Proteolytic % Siderophore Units	Antifungal activity mm dia.	mm dia.	% inhibition
MJR-1	40	30	-ve	+ + + +	14	49.69	13.34	55	8.34
MJN-2	22	17	+ +	+ + +	12.67	70.12	14	58	3.34
MJN-3	21	28	+	+ + +	12.34	71.23	14	57	5.00
MJN-4	32	29	-ve	+ + +	12	42.59	13	-	-
MJN-5	20	20	-ve	+ + +	11.34	38.58	11.67	58	3.34
MJN-6	26	35	-ve	+ + +	12	44.14	14	-	-
MJN-7	20	26	-ve	+ + +	13	44.19	12	57	5.00
MJR-8	23	33	-ve	+ +	11.67	41.67	14	58	3.34
MJN-9	42	28	+ +	+ + +	11.67	37.65	12	52	13
MJN-10	40	29	-ve	+ +	11.67	38.27	12.67	-	-
MJR-11	32	25	+ +	+ + + +	12.34	38.89	12	-	-

negative, short rod shaped, non-spore forming bacteria (Table 1 and 2).

The basal medium used was King's B medium, Ward and Raney (1954). Sands and Rovira (1970) found that penicillin (75 units/ml), novobiocin (45 μg/ml), and cycloheximide (75 μg/mL) limited the growth of nearly all fungi and bacteria except fluorescent pseudomonads.

All the strains were found to be positive for catalase and oxidase tests. This means that they showed the presence of these two catalase and oxidase enzymes. All the eleven isolates showed growth at 25°C which is the optimum temperature for growth. Nine isolates have shown growth at 4°C whereas two have shown growth at 41°C. All *Pseudomonas* sp. isolates showed aerobic growth. Eight isolates were found to be positive for denitrification test and six for positive gelatin liquification. All the eleven isolates were negative for lecithinase activity. Also none of the isolates were found to be positive for tween-80 hydrolysis (Table 2).

On the basis of these key characteristics, these bacteria were placed under the genus *Pseudomonas* especially fluorescent group. Depending upon biochemical and physiological characteristics some isolates belong to *Pseudomonas*

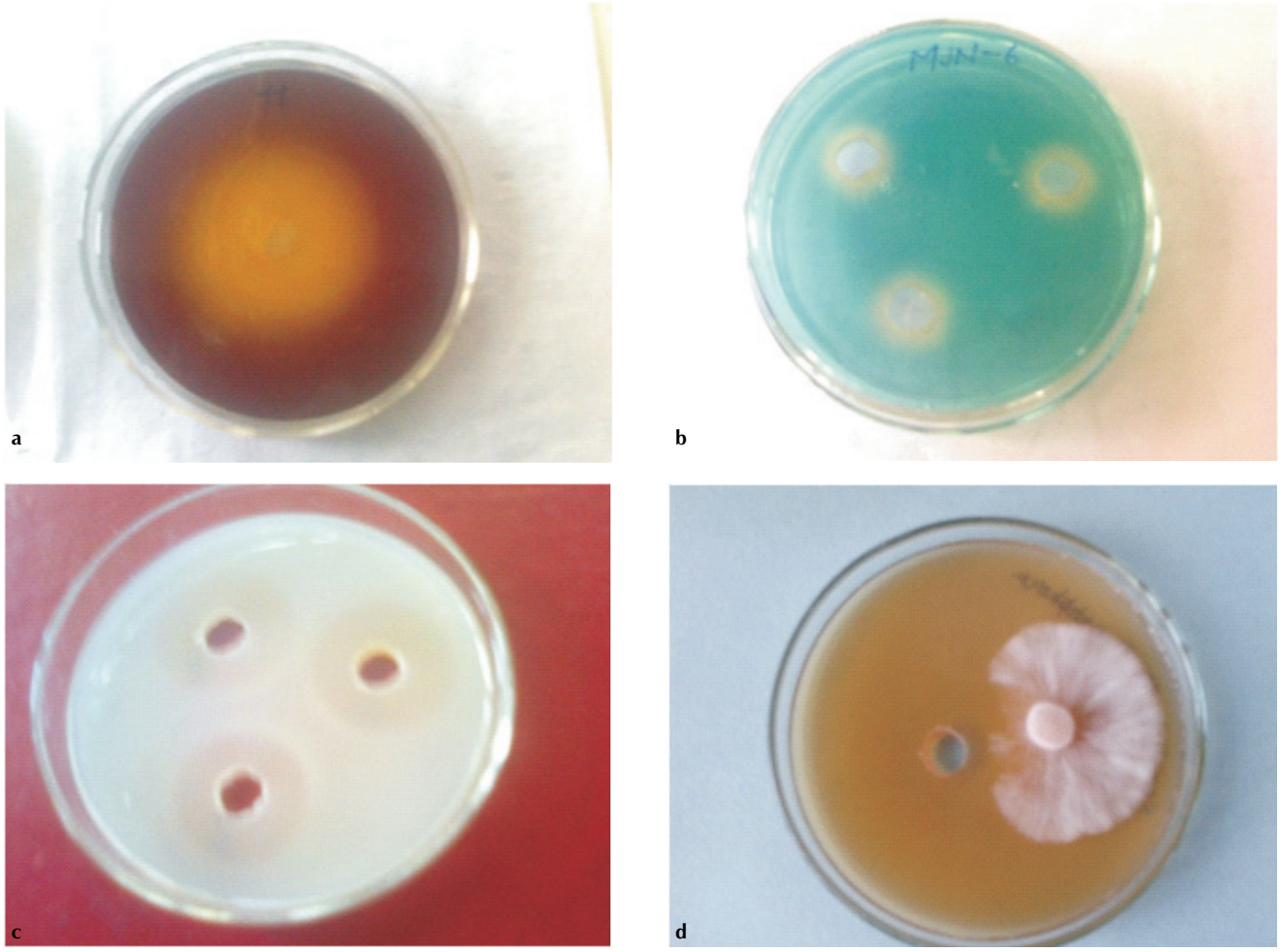

Figure 1: Phosphate solubilization by MJR-1 (a), Siderophore by MJN-6 (b), Proleolytic activities by MJN-4 (c), and antifungal activity by MJN-9 (d) against *Dematophora necatrix* shown by *Pseudomonas* sp. isolates

aeruginosa as they showed growth at 41°C and some belong to *Pseudomonas putida* and *Pseudomonas fluorescens* which showed growth at 4°C.

Indian soils on an average contain 0.05% phosphorus that constitutes 0.2% of plant dry weight. Even in phosphorus rich soils, most of this element is in insoluble form and only a small proportion (0.01%) is available to plants (Stevenson and Cole, 1999). Being an important constituent of nucleic acids, phytins, phospholipids, nucleotides, co-enzymes and enzymes, phosphorous is of great importance in the transformation of energy, transfer of heredity characters, fat and albumin formation and cell organization in plants (Mc Vickar *et al*., 1963).

Plant growth is frequently limited by an insufficiency of phosphates, which were considered one of the most important growth-limiting environmental factors also one of the major factors in replant disease. The low solubility of common phosphates, such as $Ca_3 (PO_4)_2$ hydroxyapatite and aluminum phosphate cause low phosphate availability. However, because some bacteria can solubilize insoluble phosphates, they may promote plant growth (Rodriguez and Fraga, 1999).

Most of the isolates of *Pseudomonas* sp. produced ammonia which is a common trait of these PSB. Maximum ammonia production was shown by MJR-1 and MJR-11in the range of ++++. Present results are comparable with the results of Ahmad *et al*. (2008) which reported that the plant growth promoting rhizobacteria were found to produce ammonia. Chaiharn *et al*. (2008) also reported the production of ammonia by phosphate solubilizing microorganisms and more than 64% of the isolates produced ammonia.

Pathogen antibiosis and cyanide production are considered indirect plant growth promoting properties. Among apple associated P-solubilizing bacterial isolates, these properties are unequally distributed with no relation between them indicating in general fungal antagonism is not due to cyanogenesis. HCN production has been shown both as a beneficial and harmful property for plants (Rudrappa *et al.,* 2008 and Selvakumar *et al*., 2008).

The isolates of *Pseudomonas* sp. showed HCN production in the range of (+) to (++). In fact, this is the less extended plant growth promoting (PGP) related properties among the P-solubilizing bacterial isolates reported in the present study. The low number of P-solubilizing isolates possessing this property in apple rhizosphere could indicate that this may not be an important PGP-property for this plant.

Phosphate solubilizer produce clear yellow zones around the well on Pikovaskya's medium containing insoluble phosphate (5 gm/l) such as tri-calcium phosphate (Fig.1a). 11 isolates capable of solubilizing TCP were isolated from rhizospheric soil of apple growing area of Himachal Pradesh. Phosphate solubilization was most frequently encountered in these rhizobacteria. The maximum diameter of yellow zone was shown by MJN-9 i.e. 42mm followed by MJR-1 (40mm) and minimum was shown by MJN-5 and MJN-7 (20mm each) (Table3 and Fig1a.). The maximum solubilization of inorganic phosphorus and maximum release of orthophosphate (Pi) in supernatant was shown by MJN-6 (350μg/ml) while minimum was showed by MJN-2 (170 μg/ml) shown in Table 3. *Pseudomonas* isolates showed production of phosphate solubilizing activity in the range of 199.5 to 413.4 ìg/ml available inorganic phosphate (Pi) and siderophore production in the range of 20-21 mm in plate assay and 67.27 %SU in liquid assay (Sharma *et al.*2014).

Siderophore production was another important trait of PSB. All the *Pseudomonas* sp. isolates showed orange halos on CAS agar plates (Fig.1b). Qualitatively, maximum siderophore production was shown by MJR-1(14mm) followed by MJN-7 (13mm). Other isolates showed less production of siderophore. Quantitatively, maximum % siderophore units were produced by MJN-3 (71.23) followed by MJN-2 (70.12) and least was shown by MJN-9 (37.65) shown in Table 3. Iron is a limiting bioactive metal in soil and essential for the growth of soil micro-organisms. Iron concentration in soil is low (10^{-7}M) enough to limit the growth of soil micro-organisms (10^{-8} – 10^{-6} M) (Gurinot, 1994).

As one of the very important group of PGPR for sustainable agriculture, PSB tolerances to extreme climate are of special interest for bacteria to be used as biofertilizer in arid and semi-arid regions.

All the *Pseudomonas* sp. isolates were shown to produce halo zones on skim milk agar plates that showed protease activity (Fig. 1c). Some of the tested isolates could exhibit more than 2 or 3 plant growth promoting traits, which may promote plant growth directly or indirectly. In agriculture these proteases producing microorganisms have role as biological control agents for eradication of some fungal plant pathogen.

Pseudomonas sp. isolates were screened out for the production of antifungal activity by well plate assay method against root rot causing fungi (*Dematophora necatrix*) in apple (Table 3 & Fig 1d). Antifungal activity against plant pathogen *Dematophora necatrix* has been shown by nine *Pseudomonas* sp. isolates in the range of 3.34 to 8.34 % I inhibition. Maximum inhibition was shown by MJN-9 (13%I) followed by MJR-1 (8.34%I) and minimum inhibition was found in MJN-2, MJN-5 and MJR-8 (3.34%I). Other four isolates did not show any inhibition against the test pathogen.

The results are in consistent with the findings of several research workers who demonstrated the use of *P. fluorescens* strains against various fungal pathogens (Radjacommare *et al.,* 2004). In addition to disease management, *P. fluorescens* strains were found to increase plant growth and yield in potato, radish, sugar beet, and mango and groundnut (Vivekananthan *et al.,* 2004; Saravanakumar and Samiyappan, 2008).

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