Evaluation of plant growth promoting (PGP) efficacy of rhizospheric *Pseudomonas* strains

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ABSTRACT

Plant growth promoting (PGP) potential of *Pseudomonas* species was evaluated from the normal and replant rhizospheric soils of apple orchards of district Shimla, HP. Total of twelve isolates were tested for multifarious PGP traits viz phosphate solubilization, production of plant growth regulators, antifungal metabolites and siderophores. The strains were further studied for their ability to produce plant growth promoting activities such as plant growth hormones production viz auxins, gibberellins and cytokinins, phosphate solubilizing activity, siderophore activity, protease enzyme activity, ammonia and HCN production. Auxins, gibberellins and cytokinins produced by isolates were in the range of 10-98, 35-105 and 19-82 µg/ml respectively. *Pseudomonas* isolates showed production of phosphate solubilizing activity in the range of 10 to 84 µg/ml and 15-48 mm in plate assay, siderophore production in the range of 14-28 mm in plate assay and 11.64-53.18 per cent SU in liquid assay. All twelve strains of*Pseudomonas* were also evaluated for their antifungal activity against plant pathogenic fungi. Maximum inhibition was recorded in *Dematophora necatrix* (50-60%) followed by *Pythium ultimum* (30-40%), *Phytopthora cactorum* and *Fusarium cactorum* by all *Pseudomonas* strains. The results suggested that all the isolates possessed multiple PGP traits thus can be further explored for their efficacy as effective PGPR (plant growth promoting rhizobacteria).

Keywords: PGPR; IAA; HCN; inoculation; *Pseudomonas*; soil

INTRODUCTION

Soil exhaustion or apple replant disease is a serious problem which suppresses growth and decreases yield of apple trees in all major fruit growing areas of the world (Hoestra 1968, Szczygieł and Zepp 1993, Švirinas and Lanauskas 2000, Pacholak et al 2004). Symptoms include death of fine feeder roots, stunted growth above and below ground and reduced fruit yields. A diversity of pathogens and parasites has been implicated as causal agents of replant disease and conflicting evidence abounds in the literature as to the importance of these agents in disease development (Jaffee et al 1982, Traquair 1984, Dullahide et al 1994). Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that actively colonize plant roots and increase plant growth and yield (Wu et al 2005). PGPR especially fluorescent *Pseudomonas* spp are highly versatile, diverse and efficient phosphate solubilizer. Fluorescent *Pseudomonas* spp are most promising group of PGPR bacteria that are involved in plant growth enhancement and plant disease control (Ahmadzadeh et al 2006). *Pseudomonas fluorescens* is considered as biological biocontrol agent against various plant related diseases including root diseases (Ursula et al 2000). One of the emerging research areas for the control of different phytopathogenic agents is the use of biocontrol PGPR which are capable of suppressing or preventing the phytopathogen damage (Nihorimbere et al 2011). The chemical fertilizers and pesticides are both hazardous to animals and humans and may persist and accumulate in natural ecosystem (Musa 1976). An answer to this problem is replacing chemicals with biological approaches which are considered more environment-friendly in the long term. The large scale application of indigenous plant growth promoting fluorescent *Pseudomonas* sp may be able to manage replant problem of fruit crops especially apple. Among different PGPR *P fluorescens* is a widely known biocontrol agent against many soil borne plant pathogens. Their applicability as biocontrol agents has drawn wide attention because of the

production of secondary metabolites such as siderophore, antibiotics, volatile compounds, HCN, enzymes and phytohormones. Thus the present study of *Pseudomonas* spp along with their plant growth promoting potential is important not only for understanding their ecological role in the rhizosphere and the interaction with plants but also for any biotechnological applications as plant growth promoting rhizobacteria.

MATERIAL and METHODS

Soil samples from the rhizosphere of apple orchards were collected from both normal and replant areas in Shimla district of Himachal Pradesh. Isolation and screening of fluorescent *Pseudomonas* species was done from the rhizosphere; root samples were shaken vigorously to remove loosely adhering soil; 4.5 ml of sterile physiological water was added to 0.5 g of rhizospheric soil and the mixture was shaken at 120 rpm for 2 minutes. Serial dilutions were prepared from the soil extract and 0.1 ml of each dilution was seeded on to King's B medium (King et al 1954). After 48 h incubation at 28 ± 2°C well separated individual colonies with yellow green and blue white pigments were marked and detected by viewing under UV light.

In vitro assay of fluorescent *Pseudomonas* isolates was done for plant growth promoting traits (Sharma et al 2014). All twelve isolates were screened out both qualitatively andquantitatively for the presence of PGPR traits viz production of plant growth regulators, siderophore, ammonia, HCN, phosphate solubilization and antifungal activity by using their standard methods.

In vitro antagonism against phytopathogenic fungi: Antifungal activity was observed by the formation of inhibition zone of mycelial growth based on agar diffusion of extracellular bacterial metabolite. All *Pseudomonas* isolates were tested for their ability to inhibit the growth of soil-borne phytopathogenic fungi viz *Pythium ultimum*, *Fusarium oxysporum*, *Dematophora necatrix* and *Phytophthora cactorum* by antifungal activity of each test isolate of *Pseudomonas* sp checked by standard well/bit plate assay method (Vincent 1947). Petri plates containing sterile potato dextrose agar were inoculated in the centre with a 5 mm disc of fungal culture grown for 7 days and in the periphery the bacterial strains were inoculated perpendicularly by single streak. Plates were incubated at 28 ± 2°C for 5 days. A bacterial isolate was considered positive for inhibition of fungi when the growth of pathogen under test was absent.

Assay for siderophore production: Siderophore production by *Pseudomonas* isolates was assayed qualitatively by observing orange halos production around the bacterial colony on CAS agar plates and quantitatively in liquid medium as described by Schwyn and Neilands (1987).

Ammonia and HCN production: Bacterial isolates were screened for ammonia production by adopting the method of Lata and Saxena (2003) and hydrogen cyanide (HCN) production was detected by the method given by Bakker and Schippers (1987).

Detection of phosphate solubilising activity: Bacterial isolates were screened on Pikovskaya's agar plates for phosphate solubilization index with known amount of inert phosphorus ($Ca_3(PO_4)_2$) (Pikovskaya 1948). Phosphate solubilisation was expressed in terms of mm diameter of yellow colored zone produced around the well.

Protease activity: All *Pseudomonas* spp were screened out for proteolytic activity by well plate assay (qualitative) method on skim milk agar plates. 100 µl of 72 h old cell free culture supernatant of each bacterial strain was added to each well already cut on skim milk agar plate in which 1 per cent of separately autoclaved skim milk was added to nutrient agar medium. Plates were incubated at 28 ± 2°C for 24-48 h. Proteolytic activity was expressed in terms of mm diameter of clear zones produced around the well (Kaur et al 1989).

Production and estimation of plant growth regulators

Auxins: Measurement of auxins was done by quantitative colorimetric method with slight modifications. Two to three drops of orthophosphoric acid were added to 2 ml supernatant and 4 ml of salper reagent was added (1 ml of 0.5 M $FeCl_3$ in 50 ml of 30% $HClO_4$ prepared fresh). This mixture was incubated for 1 h in dark. Absorbance was measured at 535 nm. Concentration of indole acetic acid (IAA) was estimated by preparing calibration curve using IAA as standard (10-100 µg/ml) (Gordon and Paleg 1957).

Gibberellins: The method of Holbrook et al (1961) with slight modifications was used for the determination of gibberllins. To 15 ml of supernatant 2 ml of zinc acetate reagent (21.9 g zinc acetate + 1 ml of glacial acetic acid and volume made to 100 ml with distilled water) was added. After 2 minutes 2 ml of potassium ferrocyanide (10.6% in distilled water) was added and was centrifuged at low speed (2000 rpm) for 15 minutes. To 5 ml of supernatant 5 ml of 30 per cent HCl was added and mixture was incubated at 20°C for 75 minutes. For blank 5 ml of 5 per cent HCl was used. Absorbance was read at 254 nm and concentration of gibberellins was calculated by preparing standard curve by using gibberellic acid (GA_3) as standard (100-1000 µg/ml) (Holbrook et al 1961).

Bioassay of Cytokinins: Radish cotyledons expansion bioassay test was employed for estimation of cytokinin like substances. The radish seeds (*Raphanus sativus* L cv Japanese White) were germinated in total darkness for 48 h at 28°C. After removing the seed coat smaller cotyledons were transferred to sterilized Petri dishes containing the test solution/distilled water (control)/ standard (kinetin) on filter paper strips. 12 cotyledons were placed in each Petri dish and included at 25°C under fluorescent light for 3 days. Cotyledons on filter paper strips were blotted, dried and weighed. The bioassay response (final weight/initial weight) was expressed as increase in weight of cotyledons. Concentration of cytokinins present in the extract was calculated by preparing standard curve by using kinetin as standard (10-100 µg/ml) (Letham 1971).

RESULTS and DISCUSSION

Every isolate tested showed at least one of the growth promotion traits that were investigated. The isolates in this study presented several enviable features for PGPR and multiple action mechanisms which suggest their potential for growth promotion. They all produced fluorescent pigment belonging to genus *Pseudomonas* especially fluorescent group. The versatile, predominant and desirable organisms viz *Pseudomonas* spp were isolated. It may be attributed to specific choice of media employed for isolation viz nutrient agar and King's B. Several others hence supported to use King's B medium for isolation of fluorescent *Pseudomonas* sp (King et al 1954). For the isolation of pseudomonads and fluorescent *Pseudomonas* sp TSA and King's medium B agar (Raaijmakers and Weller 1998) were used.

Table 1. Plant growth promoting (PGP) characterization of various *Pseudomonas* species

Isolate	Plant growth promoting (PGP) activities								
	HCN production	Ammonia production	Siderophore (mm diameter)	SU (%)	Phosphate solubilization (mm diameter)	Protease (mm diameter)	Auxin (µg/ml)	Gibberellins (µg/ml)	Cytokinins (µg/ml)
An-1-Si	++++	++++	18	47.06	39	-	57	70.30	23.00
An-2-Si	++	+++	20	53.18	38	10	47	88.20	42.00
An-3-Mg	++	+++	15	46.94	29	11	32	90.30	35.00
An-5-Mg	++++	+++	28	30.25	48	11	63	35.00	50.00
An-7-Mg	++++	++++	26	48.74	37	16	31	54.10	69.00
An-9-Si	++	+++	23	49.70	37	17	45	64.00	75.00
An-10-Si	+	++++	20	47.78	40	10	30	85.00	82.00
An-12-Si	++	++++	18	36.25	15	10	58	100.00	39.00
Ar-13-Mg	++++	+++	14	49.58	21	12	59	43.00	19.00
Ar-14-Mg	+++	++++	16	40.45	39	10	50	46.00	38.00
Ar-18-Mg	++++	++++	22	47.54	35	14	20	66.00	49.00
Ar-19-Mg	+++	+++	16	11.64	37	12	45	75.00	50.00
$CD_{0.05}$			1.742	1.489	1.695	1.573	1.695	1.695	1.695

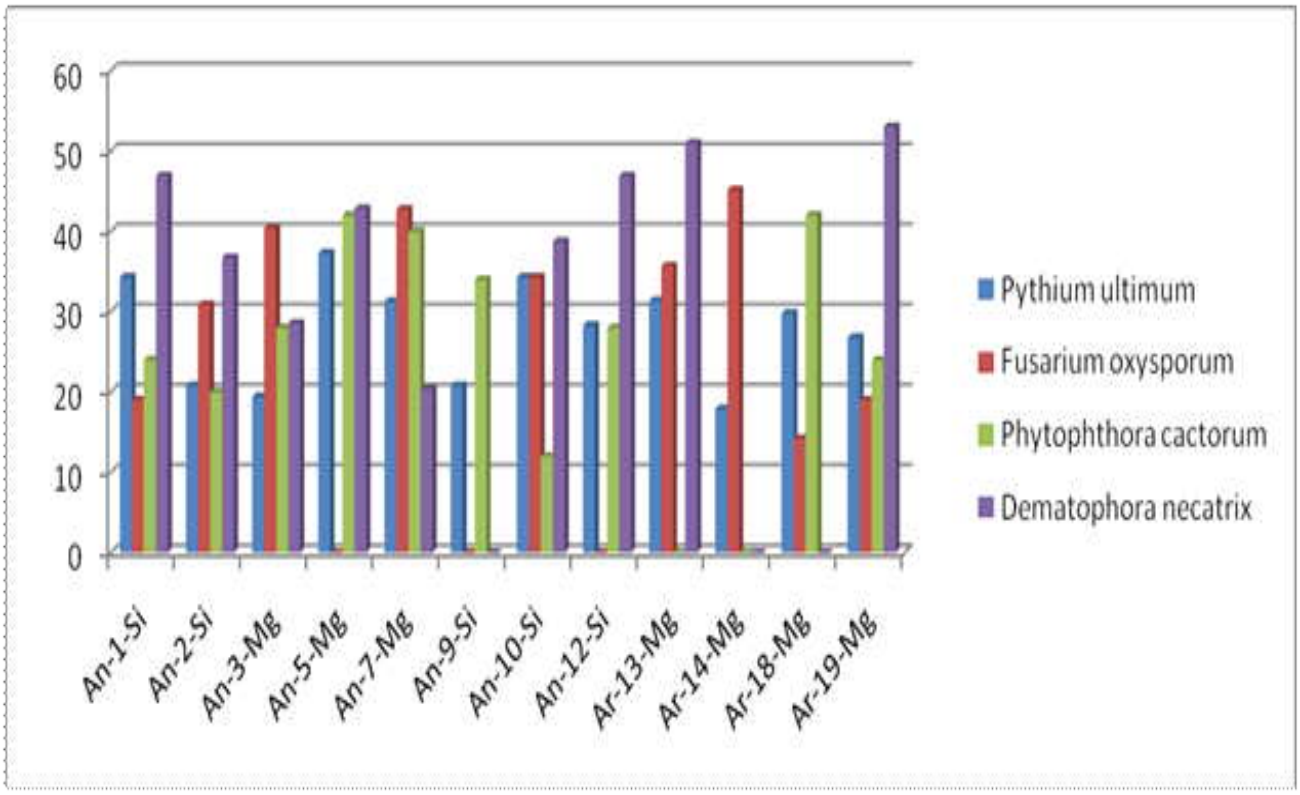

Fig 1. Antifungal activity of *Pseudomonas* strains against different indicator test fungi isolated from replant sites of apple

The strains were further studied for their ability to produce plant growth promoting activities such as plant growth hormone production viz auxins, gibberellins and cytokinins, phosphate solubilizing activity, siderophore activity, protease enzyme activity, ammonia production and HCN production. All the isolates showed HCN and ammonia production and eleven isolates showed protease production. All the isolates showed auxins, gibberellins and cytokinins produced by isolates in the range of 10-98, 35-105 and 19-76 µg/ml respectively; production of phosphate solubilizing activity in the range of 10 to 84 µg/ml and 15-48 mm in plate assay, siderophore production in the range of 16-32 mm in plate assay and 11.64-53.18 per cent SU in liquid assay and antifungal activity against plant pathogenic fungi. Maximum inhibition was recorded in *D necatrix* (50-60%) followed by *P ultimum* (30-40%), *P cactorum* and *F cactorum* by all *Pseudomonas* strains (Fig 1). The results suggested that all the isolates possessed multiple PGP traits thus can be further explored for their efficacy as effective PGPR (Table 1). This preliminary investigation shows that the fluorescent *Pseudomonas* strains are able to promote plant growth. The production of secondary metabolites such as IAA, HCN, salicylic acid, chitinase and siderophore by fluorescent pseudomonads is important feature in plant disease suppression of root rot and enhancement of plant growth (Gade 2013).

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