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Biocontrol potentialities of native *Pseudomonas* isolates of Himachal Pradesh against plant pathogenic fungi *Dematophora sp.*, *Fusarium sp.*, *Pythium sp.* and *Sclerotium sp.* of apple rhizosphere

Sheetal Rana, Ranjna Sharma and Mohinder Kaur

Abstract
Twenty indigenous fluorescent pseudomonads were isolated from the replant sites of apple from three district of Himachal Pradesh i.e. Chamba, Kullu and Mandi. The research work was carried out with the aim to characterize *Pseudomonas* sp. biochemically and study biocontrol potential against different plant pathogens i.e. *Dematophora sp.*, *Fusarium sp.*, *Pythium sp.* and *Sclerotium sp.* Among the isolates tested, Ar-3-Nag showed maximum % inhibition against *Dematophora sp*., *Fusarium sp.* and *Sclerotium sp.* i.e. 37.5, 34.20 and 45.71 respectively. Against *Pythium sp.* maximum % inhibition was showed by An-5-Kul i.e. 37.5%. Five isolates i.e. An-4-Kul, An-5-Kul, An-11-Kul, Ar-3-Nag and PN-6-San showed inhibition of mycelial growth against all four fungus pathogens. The result shows that these *Pseudomonas* isolates were very effective biocontrol agents and should be exploited for biocontrol applications.

Keywords: *Pseudomonas sp*., replant site, apple, biocontrol

Introduction
The new trees often grow poorly when fruit growers renovate and replant apple orchards and fail to meet standards for early yields or profitability. This problem is known as replant disease of apple (Mai and Abwai, 1981) [12]. Replant disease has been linked with abiotic problems such as soil nutrients loss, compaction or acidification, and the phytotoxic residues of arsenic or old roots. Biotic problems such as parasitic nematodes or fungal and bacterial infections of tree roots have also been involved.

Pre-plant soil fumigation is the primary method used for replant disease control because of presumed uncertainty regarding replant disease etiology (Willet *et al*., 1994) [22]. While fumigants consistently provide effective control of disease (Braun *et al*., 2010; Covey *et al*., 1979) [2, 3], there are disadvantages inherent in the use of such broad-spectrum biocides, including implementation difficulties, high costs and potential risks to human health. In addition, the depletion of active chemistries, such as methyl bromide, gives rise to concerns about the widespread use and long-term availability of currently registered fumigants (Helliker, 2002; Tewoldemedhin *et al*., 2011) [7, 21], and there is a need for more environment safe strategies for the management of non-fumigant diseases.

There is significant interest in finding alternate control methods for use in integrated pest management strategies for crop diseases in response to environmental and health concerns regarding extended use of pesticides (Kloepper *et al*., 1989a) [9].

Fluorescent Pseudomonads are considered the most promising and versatile plant-growth promoting rhizobacteria involved in plant disease biocontrol. They contain secondary metabolites such as antibiotics, phytohormones, hydrogen cyanide (HCN), volatile compound, and siderophores. These bacteria's growth-promoting capacity is mainly due to the synthesis of indole-3-acetic acid (Patten and Glick, 2002) [15], siderophores (Schippers *et al*., 1990) [19], and antibiotics. Root-colonizing fluorescent Pseudomonads develop a variety of antifungal and antimicrobial extracellular metabolites, some of which play a deciding role in the suppression of diseases. Important antibiotic compounds which have been shown to contribute significantly to biocontrol include 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, cyclic lipopeptides, phenazines, pyrrolnitrin, and hydrogen cyanide. The purpose of this study was to isolate, screen and characterize antagonistic bacteria from apple soil replant rhizosphere for their biocontrol activities.

Material and Methods

Isolation and biochemical characterization of fluorescent *Pseudomonas* isolates

Fluorescent *Pseudomonas* isolation was achieved by means of serial dilution technique. Plates were incubated for 48 h at 28 °C. After incubation, well-separated individual colonies were labelled and detected under UV light with yellow green and blue white pigments. Gram staining and pigment production in various fluorescent *Pseudomonas* isolates, oxidase, catalase, tween-80 hydrolyzation, lecithinase activity and gelatine liquefication were also carried out in accordance with the protocol (Rana *et al*., 2015) [17, 18]. Tests on oxidase and catalase were performed to confirm the aerobic existence of pseudomonads.

Characterization of antimicrobial traits in fluorescent pseudomonads

Siderophore production

For quantitative estimation of siderophore, chrome azurol-S (CAS) liquid assay method was used (Schwyan and Neilands, 1987) [20]. 0.5 ml of 72 h old cell free supernatant was mixed with 0.5 ml CAS assay solution (1.5 ml of 1 mM $FeCI_3$, $6H_2O$ in 10 mM HCl + 7.5 ml of 2 mM CAS stock solution dissolved 0.0219 g HDTMA in 50 ml distilled water and then mix in 100 ml cylinder, add 4.307 g piperazine in 30 ml distilled water (pH 5.6) and final volume was made to 100 ml with distilled water, 10 µl shuttle solution (0.2 M 5-sulfosalicylic acid) was added. Colour intensity of the solution was recorded at 630 nm against reference after 10 minutes at room temperature. Siderophore production was observed in terms of reduction in blue colour as per cent siderophore units (%SU).

$$\%SU = \frac{Ar\text{-}As}{Ar} X100$$

Ar = Absorbance of reference at 630 nm
As = Absorbance of supernatant at 630 nm

Protease Activity

Pseudomonas sp. isolates were screened out for proteolytic activity by well plate assay (Kaur *et al.*, 2020) [8] on skim milk agar plates. 100µl of 48 h. old cell free supernatant of each *Pseudomonas* sp. isolate was added to each well already cut on the prepoured skim milk plates. These plates were then incubated at 28±2ºC for 48 h. and observed for clear zone production around the well. Proteolytic activity was expressed in terms of mm diameter of clear zones produced around the well.

Hydrogen cyanide production

Sterilized filter paper strip saturated with a solution containing picric acid, 0.5%, and sodium carbonate (2.0%) was put inside the NA slants adjusted with glycine (4.4 g / l) and FeCl3 6H2O (0.3 mM) already inoculated with test bacteria and hermetic sealing. Changing the filter paper color in 5 days from yellow to brown was considered a positive reaction (Bakker and Schippers 1987) [1].

Ammonia Production

For the detection of ammonia production, the method of Lata and Saxena, (2003) [11] was used. *Pseudomonas* sp. isolates were grown in tubes with 5 ml of peptone water. Tubes were incubated over 4 days at 28±2°C. After 4 days, each tube was fitted with 1ml of Nessler's reagent. The appearance of very light brown (+) indicates small amounts of production of ammonia, and light brown (+ +) indicates large amounts of production of ammonia to orange brown (+++).

Antagonistic activity

Antifungal activity of each test isolate of *Pseudomonas* sp. against *Dematophora sp., Fusarium sp., Pythium sp.* and *Sclerotium sp.* was checked by well plate assay method (Rana *et al*., 2015) [17, 18]. On one side of prepoured sterilized malt extract agar (MEA) plates, 4-day-old culture bit of indicator fungi was placed.100µl of 72 h old cell free supernatant was applied to well on the other side.

Plates were incubated for 4 days at 28±2°C and observed for inhibition of mycelial growth produced around the well. Culture bit of indicator fungus was kept in MEA plate centre for control and incubated at 28±2°C for 4 days. Antifungal activity expressed in terms of percent inhibition growth of of fungal mycelia as calculated from the equation:

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

Where I = Percent inhibition, C = Radial growth of fungus in control, and Z= Radial growth of fungus in treatment.

Results and Discussion

Twenty Fluorescent *Pseudomonas* isolates were collected from the replant sites of apple orchards from Chamba, Kullu and Mandi districts of Himachal Pradesh. Isolation was done using King's B medium by serial dilution method. All the isolates showed Gram negative reaction, rod shape, Pigment Production and fluorescence under UV light which confirmed that all the isolates belong to fluorescent Pseudomonads group. Further characterization of them revealed that all the isolates were oxidase and catalase positive. Nine isolates showed tween 80 hydrolysis and nineteen isolates showed lipase and lecithinase test (Table 1).

The fluorescent *Pseudomonas* isolates are known to produce many secondary metabolites such as HCN and Ammonia, Siderophores and Protease enzyme which has antagonistic properties against many phytopathogens.

All isolates were positive for HCN and ammonia. All the isolates of fluorescent *Pseudomonas* sp. were positive for siderophore and protease production (Table 2). As an initial step in the identification and exploitation of bioagents, collection and characterization of fluorescent pseudomonad isolates were done from replant site of apple orchards from Chamba, Kullu and Mandi district of Himachal Pradesh. In addition, these isolates were characterized and the results of these isolates were correlated with the identification and screening of pgpr traits (Rana *et al* 2014; Meera and Balabaskar 2012; Deshwal and Kumar, 2013) [14, 4]. Fiddaman and Rossall (1993) [5] documented the antimicrobial existence of these secondary metabolites, which mentioned the fungal cell-wall degradation activity of HCN that was derived from *Pseudomonas fluorescens*. Manidipa *et al*. (2013) [13] reported that in *Pseudomonas* the development of antibiotics, siderophores, volatile compounds, hydrocyanic acid (HCN), enzymes, and phytohormones effectively regulated rice fungal and bacterial diseases.

Table 1: Physiological and biochemical characteristics of fluorescent *Pseudomonas* isolates

Site	Isolates	Tween 80 hydrolysis	Lecithinate test	Extracellular production of Lipase	Oxidase	Catalase
Chamba	AN-2-Cha	+	+	+	+	+
	AR-4-Cha	-	+	+	+	+
Kullu	An-4-Kul	-	+	+	+	+
	An-5-Kul	-	+	+	+	+
	An-6-Kul	-	+	+	+	+
	An-11-Kul	-	+	+	+	+
	An-15-Kul	-	-	-	+	+
Mandi	AN-1-Nag	+	+	+	+	+
	AN-2-Nag	+	+	+	+	+
	AN-3-Nag	-	+	+	+	+
	AN-4-Nag	+	+	+	+	+
	AN-8-Nag	+	+	+	+	+
	AN-9-Nag	Slightly +	+	+	+	+
	AR-3-Nag	+	+	+	+	+
	PN-3-San	-	+	+	+	+
	PN-6-San	+	+	+	+	+
	PN-7-San	Slightly +	+	+	+	+
	PR-1-San	-	+	+	+	+
	PR-3-San	-	+	+	+	+

Table 2: Characterization of fluorescent pseudomonads isolates for antimicrobial traits

Site	*Pseudomonas* isolates	% siderophore unit** (SU %)	Proteolytic activity Well Plate assay (mm dia.)	HCN Colour change (yellow to brown)	Ammonia Production
Chamba	AN-2-Cha	29.82	26	+	+++
	AR-4-Cha	60.53	18	+	+++
Kullu	An-4-Kul	39.14	21	+	++++
	An-5-Kul	70	18	+	+++
	An-6-Kul	30.59	21	++	++
	An-11-Kul	37.82	22	++	++++
	An-15-Kul	25.65	19	+	++
Mandi	AN-1-Nag	47.32	29.5	+	+++
	AN-2-Nag	48.39	30	+	+++
	AN-3-Nag	20.98	24	+	+++
	AN-4-Nag	41	22	+	+++
	AN-8-Nag	11.60	29.5	+	+++
	AN-9-Nag	24.55	22.5	+	+++
	AR-3-Nag	45.53	14	+	+++
	PN-3-San	43	17	+	++++
	PN-6-San	60	23	+	+++
	PN-7-San	54	24	+	+++
	PR-1-San	39	20	+	+++
	PR-3-San	32	21	+	+++

Efficacy of 20 indigenous fluorescent pseudomonads were studied under *in vitro* by screening against *Dematophora sp.*, *Fusarium sp.*, *Pythium sp.* and *Sclerotium sp.* using the dual culture method. The results on inhibition of mycelial growth of *Dematophora sp.*, *Fusarium sp.*, *Pythium sp.* and *Sclerotium sp.* was recorded and presented here under. Among the isolates tested, Ar-3-Nag showed maximum % inhibition against *Dematophora sp*. and *Fusarium sp* i.e. 37.5 and 34.20 respectively. Against *Pythium sp.* maximum % inhibition was showed by An-5-Kul i.e. 37.5% and against *Sclerotium sp.* maximum % inhibition was showed by Ar-3-Nag i.e. 45.71 (Fig 1). Five isolates i.e. An-4-Kul, An-5-Kul, An-11-Kul, Ar-3-Nag and PN-6-San showed inhibition of mycelial growth against all four fungus pathogens.

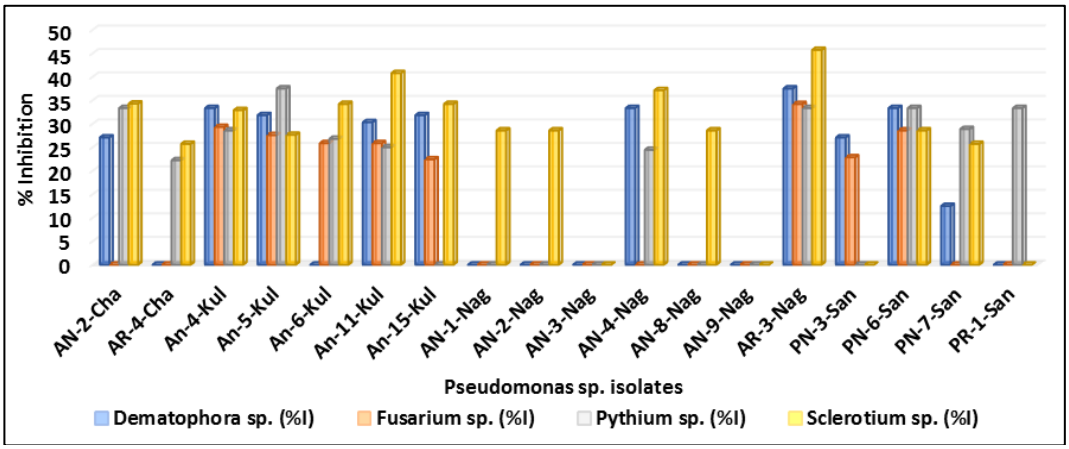

Fig 1: Against *Pythium sp.* maximum % inhibition was showed by An-5-Kul i.e. 37.5% and against *Sclerotium sp.* maximum % inhibition was showed by Ar-3-Nag i.e. 45.71

Fluorescent *Pseudomonas* colonize the rhizosphere and protect plants against agronomic fungal diseases such as black root - rot of tobacco, root - rot of wheat, damping - off of sugar beet, root - rot of pea and therefore has been suggested as a possible biological control agent. Fluorescent *Pseudomonas* also exhibits significant antifungal activity in rice and sugarcane rhizosphere against *Rhizoctonia bataticola* and *Fusarium oxysporum*, primarily through the formation of antifungal metabolites (Kumar *et al*., 2004) [10].

Pseudomonas exhibits biocontrol ability *in vivo* and *in vitro* conditions against phytopathogenic fungi from chickpea rhizosphere. *Pseudomonas putida* has root-rot disease complex of chickpea biocontrol potential by demonstrating antifungal activity against *Macrophomina phaseolina.* Fluorescent *Pseudomonas* also inhibits *Phytophthora capsici*, the causal organism of foot rot disease (Paul and Sarma, 2006) [16]. *Pseudomonas aeruginosa* Sha8 produces toxic volatile compound which inhibit the growth of both *Fusarium oxysporum* and *Helmithosporium sp*. (Hassanein *et al*., 2009) [6]. To conclude, *Pseudomonas* sp. apparently predominate as multipotential PGPR with biocontrol traits irrespective of the rhizosphere; a combination rarely explored in known beneficial rhizobacteria.

References

1. Bakker AW, Schippers B. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas spp*. Mediated plant growth stimulation. Soil Biology and Biochemistry. 1987; 19:451-457.
2. Braun PG, Fuller KD, McRae K, Fillmore SAE. Response of “HoneycrispR” apple trees to combinations of pre-plant fumigation, deep ripping, and hog manure compost incorporation in a soil with replant disease. Horticulture Science. 2010; 45:1702-1707.
3. Covey R, Benson NR, Haglund WA. Effects of soil fumigation on the apple replant disease in Washington. Phytopathology. 1979; 69:684-86.
4. Deshwal VK, Kumar P. Effect of heavy metals on growth and PGPR activity of Pseudomonads and production of plant growth promoting substances by Pseudomonads. J Acad Indus Res. 2013; 2(5):286-290.
5. Fiddaman PJ, Rossall S. The production of antifungal volatiles by Bacillus subtilis. J Appl Bacteriol. 1993; 74(2):119-126.
6. Hassanein WA, Awny NM, El-Mougith AA, Salah El-Dien SH. The antagonistic activities of some metabolites produced by *Pseudomonas aeruginosa*. J App. Sci. Res. 2009; 5(4):404-414.
7. Helliker PE. California Management Plan: 1,3-Dichloropropene. Sacramento,C A: California Department Pesticides Regulation, 2002, 4.
8. Kaur M, Rana S, Kumari M. Purification of Industrially Important Proteases from *Pseudomonas* Strain and Its Kinetic Studies. Int. J. Curr. Microbiol. App. Sci. 2020; 9(01): 900-909. doi: https://doi.org/10.20546/ijcmas.2020.901.100
9. Kloepper JW, Lifshitz R, Zablotowicz RM. Free living bacterial inocula for enhancing crop productivity. Trends Biotechnology. 1989a; 7:39-43
10. Kumar V, Behl RK, Narula N. Establishment of phosphate solubilizing strains of *Azotobacter chroococcum* in rhizosphere and their effect on wheat under greenhouse condition. Microbial. Res, 2004, 87-93.
11. Lata, Saxena AK. Characterization of plant growth promoting rhizobactria. In: Training manual on Biofertilizer Technology. AK Saxena (*eds*.). IARI Delhi, 2003, 24-25.
12. Mai WF, Abawi GS. Controlling replant disease of stone fruits in north-eastern United States by preplant fumigation. Plant Disease. 1981a; 65:859-864.
13. Manidipa R, Dutta SG, Venkata RC. Pseudomonads: Potential biocontrol agents of rice diseases. Res J Agric Forestry Sci. 2013; 1(9):19-25.
14. Meera T, Balabaskar P. Isolation and characterization of *Pseudomonas fluorescens* from rice fields. Int J Food Agric Veterinary Sci. 2012; 2(1):113-120.
15. Patten C, Glick B. Role of *Pseudomonas putida* indole acetic acid in development of host plant root system. Applied and Environmental Microbiology. 2002; 68:3795-3801.
16. Paul D, Sarma YR. Antagonistic effects of metabolites of *Pseudomonas fluorescens* strains on the different growth phases of *Phytophthora capsici*, foot rot pathogen of black pepper (*Piper nigrum* L). Archives of Phytopathol. Plant Protect. 2006; 39(2):113-118.
17. Rana S, Sharma R, Kaur M. Biochemical characterization of fluorescent Pseudomonas species isolated from normal and replant sites of apple orchard. International Journal of Farm Sciences. 2015; 5(3):165-170.
18. Rana S, Sharma R, Sharma YP, Kaur M. Isolation and Characterization of Novel Apple Rhizosphere Bacterium as Fungal Antagonist. Journal of Pure And Applied Microbiology. 2015; 9(3)-10(4):3189-3193.

19. Schippers B, Bakker A, Bakker P, Van PR. Beneficial and deleterious effects of HCN producing *Pseudomonads* on rhizosphere interactions. Plant and Soil. 1990; 129:75-83.
20. Schwyn B, Neilands JB. Universal chemical assay for the detection and determination of siderphores. Analytical Biochemistry. 1987; 28(8):751-759.
21. Tewoldemedhin YT, Mazzola M, Mostert L, McLeod A. *Cylindrocarpon* species associated with apple tree roots in South Africa and their quantification using real-time PCR. European Journal of Plant Pathology. 2011; 129:637-51.
22. Willett M, Smith TJ, Peterson AB, Hinman H, Stevens RG. Growing profitable apple orchards in replant sites: an interdisciplinary team approaching Washington State. Horticulture Technology. 1994; 4:175-81.