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Phytohormones Production and Phosphate Solubilization Capacities of Fluorescent *Pseudomonas* sp. Isolated from Shimla Dist. of Himachal Pradesh

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ABSTRACT

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Fluorescent *Pseudomonas* species are the diverse group of soil microorganisms that are involved in plant growth enhancement through various direct or indirect mechanisms. The present study was conducted to investigate the phytohormone production and other plant growth promoting traits of indigenous fluorescent *Pseudomonas* sp. isolated from apple rhizosphere. All the eight isolates produced plant growth regulators viz., auxin, gibberellins and cytokinins in the range of 11-22 µg/ml, 25.92-43.32 µg/ml and 17-30 µg/ml respectively. The maximum production of auxin (22 µg/ml) and gibberellins (43.32 µg/ml) was showed by isolate P7 whereas M and P11 isolates showed maximum production of cytokinins (30 µg/ml). The isolate K and P17 showed maximum (46 µg/ml) inorganic phosphate solubilization and maximum siderophore production of 43.32 % SU was showed by isolate P7. All the isolates showed ammonia production and only six isolates (viz., K, L, M, P3, P7 and P17) showed HCN production. Finally, these best selected fluorescent *Pseudomonas* isolates could be used for plant growth promotion and development of mixed bioformulation. The augmentation of such plant growth promoting fluorescent *Pseudomonas* in the ecosystems will ensure a healthy micro climate around the root environment by replacing chemical fertilizers.

Introduction

Soil microorganisms that colonize roots and promote plant growth represents a subset of rhizosphere bacteria called plant growth promoting rhizobacteria (PGPR). In recent years, the use of PGPR to promote plant growth had increased in various parts of world (Zahir *et al.*, 2003). PGPR can affect plant growth by production and release of secondary metabolites, preventing deleterious effects of phytopathogenic organisms in the rhizosphere and/or facilitating the availability and uptake of certain nutrients from the root

environment. The use of PGPR is steadily increasing in agriculture and offers an attractive way to replace chemical fertilizers, pesticides and supplements (Ashrafuzzaman *et al.*, 2009).

Fluorescent *Pseudomonas* species are the diverse group of soil microorganisms able to synthesize different secondary metabolites with diverse biological activities. Plant hormones produced by *Pseudomonas* include auxins (indole acetic acid), cytokinins,

ethylene 2, 3 butanediol and acetoin (Lambrecht *et al.*, 2000). They are involved in the growth and development of plants. The production of plant growth regulators especially auxins induces additional root hair and lateral root formation hence thereby enhancing the plants ability to take up nutrients from soil and increase yield (Tien *et al.*, 1979). The several species of *Pseudomonas* produce phytohormones or growth regulators that cause crops to have greater amounts of fine roots which have the effect of increasing the absorptive surface of plant roots for uptake of water and nutrients. The potential for auxin biosynthesis by rhizobacteria can be used as a tool for the screening of effective PGPR strains.

Many micro-organisms species producing phytohormones were able to form gibberellins and kinetin, either together with auxin or alone. Gibberellic acid and gibberellin like compounds were identified in cultures of *Azotobacter chroococcum*, *A. baijerinckii*, *A. vinelandii*, *A. paspali*, various pseudomonads and bacilli. Some strains of pseudomonads produced more types of auxins and gibberellins in large concentrations, together with kinetins (Azcon and Barea, 1975). Thus considering the importance of above mentioned topics, in the present study an attempt has been made to screen fluorescent *Pseudomonas* sp. for phytohormone production and phosphate solubilization that could induce plant growth and can be used for bioformulation development.

Materials and Methods

Isolation of fluorescent *Pseudomonas* sp.

Isolation of fluorescent *Pseudomonas* sp. was made from apple rhizosphere of Shimla district of Himachal Pradesh (India). Isolation of fluorescent *Pseudomonas* sp. was made by following the serial dilutions and pour plate

method using the specific King's B medium (King *et al.*, 1954). The composition of the medium was (g/l⁻¹): Peptone, 20.0; K₂ HPO₄, 1.5; MgSO₄.7H₂O, 1.5; Glycerol, 15.0 ml. Plates were incubated at 28⁰ +2⁰C for 48 hr. After incubation, well separated individual colonies with yellow green pigments were marked and detected by viewing under UV light.

The individual colonies were picked up with sterile loop and transferred to fresh King's B slants and the pure cultures so obtained were stored in refrigerator at 4⁰C for further use. These were again identified on the bases of morphological, biochemical and physiological tests and growth at optimum temperature i.e 4°C and 41°C.

Siderophores detection and quantification

Siderophore production by fluorescent *Pseudomonas* isolates was detected by observing orange halos production on CAS agar plates after 72 hr of growth. For quantitative estimation of siderophores, chrome azurol-S (CAS) liquid assay method (Schwyn and Neilands, 1987) was used. The siderophore content was expressed as percentage siderophore units (% SU).

Estimation of phosphate solubilization

Phosphate solubilising activity was estimated on pikovskaya's agar plates with known amount of inert phosphorus (Ca₃ (PO₄)₂) (Pikovskaya's, 1948). The composition of the medium was (g/l⁻¹): Glucose, 10.0; Ca₃ (PO₄)₂, 5.0; (NH₄)₂SO₄, 0.5; MgSO₄.7H₂O, 0.2; MnSO₄, 0.1; FeSO₄, 0.0001; Yeast extract, 0.5; Bromocresol purple, 0.1. Quantitative estimation of phosphate solubilization was done by spectrophotometric method (Dickman and Bray, 1940; Bray and Kurtz 1945; Olsen *et al.*, 1954) and the results were expressed as µg/ml.

Detection of ammonia and HCN

Fluorescent *Pseudomonas* isolates were tested for ammonia production according to Lata and Saxena (2003) and King's B medium amended with 0.44% Glycine was used for detection of hydrogen cyanide following the method of Bakker and Schippers (1987)

Determination of auxins and produced by fluorescent *Pseudomonas* sp.

Quantitative estimation of auxins was done by colorimetric method (Gorden and Weber, 1951) with slight modifications i.e. 2 to 3 drops of orthophosphoric acid was added to 2 ml supernatant and 4 ml of salper reagent (1 ml of 0.5M FeCl₃ in 50 ml of 30% HClO₄). This mixture was incubated for 60 minutes in dark.

Absorbance was measured at 535 nm. Concentration of auxins was estimated by preparing calibration curve using IAA as standard (10-100 µg/ml).

Determination of gibberellins produced by fluorescent *Pseudomonas* sp.

The gibberellins were estimated calorimetrically by the method of Holbrook *et al.*, (1961). 15 ml of supernatant, 2 ml of zinc acetate reagent (21.9 g zinc acetate + 1 ml of glacial acetic acid and volume was made upto 100 ml with distilled water) was added. 2 ml of potassium ferrocyanide (10.6% in distilled water) was added after 2 minutes and was centrifuged at 2000 rpm for 15 minutes. To 5 ml of supernatant 5 ml of 30 per cent HCl was added and mixture was incubated at 20⁰C for 75 min.

Absorbance was read at 254 nm and concentration of gibberellins was calculated by preparing standard curve by using gibberellic acid as standard (10-100 µg/ml).

Determination of cytokinins produced by fluorescent *Pseudomonas* sp.

The production of cytokinins was estimated by radish cotyledons expansion bioassay test (Latham, 1971). The seeds of *Raphanus sativus* were germinated in total darkness for 48 hr. at 28⁰C. The bioassay response (final weight-initial weight) was expressed as increase in weight. Concentration of cytokinins present in the extract was calculated by preparing standard curve by using kinetin as standard (10-100 µg/ml).

Results and Discussion

Eight bacterial isolates from apple rhizosphere were isolated. All isolates were Gram negative bacteria. These bacterial isolates were predominantly rod-shaped and fluorescent with irregular colonies though a few of them were circular (M and P17). All the bacterial isolates were large with an entire margin and produces yellowish to dark green pigmentation on King's B agar (Table 1). All the isolates viz., K, L, M, P3, P7, P11, P12 and P17 exhibited green fluorescence under UV light. The versatile and predominant fluorescent *Pseudomonas* sp. was isolated which may attribute to specific selection of King's B medium employed for isolation. Several others hence supported the use of King's B medium for isolation of fluorescent *Pseudomonas* sp. (King *et al.*, 1954).

The physiological and biochemical characteristics of all the isolates were given in table 2. All the identified isolates showed positive reactions for catalase and oxidase test confirming them to be *Pseudomonas* sp. (Bergey's Manual of Determinative Bacteriology, 1974). The isolates K, L, P7 and P12 were found to be positive for gelatin hydrolysis whereas M, P3, P11 and P17 were not able to hydrolyse gelatin. Our study corroborate with Angayarkanni *et al.*, (2005)

who reported that *P. fluorescens* can dissolve solid gelatin into a liquid form with the presence of an enzyme responsible for gelatin hydrolysis in room temperature. Some species such as *P. fluorescens* strains are capable of denitrification and able to grow anaerobically in nitrate media. In our study six isolates (K, P3, P7, P11, P12 and P17) were capable of denitrification however, negative responses were also identified for some *Pseudomonas* isolates such as for Tween 80 hydrolyses, growth at 4°C and 41°C.

All the isolates were able to grow at a temperature of 25°C. Todar (2004) reported that incubation temperature around 30°C favours the growth of denitrifying biotypes of *P. fluorescens*, while temperatures above 37°C may be conducive for other *Pseudomonas* species. The results of this study indicate that the isolates may belong to *P. fluorescens*, *Pseudomonas putida* and *Pseudomonas aeruginosa* group. Reynolds (2004) also characterized and identified *P. fluorescens* on the bases of different biochemical tests like oxidase, catalase, gelatin hydrolysis, nitrate reduction and performing growth at different temperature.

All the fluorescent *Pseudomonas* isolates were screened for their ability to perform multiple plant growth promoting traits. All the isolates exhibited variation in production of different growth promoting activities. In our study, all the isolates of fluorescent *Pseudomonas* sp. isolated from apple rhizosphere showed production of siderophores and were able to solubilise phosphate. All the isolates were screened out for production of siderophores by qualitative and quantitative method. The results revealed that the maximum siderophore production was showed by isolate P7 (43.32 % SU) and the minimum by two isolates M and P17 (25.92 % SU) (Table 3). Under plate assay method, the maximum siderophore production in terms of orange zone was also showed by

P7 (22 mm) followed by seven isolates that showed siderophore production in the range of 11 mm to 15 mm diameter. Our results corroborate with Ganesan and Sekar (2012) who also reported the production of siderophores from fluorescent *Pseudomonas* sp. Fluorescent *Pseudomonas* produced extracellular siderophore which efficiently complex environmental iron, making it less available or unavailable to pathogen and native microflora (Dave and Dube, 2000).

The indigenous fluorescent *Pseudomonas* sp. was also screened out for production of phosphate solubilization by qualitative and quantitative method. All results were documented in table 3. The maximum production of phosphate solubilisation in terms of yellow zones (mm diameter) on Pikovskaya's medium by plate assay method was showed by isolate M and P11 (30 mm) whereas all other isolates showed phosphate solubilization in the range of 17 to 27 mm diameter. The result also showed that maximum solubilization of inorganic tricalcium phosphate was showed by two isolates viz., K and P17 (46 µg/ml) followed by all other isolates which showed medium range of release of phosphorus i.e. 23 to 37 µg/ml. These results were supported by Verma and Kaur (2015) who isolated ten fluorescent *Pseudomonas* sp. from apple rhizosphere that had the ability to solubilise phosphate and the amount of P solubilized varied between all the isolates.

All the isolates of fluorescent *Pseudomonas* were screened for HCN and ammonia production. All the isolates were found to be positive for ammonia production whereas only six isolates viz., K, L, M, P3, P7 and P17 showed HCN production (Table 3). The isolate P11 and P12 did not showed HCN production. Verma *et al.*, (2016) also reported the production of ammonia and HCN by fluorescent *Pseudomonas* sp. Microbial production of HCN has been suggested as an

important antifungal feature to control root fungal pathogen as cyanide act as a general metabolic inhibitor to avoid predation or competition. The host plants are generally not

harmfully affected by inoculation with HCN producing bacteria and rhizobacteria can operate as biological control agents (Ramette *et al.*, 2003).

Table.1 Morphological characteristics of fluorescent *Pseudomonas* sp. isolated from apple rhizosphere

Isolates	Shape	Elevation	Edge	Opacity	Pigment
K	Irregular	Flat	Entire	Transparent	Dark Green
L	Irregular	Raised	Entire	Transparent	Yellowish
M	Circular	Raised	Entire	Transparent	Yellowish
P3	Irregular	Flat	Entire	Transparent	Yellowish
P7	Irregular	Flat	Entire	Transparent	Yellowish
P11	Irregular	Flat	Entire	Transparent	Yellowish
P12	Irregular	Raised	Entire	Transparent	Yellowish
P17	Circular	Raised	Entire	Transparent	Yellowish

Table.2 Physiological and biochemical characteristics of fluorescent *Pseudomonas* sp. from apple rhizosphere

Isolates	Gelatin Liquification	Denitrification	Catalase	Tween 80 hydrolysis	Oxidase test	4°C	25°C	41°C	Lecitinase
K	+	+	+	+	+	-	+	+	+
L	+	-	+	-	+	+	+	-	-
M	-	-	+	-	+	+	+	-	-
P3	-	+	+	+	+	+	+	-	-
P7	+	+	+	+	+	+	+	+	-
P11	-	+	+	-	+	+	+	+	-
P12	+	+	+	-	+	+	+	+	-
P17	-	+	+	-	+	+	+	+	-

Table.3 Characterization of fluorescent *Pseudomonas* sp. for plant growth promoting traits

Isolates	Siderophore		Phosphate Solubilization		HCN	Ammonia
	Qualitative	Quantitative	Qualitative	Quantitative	Change of color (yellow to brown)	
	Plate (mm dia)	% SU*	Plate (mm dia)	(Pi µg/ml) **	Paper strip	Culture broth
K	11	33.33	19	46	++	++++
L	12	33.33	17	23	+	+++
M	13	25.92	30	35	+	+++
P3	15	37.03	25	37	+++	++
P7	22	43.32	22	26	+++	+++
P11	13	33.33	30	45	-	+
P12	12	40.74	27	35	-	+
P17	13	25.92	19	46	+++	+++

*The siderophore unit (% SU) expressed as percent reduction in blue color of chrome azurol-S as compared to reference i.e. % SU= (Ar-As)/Ar×100; where, Ar = Absorbance of reference solution at 630 nm; As = Absorbance of test solution at 630 nm

**Phosphate solubilizing activity expressed in terms of tricalcium phosphate solubilization which in turn represents µg/ml of soluble inorganic phosphate(Pi) in supernatant as calibrated from the standard curve of KH₂PO₄ (10-100 µg/ml).

Table.4 Screening of *Pseudomonas* sp. for the production of plant growth regulators

Isolates	Plant growth regulators (µg /ml)		
	Auxin*	Gibberellins**	Cytokinins***
K	11	33.33	19
L	12	33.33	17
M	13	25.92	30
P3	15	37.03	25
P7	22	43.32	22
P11	13	33.33	30
P12	12	40.74	27
P17	13	25.92	19

Extracellular production of plant growth regulators i.e. Auxins*, Gibberellins** and Cytokinins*** expressed in terms of concentration (µg/ml) in 72h old supernatant as calibrated from the standard curve of indole acetic acid (IAA); 10-100 (µg/ml), gibberellic acid (GA₃); 100-1000 (µg/ml), kinetin; 10-100 (µg/ml)

All the fluorescent *Pseudomonas* sp. was characterized for the production of plant growth regulators viz., auxins, gibberellins and cytokinins by their specific spectrophotometric and bioassay methods. The results showed that all the isolates of *Pseudomonas* sp. produced plant growth regulators whereas the level of production of plant growth regulators varies among all the bacterial isolates (Table 4). The isolate P7 produced maximum amount of auxin viz., 22 µg/ml, whereas all the other isolates produced auxins between 11 and 15 µg/ml (Table 4). Similar results were also observed by Verma *et al.*, (2016) in which all the screened fluorescent *Pseudomonas* sp. produced auxin in concentration of 2.0 to 14.0 µg/ml. Similarly Sharma *et al.*, (2014) also reported production of auxin by fluorescent *Pseudomonas* sp. which varied among all the isolates. All the eight isolates of fluorescent *Pseudomonas* sp. showed the production of gibberellins. The gibberellins production was found in the range of 25.92-43.32 µg/ml (Table 4). The maximum gibberellins production was observed in P7 (43.32 µg/ml) followed by P12 (40.74 µg/ml). All the isolates also produced cytokinins in the range of 17- 30 µg/ml (Table 4). The minimum production of auxin was showed by isolate L (17 µg/ml). Thakur *et al.*, (2013) also observed the production of gibberellins and

cytokinins by fluorescent *Pseudomonas* isolates in the range 15.20 to 179.48 µg/ml and 51.20 to 179.48 µg/ml respectively. This study is assumed to be important as the agriculturally beneficial fluorescent *Pseudomonas* sp. could be one of the potential candidates in the development of biofertilizer and these isolates could be explored further under field condition.

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