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Evaluation of Fluorescent *Pseudomonas* sp. for their Activity against Plant Pathogenic Fungi in Replant Sites of Apple and Pear

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The aim of the study was to assess *Pseudomonas sp*. for their plant growth and antagonistic activity against most probable fungal pathogens associated with apple and pear plants viz., *Dematophora sp*., *Fusarium sp*., *Pythium sp.* and *Sclerotium sp.* These isolates were phenotypically and biochemically characterized from the rhizosphere of normal and replant sites of apple and pear from different locations of two districts of H.P i.e. Chamba and Mandi. The isolates of *Pseudomonas* sp. were screened out for the direct and indirect plant growth promoting activities viz., siderophores, phosphate solubilizing, HCN, ammonia, lytic enzymes and plant growth regulators viz., auxins, cytokinins and gibberellins so as to screen potential strains to be further developed and used as inoculants for management of replant problem of apple and pear. Out of twenty nine *Pseudomonas* sp. isolates, seven isolates were selected on the basis of overall best PGPR activities including antifungal activity for the extraction and isolation of antifungal metabolite DAPG (2,4-diacetylphloroglucinol) from them.

Introduction

Biocontrol with antagonistic bacteria (such as fluorescent Pseudomonads) is deemed to be an alternate approach to agrochemicals which are detrimental to human health and environment. Fluorescent Pseudomonad species such as *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Pseudomonas aureofaciens*, *Pseudomonas putida* and *Pseudomonas pyrrocinia* have shown varying degrees of antagonism to demonstrate antifungal activity (Hu*et al.,* 2005) because of the production of indole-3-acetic acid, siderophores and antibiotics such as phenazine-1-carboxylic acid (PCA), pyocyanin, 2-acetamidophenol, pyrrolnitrin, pyoluteorin, PCN, 2,4 diacetylphloroglucinol, viscosinamide and tensin (Nielsen *et al*,. 2000, Hammer *et al.,* 1999). The incorporation of their important features such as antifungal antibiotics production,

siderophore iron chelation, lytic enzymes, plant growth regulators, phosphate solubilization, ammonia and HCN production with ecological strain fitness will become essential for the design of effective, safe and reliable novel bioagents (Rana *et al.,* 2016).

The development of different strategies using a mixture of PGPR strain especially fluorescent *Pseudomonas* sp. would be an important emerging area in crop growth promotion, protection and also in establishment of newly planted trees on old sites of apple and pear. It may reduce the losses caused by replant problem in economically important fruit crops especially apple.

The large scale applications of PGPR to crops as inoculants would be attractive. So isolation, preliminary and genetic characterization of indigenous fluorescent *Pseudomonas* sp. is very important. Current work has therefore been carried out to find best bacterial isolates effectively control the phytopathogens and promote plant growth in replant site of apple and pear.

Materials and Methods

Sample collection

Isolation of fluorescent *Pseudomonas* sp. was done from soil samples collected from the rhizosphere of apple and pear in normal and replant sites of Chamba and Mandi districts of H.P.

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., Penicillin-G (75,000 units/l), Cycloheximide (75 mg/l) and Novobiocin (45 mg/l). Plates were incubated at 28 ± 2℃ for 24 – 48 h (Rana *et al.,* 2014).

Screening of *Pseudomonas* sp. Isolates for *in vitro* production of plant growth promoting activities

All isolates were screened for the production of siderophores, phosphate solubilization, HCN, ammonia, lytic enzymes and plant growth regulators. Quantitative detection of siderophore production in liquid medium of Chrome-azurol-S (CAS) was carried out and change in the colour of reaction mixture was observed from dark blue to orange or pink (Schwyn and Neilands, 1987). Phosphorous estimation (Sharma *et al.,* 2014) was done quantitatively in PVK broth supplemented with 5.0 g/l tri-calcium phosphate (TCP). Estimation of hydrocyanic acid (HCN) production by *Pseudomonas* sp., a color change was observed from yellow to orange brown to dark brownin the sodium picrate containing filter paper strip (Bakker and Schippers, 1987). The method of Lata and Saxena, (2003) was used for the estimation of ammonia production. All *Pseudomonas* sp. isolates were screened out for proteolytic activity by well plate assay (Kaur *et al*., 1988) on skim milk agar plates. Proteolytic activity was expressed in terms of mm diameter of clear zones produced around the well. Quantitative measurement of auxins was done by colorimetric method (Gorden and Paleg, 1957) with slight modification. The gibberellins were estimated colorimetrically by the method of Holbrook *et al.,* (1961). For bioassay of cytokinins, the radish cotyledon expansion test was employed (Letham, 1971). The bioassay response (final weight - initial weight) was expressed as increase in weight of cotyledons. Antifungal activity was checked by well plate assay method (Vincent, 1947) using dual culture technique. Plates were incubated at 28 ± 2℃ for 4 days and observed for inhibition of mycelial growth produced around the well. For control, culture bit of indicator fungus was kept in the centre of MEA plate and incubated at 28 ± 2℃ for 4days.

Extraction of antifungal metabolite from selected *Pseudomonas* sp. Isolates

Out of twenty-nine isolates seven *Pseudomonas* sp. isolates were selected on the basis of overall best PGPR activities for the extraction of antifungal metabolite. Isolates were grown in 25 ml of pigment production media broth, incubated for five days at 30°C and centrifuged at 3500 rpm for 5 minutes. The collected supernatant of each *Pseudomonas* sp. was acidified to pH 2.0 with 1N HCL.

Extraction was done with equal volume of ethyl acetate (Rana S *et al.,* 2014). Ethyl acetate fraction of each *Pseudomonas* isolates was reduced to dryness in Vacuum. Residue were redissolved in methanol separately. 10µl samples was chromatographed on Silica-G TLC plates and Chloroform:methanol (9:1) solvent system was used. TLC was visualized under UV and sprayed with Para-anisaldehyde and compared bands with reference compound. Antifungal activity of each crude extract was checked by well plate assay method.

Results and Discussion

Pseudomonas and many soil microorganisms developed antifungal and antibacterial compounds such as antibiotics, iron-chelating siderophores, cyanides and enzymes such as gluconase, cellulite and chitinolytic enzymes which inhibit fungal growth by damaging cell walls (Voisard *et al.,* 1989, Sindhu and Dadarwal, 2001).

Variable compounds with antifungal and antibacterial nature were produced by *Pseudomonas* sp. and many soil microorganisms such as antibiotics, iron chelating siderophores, cyanide and enzymes such as gluconase, cellulytic and chitinolytic enzymes (Voisard *et al.,* 1989). The activity of these compounds was reported among antifungal mechanisms by which *Pseudomonas* strains inhibited the fungal growth through damaging of cell walls (Sindhu and Dadarwal, 2001).

In the present study, bacterial isolates from the apple and pear rhizosphere were found to be fluorescent, pigmented (greenish, yellowish green and some also produced dark brownish pigment along with fluorescence), transparent to translucent, with maximum isolates being coccobacillus and irregular in shape while few were rods. All the isolates were gram-negative, negative spore, and motile (Table 1). Almost all the isolates were positive for catalase, oxidase, gelatin liquification, denitrification (Table 2). Maximum % of siderophore production which was estimated by CAS assay was showed by PN-11-San i.e. 47% siderophore unit followed by PN-12-San i.e. 45% siderophore unit.Phosphorus (P) is an essential macronutrient for biological growth and development (Fernandez *et al.,* 2007).

Quantitative estimation of phosphate solubilization revealed that the *Pseudomonas* sp. isolates PR-2-San followed by PN-7-Cha have a higher capacity to release 355 µg P ml^{-1} and 345µg P ml^{-1}, in PVK-TCP culture media, respectively.

Bacteria from the rhizosphere protect crops by producing secondary metabolites or extracellular lytic enzymes. Several studies have shown that exposure to lytic enzymes such as chitinases, proteases or glucanases of selected phytopathogenic fungi can lead to degradation of the structural matrix of fungal cell walls (Dunne *et al.,* 1997b). *Pseudomonas* sp. are also able to synthesize HCN to which these *Pseudomonas* sp. are themselves resistant that may be linked to the ability of these strains to inhibit some pathogenic fungi.

Table.1 Morphological characterization of fluorescent *Pseudomonas sp*. Isolates from pear rhizosphere

Site	Isolates	Colony Morphology				Gram reaction	Spore staining	Pigment	Levan /Slime Production
		Shape	Elevation	Edge	Opacity				
Chamba	AN-1-Cha	Irregular	Flat	Entire	Transparent	-	-	Yellowish green	-
	AR-5-Cha	Irregular	Flat	Entire	Transparent	-	-	Yellowish green	-
	PN-7-Cha	Irregular	Flat	Entire	Transparent	-	-	Yellowish green	-
	PN-8-Cha	Irregular	Flat	Entire	Transparent	-	-	Yellowish green	-
Mandi	PN-11-San	Irregular	Raised	Entire	Translucent	-	-	Dark green	Mucoid
	PN-12-San	Rods	Flat	Entire	Translucent	-	-	Grayish	-
	PR-2-San	Coccobacillus	Raised	Entire	Translucent	-	-	Grayish	-

Table.2 Physiological and biochemical characteristics of fluorescent *Pseudomonas* isolates

Site	*Pseudomonas* Isolates	Catalase test	Oxidase test	Aerobic/ Anaerobic growth	Growth at 4°C, 25°C and 41°C			Gelatin liquification	Denitrification test
					4°C	25°C	42°C		
Chamba	AN-1-Cha	+	+	Aerobic	-	+	+	+	+
	AR-5-Cha	+	+	Aerobic	-	+	+	+	+
	PN-7-Cha	+	+	Aerobic	+	+	+	+	+
	PN-8-Cha	+	+	Aerobic	-	+	+	+	-
Mandi	PN-11-San	+	+	Aerobic	-	+	+	+	+
	PN-12-San	+	+	Aerobic	+	+	+	+	-
	PR-2-San	+	+	Aerobic	-	+	-	+	-

Table.3 Plant growth promoting activities of fluorescent *Pseudomonas* isolates

Pseudomonas isolates	Site	Siderophores (%SU)	Phosphate Solubilization (µg/ml)	Proteolytic (mm)	HCN	Ammonia	Auxins (µg/ml)	Gibberellins (µg/ml)	Cytokinins (µg/ml)
AN-1-Cha	Chamba	28.39	290	25	+	+++	7	510	394
AR-5-Cha	Chamba	32.14	115	28	+	++++	10	490	391
PN-7-Cha	Chamba	40.71	345	28	+	+++	7	550	450
PN-8-Cha	Chamba	35.35	225	25	+++	+++	4	550	502
PN-11-San	Mandi	47	285	24	++++	++++	3	365	232
PN-12-San	Mandi	45	300	23	+++	++++	12	470	240
PR-2-San	Mandi	39	355	22	+++	+++	3	490	570

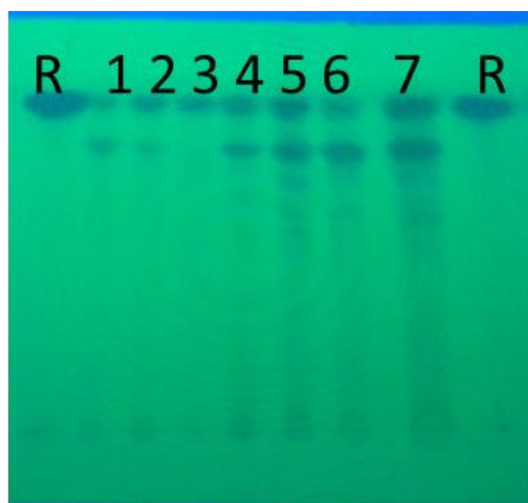
Table.4 Antifungal activity shown by fluorescent *Pseudomonas* isolates against different plant pathogenic fungi in terms of per cent inhibition

Pseudomonas isolates	Site	*Dematophora* sp.(% I)	*Fusarium* sp. (%I)	*Pythium* sp. (%I)	*Sclerotium* sp.(%I)
AN-1-Cha	Chamba	45.83	28.57	22.22	37.14
AR-5-Cha	Chamba	27.08	0.00	22.22	28.57
PN-7-Cha	Chamba	29.16	22.85	22.22	34.28
PN-8-Cha	Chamba	27.08	28.57	26.66	28.57
PN-11-San	Mandi	0.00	34.28	22.22	28.57
PN-12-San	Mandi	33.33	22.85	33.33	37.14
PR-2-San	Mandi	37.5	40	33.33	31.42

Table.5 Antifungal activity of extracted metabolite against *Pythium* sp

Pseudomonas isolates	Site	% Growth inhibition
AN-1-Cha	Chamba	22.87
AR-5-Cha	Chamba	34.37
PN-7-Cha	Chamba	23.75
PN-8-Cha	Chamba	28.12
PN-11-San	Mandi	27.27
PN-12-San	Mandi	37.27
PR-2-San	Mandi	33.36

Fig.1 Thin layer chromatographic analysis (Under UV) of extracted antifungal metabolite 2,4-diacetylphloroglucinol (Rf 0.86) from fluorescent *Pseudomonas* sp. Lane (R) Reference, (1) AN-1-Cha, (2) AR-5-Cha, (3) PN-7-Cha, (4) PN-8-Cha, (5) PN-11-San, (6) PN-12-San, (7) PR-2-San, on Silica gel G by using solvent system chloroform: methanol (9:1)



Maximum Proteolytic activity was observed in case of *Pseudomonas* sp. isolates AR-5-Cha and PN-7-Cha (28mm dia.). *Pseudomonas* sp. isolate PN-11-San showed maximum HCN production followed by PN-12-San, PR-2-San and PN-8-Cha. Similarly, AR-5-Cha, PN-11-San and PN-12-San showed maximum amount of ammonia production.

Different micro-organisms have been recognized to exude different plant growth promoters. Quantitative estimation of plant growth regulators revealed that PN-12-San showed maximum auxin production i.e. 12 µg/ml. PN-7-Cha and PN-8-Cha showed maximum production of gibberellins i.e. 550 µg/ml. Maximum cytokinin production was observed in case of PN-8-Cha i.e. 502 µg/ml (Table 3). Dual culture studies revealed that all the bacterial isolates effectively control the phytopathogens (Table 4). Against *Dematophora* sp. AN-1-Cha showed maximum % inhibition i.e. 45.83 %I, similarly PR-2-San, PN-12-San and PR-2-San; AN-1-Cha and PN-12-San showed maximum %inhibition against *Fusarium* sp. i.e. 40%I, *Pythium* sp. i.e. 33.33%I and *Sclerotium* sp. i.e. 37.14%I respectively. In case of PN-11-San and AR-5-Cha no inhibition was observed against *Dematophora* sp. and *Fusarium* sp. respectively.

Bacteria that produce DAPG play a key role in agriculture, and their potential for use in sustainable agriculture is promising. Production of antibiotics is a primary mechanism of pathogen inhibition (Weller, 1988). DAPG is produced by fluorescent *Pseudomonas* sp. of diverse geographic origin that has common ability to suppress one or more root and seedling diseases of crop plants caused by soil-borne pathogens. Seven *Pseudomonas* isolates were chosen overall on the basis of best plant growth promoting activities for the extraction of active antifungal metabolite i.e. may belong to 2,4-diacetylphloroglucinol. Thin layer chromatography showed orange coloured amount of spots that is the indication of antifungal activity. The Rf value shown by the ethyl acetate extracted metabolite of *Pseudomonas* isolates was 0.86 (Fig. 1). These results gave an idea that extracted antifungal metabolites may be related to the group of antibiotic i.e. 2, 4-diacetylphloroglucinol. Crude extract of all seven *Pseudomonas* isolates showed percent growth inhibition against *Pythium* sp. Maximum percent growth inhibition was shown by PN-12-San (37.37%I) andAR-5-Cha (34.37%I) (Table 5). This research describes the multifarious role played by *Pseudomonas* sp. with potentials encouraging plant growth. The option of such bacteria will increase their utility in sustainable organic farming as bio-inoculations further.

In conclusion the fluorescent *Pseudomonas* are effective root colonizer along with other direct and indirect plant growth promoting activities. They control deleterious fungi and bacteria due to production of many indirect PGP activities like broad-spectrum antifungal antibiotics, iron chelating siderophores, HCN, ammonia, supply of nutrients like available phosphorus, iron ions and other small molecules through P-solubilizing and lytic enzymes. Therefore, these isolates can be further used as inoculants for management of replant problem of apple and pear and can be considered as an alternative strategy to agrochemicals as they are environment friendly.

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