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Evaluation of Siderophore Production and Antimicrobial Activity by Fluorescent *Pseudomonas* Diversity Associated with Rhizosphere of Apple and Pear

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Abstract

Replant problem is very serious problem which suppresses growth and yield of apple and pear in all major fruit growing areas of the world. Fluorescent *Pseudomonas* has potential to synthesize different secondary metabolites with diverse PGPR activities which enhance soil fertility and promote plant growth. Under this study, twenty- six *Pseudomonas* strains were isolated from the rhizosphere of apple and pear plants from their normal and replant sites. All the isolates were positive for catalase, oxidase, denitrification test, lecithinase test and tween 80 hydrolysis. They were further screened for antifungal and siderophore production. Maximum siderophore production was recorded in isolates AN-1-UHF, AN-3-UHF, PN- 7-UHF and PN-13-UHF whereas, maximum antifungal activity against *Dematophora* sp., *Fusarium oxysporum*, *Alternaria* sp. and *Pythium* sp. was recorded in isolates AN-1-UHF, AN-3-UHF and PN-13-UHF. It was found that *Pseudomonas* sp. isolated from the replant sites showed comparatively less siderophore and antifungal activities. These potential isolates could be further used as biocontrol agents against various fungal pathogens in apple and pear orchards.

Highlight

- AN-1-UHF, AN-3-UHF, PN- 7-UHF and PN-13-UHF isolates showed maximum siderophore and antifungal activities.
- Replant rhizosphere soil isolates showed greater activities than replant rhizophere soil isolate.

Keywords: Siderophore production, antifungal activity, fluorescent *Pseudomonas*, replant problem

Replant site is the site which often encountered in establishing new orchards on old sites and poor growth of newly planted trees. Replant problem in apple and pear orchards appears to be related to soil and rhizosphere microflora (Catska *et al.*, 1982). This problem is distributed worldwide and is often encountered in establishing new orchards on old sites (Yao *et al.*, 1986). The disease is a complex syndrome that affects young trees in replant orchard sites and reduces growth survival, growth and yield of replanted trees. Rhizobacteria that exert beneficial effects on plant growth and development are referred to as plant growth promoting rhizobacteria (PGPR) (Ashrafuzzaman *et al.*, 2009). The exact mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include, the ability to produce plant growth regulators, asymbiotic N2 fixation, antagonism against phytopathogenic microorganisms by production of siderophores, antibiotics and cyanide solubilization of mineral phosphates and other nutrients (Sarvanakumar *et al.*, 2007). More specifically, the soil-borne fluorescent *Pseudomonas* strains have received particular attention because of their capacity to produce a wide range of enzymes and metabolites (Sarvankumar and Samiyappan 2008, Kapoor *et al.*, 2016).

The iron is the fourth most abundant in earth crust and it is largely insoluble and thus is unavailable for direct microbial assimilation. In aqueous solution iron can exists in either the ferrous (Fe^{2+}) or ferric (Fe^{3+}) form, the later being the less soluble (Neilands 1982). Organisms secrete iron binding ligands (siderophores), which can bind the ferric iron and make available to the host and microorganisms (Sujatha and Ammani 2013). Siderophores are microbial produced iron chelating compounds that can increase and regulate the availability of iron in plants rhizosphere. Now these compounds have come under investigation in plant nutrition along with disease suppression (O'Sullivan and O'Gara 1992). The possible solution to the management of replant problem of horticulture crops is to introduce appropriate novel and important indigenous best plant growth promoting bioagent conferring maximum plant growth promotion and disease controlling activities against various fungal pathogens. So, isolation and preliminary characterization of indigenous fluorescent *Pseudomonas* sp. producing siderophore and antifungal activities is important because they could be potential biocontrol agents for apple and pear.

Materials and Methods

Isolation and identification

Fluorescent *Pseudomonas* sp. were isolated from rhizosphere of apple and pear in normal and replant sites of University Campus (Nauni) by serial dilution and plate count method using King's B agar media. The most predominant *Pseudomonas* sp. isolates were identified on the basis of morphological, biochemical and physiological methods viz. catalase test, oxidase test, growth at 4 °C and 41 °C, fermentation, gelatin liquification test, lecitinase test, tween 80 hydrolysis and denitrification test as prescribed in Bergey's manual of systematic bacteriology. Here, AN and AR stand for isolates from normal and replant sites of apple orchard, respectively, whereas, PN and PR stand for isolates from normal and replant sites of pear orchard, respectively.

Catalase test

Culture tubes containing trypticase soy agar medium were inoculated with *Pseudomonas* cultures. An uninoculated trypticase soy agar slant was kept as control. The cultures were incubated at 300C for 28-48h. 3-4 drops of hydrogen peroxide was allowed to flow over the growth of each slant culture. Catalase positive culture produced bubbles of gas within one minute after addition of hydrogen peroxide.

Oxidase Test

Small pieces of filter paper were soaked in 1% aqueous tetra methyl- p- phenylenediamine dihydrochloride. Some of fresh young culture was scarped with a clean platinum wire and rub on the filter paper. A blue color with in ten seconds was positive oxidase test, while no change in color was recorded as negative test.

Growth at 4°C and 41°C

0.5 ml of overnight culture was inoculated in test tubes containing 10ml nutrient broth. The tubes were incubated at 4 °C and 41 °C. The growth was observed at absorbance 540nm.

Fermentation metabolism

Fermentation metabolism was used for testing the presence of fermentation metabolism in *Pseudomonas* isolates. Fermentation broth was prepared by using tpticas (10g), carbohydrates (5g), sodium chloride (15g), phenol red (0.018g) for one liter at pH 7.3. Fermentation broth was taken into fermentation tubes and was inoculated with *Pseudomonas* isolates and one uninoculated tube was kept as a comparative control. All inoculated and uninoculated tubes were incubated at 30 °C for 24-48h. Change in color and appearance of bubbles showed the presence of fermentation metabolism.

Gelatin liquification test

Nutrient gelatin deep tubes were prepared and stab inoculation was made from each *Pseudomonas* isolates. Uninoculated deep tube was kept as a control. All inoculated and uninoculated tubes were incubated at 30 °C for 4-7 days. After incubation, the tubes were placed in refrigerator at 4 °C for 15 minutes. Deep gelatin tubes that remain liquefied produced gelatin and showed positive test and those tube that remain solid demonstrated negative reaction for gelatin hydrolysis.

Denitrification test

Semisolid nitrate medium was prepared by using beef extract (3 g), peptone (5 g), potassium nitrate (1 g), agar (1%) for one liter at pH 7. *Pseudomonas* cultures were inoculated in semisolid nitrate medium. One tube of semisolid non-nitrare media was also inoculated. Place inoculated tube in ice bath for 10-15 minutes so as to make semisolid medium firmer and overlaid with 0.1% agar on top. All inoculated and uninoculated tubes were incubated at 30 °C for 48h.

Hydrolysis of Tween compound

Tween medium was prepared by Peptone (10.0 g), sodium chloride (5.0 g), tween-80 (10 ml), agar (15 g) for one liter at pH 7 Tween-80 medium plates were spotting with fresh *Pseudomonas* isolates at the center and incubate at 300 for 5 days. Opaque zones surrounding microbial growth consisted of calcium salts of the free fatty acids and were taken as indication of positive lipolytic activity.

Lecithin hydrolysis

A nutrient agar with the addition of 1% NaCl and 10% (v/w) egg yolk emulsion was made. Inoculate poured dried plates of medium by streaking across the surface and incubate at the 300 for 4 days. Lecithinase activity resulted in the formation of opaque zones (mm diameter) around the region of microbial growth.

Measurement of production of siderophores

Succcinate (iron free) medium was used for the production of siderophore with slight modification. It contained K_2HPO_4 6.0 g; KH_2PO_4 3.0 g $(NH_4)_2SO_4$ 1.0 g; $MgSO_4.7H_2O$ 0.2 g in 1 liter of distilled water. 0.5 ml of old culture of each test isolate was inoculated in 100 ml medium in flasks and incubated at 30 °C for 72 h on rotary shaker conditions. Cell free supernatant was harvested by centrifugation in multifuge XIR (Thermoscientific Germany) at 10,000 rpm at 4 °C.

Qualitative assay

Siderophores production was detected by chrome azurol -S (CAS) plate assay method with slight modification (Schwyn and Neilands 1987). 72 h old culture of each test bacteria i.e. *Pseudomonas* sp. was placed on prepoured CAS plates. Plates were incubated at 30 °C for 48 h and observed for production of orange zone (mm) around the well.

Liquid assay (Quantitative)

For quantitative estimation of siderophores, CAS liquid assay method was used (Schwyn and Neilands 1987). 0.5 ml of 72 h old cell free supernatant was mixed with 0.5 ml CAS assay solution (1.5 ml of 1mM FeCI3, $6H_2O$ in 10 mM HCl + 7.5 ml of 2 mM CAS stock solution dissolved in 50 ml of HDTMA in mixing cylinder, add 30 ml piperazine solution into it and final volume was made to 100 ml with distilled water), 10 µl shuttle solution (0.2 M 5-sulfosalicylic acid) was added. Color intensity of the solution was recorded with UV-VIS SL-159 spectrophotometer at 630 nm against reference after 10 minutes at room temperature. Siderophore production was observed in terms of reduction in blue color as per cent siderophore units (%SU).

$$\% \text{ SU} = \frac{Ar - As}{Ar} \times 100$$

Ar = Absorbance of reference at 630 nm,

As = Absorbance of supernatant at 630 nm

Antifungal activity

Plant pathogenic indicator test fungi viz., *Dematophora, Fusarium oxysporum, Alternaria, Pythium* sp., *Sclerotium* sp., and *Rhizoctonia solani* were procured from the Department of Mycology and Plant Pathology (MPP). These fungal cultures were maintained on malt extract agar (MEA) at 4 °C and sub-cultured periodically at 28+0.1 °C. Antifungal activity of each test isolate of *Pseudomonas* sp. was checked by standard well plate assay method (Vincent *et al.,* 1982, Fleming *et al.,* 1975). 72h old culture bit of indicator fungi were placed on the one side of prepoured malt extract agar (MEA) plates. 100 µl of 72 h old cell free culture supernatant of each test bacterial strain was added to each well. Plates were incubated at 28+0.1 °C for 3-5 days and observed for inhibition zone produced around the well. For control culture bit of indicator fungi kept in the center of MEA plate and incubated at 28±0.1°C for 3-5-days. Antifungal activity expressed in terms of mm diameter of clear zone around the well and

Table 1: Siderophore production by isolates of fluorescent *Pseudomonas* sp. isolated from the rhizosphere of apple and pear

Plant	Isolates	Siderophore activity (mm)	%siderophore units (SU)	Plant	Isolates	Siderophore activity (mm)	%siderophore units (SU)
Apple	AN-1-UHF	24[a]	61.7[a]	Pear	PN-1-UHF	22[d]	56.5[c]
	AN-2-UHF	20[b]	58.6[b]		PN-2-UHF	20[e]	58.6[b]
	AN-3-UHF	23[a]	56.0[c]		PN-3-UHF	20[e]	56.5[c]
	AN-4-UHF	21[b]	58.6[b]		PN-4-UHF	24[c]	50.0[e]
	AN-5-UHF	23[a]	61.7[a]		PN-5-UHF	20[e]	56.5[c]
	AN-6-UHF	19[c]	53.19[d]		PN-6-UHF	21[e]	53.1[d]
	AN-7-UHF	18[c]	50.0[f]		PN-7-UHF	27[a]	60.2[a]
	AN-8-UHF	20[b]	58.6[b]		PN-8-UHF	19[f]	53.1[d]
	AN-9-UHF	20[b]	44.7[g]		PN-9-UHF	26[ab]	58.6[b]
	AR-1-UHF	19[c]	44.7[g]		PN-10-UHF	17[g]	44.7[f]
	AR-2-UHF	15[e]	51.0[e]		PN-11-UHF	23[b]	42.6[g]
	AR-3-UHF	18[d]	40.4[h]		PN-12-UHF	22[d]	50.0[e]
					PN-13-UHF	28[a]	60.8[a]
					PR-1-UHF	15[h]	38.2[h]
CD (0.05)		1.68	0.97	CD (0.05)		1.67	0.98

** Each value represents the mean of three replicates. In the same column, significant differences according to Completely Randomized Design are indicated by different letters. Same letters represent that their values are statistically at par.*

Here, Ar: Absorbance of reference solution is 0.47

Table 2: Antifungal activity by fluorescent *Pseudomonas* sp. isolated from rhizosphere of apple and pear

Plant	Isolates	Antifungal activity indicator test fungi (% Inhibition)				Plant	Isolates	Antifungal activity indicator test fungi (% Inhibition)			
		Dematophora sp.	*Fusarium oxysporum*	*Alternaria sp.*	*Pythium sp.*			*Dematophora sp.*	*Fusarium oxysporum*	*Alternaria sp.*	*Pythium sp.*
	Control*	0.00	0.00	0.00	0.00		Control*	0.00	0.00	0.00	0.00
Apple	AN-1-UHF	41.5	51.7	42.3	40	Pear	PN-1-UHF	38.5	43.1	0	35.7
	AN-2-UHF	0	0	46	0		PN-2-UHF	35.4	31.03	27	31.42
	AN-3-UHF	38.5	44.8	30.7	34.3		PN-3-UHF	38.5	27.5	23.1	0
	AN-4-UHF	35.4	38	0	0		PN-4-UHF	41.5	0	0	0
	AN-5-UHF	35.3	29.3	30.7	25.7		PN-5-UHF	0	0	0	31.4
	AN-6-UHF	0	0	0	0		PN-6-UHF	0	51.7	0	0
	AN-7-UHF	0	0	0	26		PN-7-UHF	0	48.2	33	36
	AN-8-UHF	35.34	48.2	30.8	0		PN-8-UHF	0	0	0	0
	AN-9-UHF	41.5	29.3	23.1	0		PN-9-UHF	0	0	30.7	34.2
	AR-1-UHF	0	0	0	0		PN-10-UHF	0	46.5	0	33
	AR-2-UHF	0	0	0	0		PN-11-UHF	0	0	0	0
	AR-3-UHF	41.5	0	0	31.5		PN-12-UHF	0	0	0	31.4
							PN-13-UHF	43	44.8	27	31.4
							PR-1-UHF	0	0	23.1	30.7

**Diameter of respective (control) indicator test fungi*

Fusarium oxysporum = 58 mm, *Dematophora* sp. = 65 m, *Alternaria* sp.= 52 mm, *Pythium* sp. = 70 mm

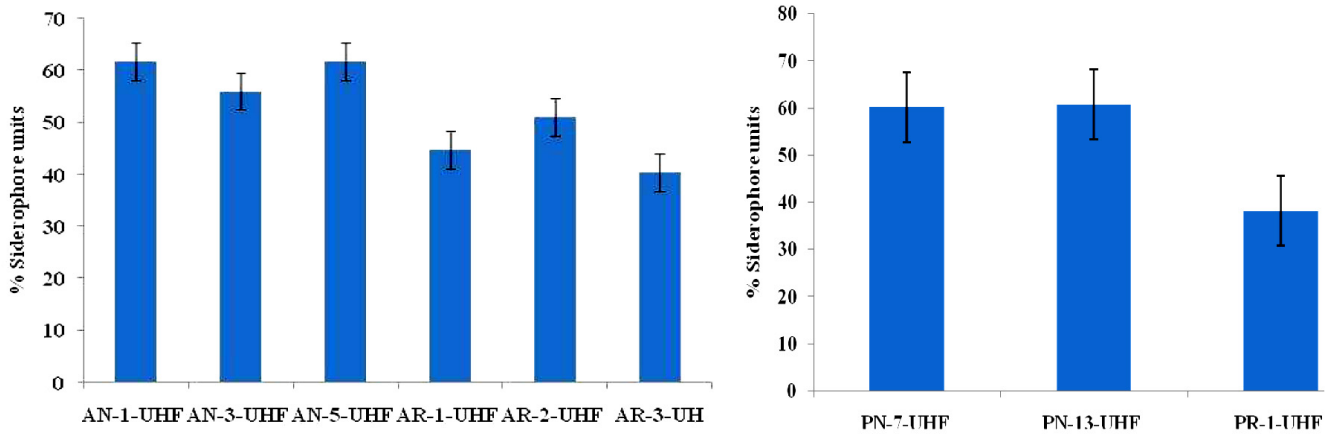

Fig. 1: Comparison of siderophore production from isolates of normal and replant sites of apple and pear orchards

also expressed in terms of % inhibition of fungal mycelium as calculating from equation:

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

C = growth of mycelium in control

Z = growth of mycelium in treatment

Statistical analysis of data

Results of the measurements were subjected to two way analysis of variance (ANOVA) and by CRD (Complete Randomized Design) Cpcsd (1.0), a programs package for the analysis of commonly used experimental designs.

Results and Discussion

Morphological, physiological and biochemical characteristics of fluorescent Pseudomonas isolates

The isolates from the rhizosphere of apple and pear were found to be fluorescent, pigmented (green & yellow, some also produced dark greenish along with fluorescence), Gram negative rod, aerobic, non spore former and motile. Colonies with circular, flat, entire, smooth morphology were obtained. All the isolates were positive for catalase, oxidase and negative for utilization of lactose, gelatin liquification, oxidative glucose, fermentation metabolism. All were positive for denitrification test, lecithinase test (10-15 mm zones) and tween 80 hydrolysis (7-8 mm zones). Seven of the *Pseudomonas* isolates showed positive growth at 4 °C, whereas, nineteen *Pseudomonas* isolates showed growth at 41 °C. Almost all the isolates showed growth at 25 °C. From these tests, it was concluded that tentatively out of twenty six isolates, twelve isolates may belong to *P. aeruginosa* group, nine isolates may belong to *P. putida* group and five isolates may belong to *P. fluorescence* group.

Siderophore production

All the isolated strains showed production of siderophore by both qualitative and quantitative assays. The results showed that in qualitative assay all the isolates of *Pseudomonas* sp. produced orange zone on CAS agar plates in the range of 15-30 mm diameter and in quantitative assay siderophores production was observed in terms of reduction in blue color in the range of 40.4-61.7 %SU (Table 1). Among apple isolates, AN-1-UHF showed statistically higher siderophore production i.e. 24 mm diameter zone and 61.7 %SU which was followed by AN-3-UHF and AN-5-UHF (23 mm) having %SU 58.6 and 61.7, respectively.

Among pear isolates, PN-13-UHF showed statistically higher siderophore production i.e. 28 mm zone and 60.2 %SU which was followed by PN-7-UHF (27 mm with 60.2 %SU). Strains isolated from replant sites of orchards showed comparatively less production of siderophores (15-19 mm, 38.2 -51 %SU) (FIGURE 1). These results suggest that quantitative assay method was more sensitive and accurate for detection of siderophores. These results revealed that siderophores produced by these strains may be beneficial in the growth promotion and disease suppression in the bacterized apple

and pear seedlings planted in replant sites as stated by Cox and Adams (1985). Many other workers also studied that the production of siderophore by fluorescent *Pseudomonas* species have great impact on plant disease which was produced by various fungi and bacteria (Loper and Buyer 1991, O'Sullivan and O'Gara 1992, Kumar and Bezbaruah 1996). There are also number of reports suggesting that plant species are capable of obtaining iron from some microbial siderophores (O'Sullivan and O'Gara 1992, Park *et al.,* 2005).

Antifungal activities

The indigenous *Pseudomonas* strains were further screened out for the production of antifungal activities in vitro against various fungal pathogens (Table 2). They were found to be effective against fungi i.e. *Dematophora* sp., *Fusarium oxysporum*, *Alternaria* sp. and *Pythium* sp. indicating that antagonistic metabolites may be broad spectrum in nature like antibiotics. The results revealed that maximum % inhibition was shown by AN-1-UHF against *Dematophora* sp. (41.5%), *Fusarium oxysporum* (51.7%), *Alternaria* sp. (42.3%) and *Pythium* sp. (40.0%). AN-3-UHF also showed antifungal activities against *Dematophora* sp., *Fusarium oxysporium*, *Alternaria* sp. and *Pythium* sp. i.e. 38.3, 44.8, 30.7 and 34.3%, respectively. AN-5-UHF also showed antifungal activities against all the fungal-pathogens. None of the replant site isolates showed antifungal activities against-fungal pathogens except AR-3-UHF, which showed antifungal activity against *Dematophora* sp. Among pear isolates, PN-13-UHF showed maximum % inhibition against *Dematophora* sp. (43.0%), *Fusarium oxysporum* (44.8%), *Alternaria* sp. (27.0%) and *Pythium* sp. (31.4%). Besides that, isolates from replant sites showed less or no inhibitions against test fungi. None of the isolates showed antifungal activity against *Rhizoctonia solani* and *Sclerotium* sp.

Pseudomonas species contains one or more than one type of secondary metabolites. It may be due to the fact that the microorganisms selected from the rhizosphere enrichment environment like apple and pear may be so diversified and may be able to produce bioactive substances and biocatalysts (Steele and Stowers 1991, Cheetham 1987). In successful cropping systems where the economic output per unit area is positive, the underlying microbiological association diversity, their interaction and the overall impact on the growth and productivity need to be understood for practical utility. The most effective mechanism that a PGPR can employ to prevent growth of phytopathogens is the synthesis of antibiotics and siderophores (Hass *et al.,* 1991, Keel *et al.,* 1992). The results are consistent with the findings of several research workers who demonstrated the use of *P. fluorescens* strains against various fungal pathogens (Sarvanakumar *et al.,* 2007, Vivekanathan *et al.,* 2004, Saravankumar and Samiyappan 2008).

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Conclusion

It can be concluded that *Pseudomonas* sp. can be used as a potential bacterial source to solve the replant problem and provide a good alternative for chemical fertilizers. These isolates can be used as efficient antagonist and to improve the growth and quality of plants.

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