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Role of fluorescent *Pseudomonas* in reduction of the use of chemical pesticides and fungicides in normal and replant sites of apple and pear

DEEP SHIKHA THAKUR, MOHINDER KAUR AND VINEET SHYAM

Department of Basic Science, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, SOLAN (H.P.) INDIA
Email : deepshikhathakur86@gmail.com; vineet.shyam@gmail.com

Phytopathogenic microbes including few fungi and bacteria have an immense effect on the agricultural productivity, greatly reducing crop yield and sometimes causing total crop loss. However, the fungicides being used have the negative effects on the environment and human health. In this context, biological control of plant diseases is gaining attention due to increased pollution concerns and is an alternative method to control pathogenic fungi. So, the current trends in agriculture are focused on reduction in the use of chemical pesticides and fungicides, compelling the search for alternatives that enhance environmental quality. Effective option is to employ the pathogen's natural enemies as biocontrol agents, which is less destructive and environmental friendly. PGPR are a group of bacteria that actively colonize plant roots and increase plant growth and yield. Among various biocontrol agents, *Pseudomonas* sp., equipped with multiple mechanisms of biocontrol of phytopathogens is being used widely. Biological control of plant pathogens is through the production of antibiotics, lytic enzyme, siderophore and HCN production. In the present study, the *Pseudomonas* spp. were isolated from the rhizosphere of apple and pear and screened for their biocontrol properties such as antifungal activity, siderophore production and HCN production. Out of 30 isolates, 15 isolates showed antifungal activity against *Fusarium* sp. and *Alternaria* spp. Maximum isolates showed siderophore production. All isolates were found to be positive for ammonia and HCN production. Taken together, results suggest that *Pseudomonas* spp. is an efficient approach to replace agrochemicals and pesticides, thereby reducing their negative effects on the environment.

Key words : Apple, Pear, *Pseudomonas*, Siderophore, Antifungal activity, Proteolytic activity

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INTRODUCTION

Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. As agricultural production intensified over the past few decades, producers became more and more dependent on agrochemicals as a relatively reliable method of crop protection helping with economic stability of their operations. However, increasing use of chemical inputs causes several negative effects, *i.e.*, development of pathogen resistance to the applied agents and their nontarget environmental impacts (Gerhardson, 2002). Furthermore, the growing cost of pesticides, particularly in less-affluent regions of the world, and consumer demand for pesticide-free food has led to a search for substitutes for these products. There are also a number of fastidious diseases for which chemical solutions are few, ineffective, or nonexistent.

As the crop production has intensified over the past few decades, producers have become more and more dependent on chemical inputs/ synthetic inputs as a relatively reliable method of crop production. This indiscriminate use of chemical inputs has several negative environmental effects. So in present scenario, one of the most acceptable and environmentally conscious approaches to solve these problems is to manipulate crop rhizosphere population by inoculating beneficial bacteria (Nelson, 2004).

Biological control is thus, being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Welbaum *et al.*, 2004). The most widely studied group of PGPB are plant growth-promoting rhizobacteria (PGPR) colonizing the root surfaces and the closely adhering soil interface, the rhizosphere (Kloepper *et*

al., 1999). Biocontrol strains often belong to the genera *Bacillus* (Nair *et al.*, 2002) and *Pseudomonas* (Mark *et al.*, 2006). Biological control of the plant diseases followed by the inoculation of these biocontrol strains has been widey reported in different crops (Sivaramaiah *et al.*, 2007; Rakh *et al.*, 2011 and Ramyasmruthi *et al.*, 2012).

The widely recognized mechanisms of biocontrol mediated by PGPB are competition for an ecological niche or a substrate, production of inhibitory allelochemicals including iron-chelating siderophores, antibiotics, and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens or abiotic stresses (Haas *et al*., 2002; Mayak *et al*., 2004). The present research studied the different biocontrol mechanisms of the fluorescent *Pseudomonas* sp. like antifungal activity, siderophore production, lytic enzymes production, ammonia and HCN production and its potential use for the biological control of plant diseases in apple and pear.

RESEARCH METHODOLOGY

Isolation and screening of the microorganisms :

Isolation of plant growth promoting bacteria (PGPR) was carried out from the rhizosphere soil collected from of normal and replant sites of apple orchards, located at the height of 2300 meters in different locations of tehsil Nankhari, district Shimla of Himachal Pradesh. Total bacterial and fluorescent *Pseudomonas* sp. were enumerated using standard spread plate technique using appropriate dilutions on nutrient agar and Kings B agar media, respectively. The probable isolates showing greenish/yellowish fluorescent or pyocyanin pigments were assumed to be *Pseudomonas sp*. The colonies were restreaked for purification and further observation of colony morphological characterization and pigment production on different media *i.e*. nutrient agar, Kings, Pikovskayas etc. The most predominant *Pseudomonas* isolates were identified on the basis of morphological, biochemical and physiological tests as prescribed in Bergey's manual of systematic bacteriology and were confirmed in the Department of Basic Sciences, Dr. Y.S Parmar University of Horticulture and Forestry, Nauni, Solan (H.P). The screening of the bacterial isolates for various plant growth promoting activities like antagonism against fungal pathogens, siderophore, lytic enzymes, ammonia and HCN, were performed by adapting the standard methods.

Antagonistic activity of bacterial isolates against test fungus:

Antifungal activity :

Antifungal activity of each test isolate of *Pseudomonas* sp. was checked by well plate assay method (Vincent, 1947) using dual culture technique.

Production of siderophores :

Succinate medium (iron free) was used for the production of siderophore with slight modification (Meyer and Abdailah, 1978). Siderophore production was estimated both qualitatively by well plate assay method and quantitatively by liquid assay method (Schwyn and Neilands, 1987).

HCN and ammonia production :

Production of hydrogen cyanide (HCN) was checked according to Bakker and Schippers (1987) and for the detection of ammonia production method of Lata and Saxena (2003) was used.

Proteolytic activity :

Production of proteases was observed by the method Fleming *et al*. (1975) on skim milk agar medium as well as by quantitative assay method of Morrihara *et al*. (1963).

Statistical analysis :

The data recorded under laboratory conditions for various parameters were subjected to statistical analysis as per method outlined by Gomez and Gomez (1984). The CD at 5 per cent level was used for testing the significant differences among the treated means.

RESEARCH FINDINGS AND ANALYSIS

The experimental findings obtained from the present study have been discussed in following heads:

Siderophore production :

Iron is an important and limiting bioactive metal in soil and essential for the growth of soil microorganisms. The iron concentration in the soil is low (10^7) enough to limit the growth of soil microorganisms (Gurinot, 1994). However, its availability to the organisms is very limited due to the rapid oxidation of ferrous (Fe^{++}) to ferric (Fe^{+++}) state. Siderophore production not only improve rhizosphere colonization of producer strain but also play an important role in stimulating plant growth directly by increasing the availability of iron in the soil surrounding the roots (Vansuyt *et al.*, 2007) and antagonism against phytopathogens (Chincholkar *et al*., 2007). Competition for iron is also a possible mechanism in agriculture to control the pathogenic fungi in the soil.

Pseudomonas sp. is best known to have the capacity to utilize siderophores produced by diverse species of microorganisms. *P. fluorescens* is one of the fluorescent *pseudomonads* that secrete pyoverdins (Meyer, 2000) for its essential requirement for iron. De Villegas *et al.* (2002) evaluated the siderophore production by *Pseudomonas aeruginosa* PSS in a conventional batch system in succinate, glucose and glutamic medium. Sarode *et al.* (2009) isolated SCW1 from wheat rhizosphere and confirmed as

Acinetobacter calcoaceticus. The strain produced catechol type of siderophores during exponential phase which was influenced by iron content of medium. Seed bacterization with siderophoregenic *A. calcoaceticus* improved plant growth in pot and field studies.

In our study, all the fluorescent *Pseudomonas* isolates from the rhizosphere of apple and pear were screened out for the production of siderophore (Table 1) by both qualitative and quantitative *i.e.* plate and liquid assay methods. The results showed that in plate assay siderophores are produced in the range 20 mm to 32 mm diameter pinkish/orange zone in Chromeazurol-S agar plates by all *Pseudomonas* isolates

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

Plant	*Pseudomonas* isolates	Siderophore activity (assay)	
		Plate	Quantitative
		Pinkish/orange zone (mm dia) *	% Siderophore unit (%SU) **
Apple	An-1-Naga	30	43.75 (41.41)
	An-2-Naga	28	52.08(46.19)
	An-3-Naga	25	50.00(45.00)
	An-4-Naga	24	54.10(47.35)
	An-1-kho	28	58.30(49.77)
	An-2-kho	24	62.50(52.24)
	An-3-kho	32	37.50(37.76)
	An-4-kho	22	58.30(49.77)
	An-1-bagh	26	58.30(49.77)
	An-2-bagh	28	62.50(52.24)
	An-3-bagh	26	68.7(55.98)
	An-4-bagh	28	56.25(48.59)
	An-1-panch	29	54.10(47.35)
	An-2-panch	0	52.08(46.19)
	An-3-panch	22	47.91(43.80)
	An-4-panch	0	45.80(42.17)
	An-1-nali	28	50.00(45.00)
	An-2-nali	0	68.70(55.98)
	An-3-nali	28	60.41(51.01)
	Ar-1-Kho	28	52.08(46.19)
	Ar-2-Bagh	22	20.83(27.15)
	Ar-3-Nali	22	41.60(40.16)
$CD_{0.05}$		2.04	0.868 (.508)
Pear	Pn-1-panch	25	56.25 (48.59)
	Pn-2-panch	26	41.60 (40.16)
	Pn-3-panch	28	52.08 (46.19)
	Pn-1-kho	25	47.91 (43.71)
	Pn-2-kho	26	35.41 (36.52)
	Pr-1-panch	23	41.60 (40.16)
	Pr-2-panch	21	39.50 (38.94)
	Pr-3-kho	20	20.83 (27.16)
$CD_{0.05}$		3.72	0.95(0.55)

Siderophore activity expressed in terms of mm diameter of pinkish/orange zone produced around the well on chromeazurol–S agar plates at 28ºC for 48 hrs; 0 indicates no activity. **The % siderophore units are defined as percent reduction in blue colour of chromeazurol–S as compared to reference *i.e* (% SU) = $\frac{A_r - A_s}{A_s} x100$ where Ar = Absorbance at 630nm of reference *i.e*. 0.48; As =Absorbance at 630 nm of test , # Values in Paranthesis are arc sin Transformed values

and in quantitative assay siderophore production was observed in terms of reduction in blue colour in the range of 20.83 to 68.70 per cent siderophore units (% SU).

Antifungal activity :

Pseudomonas fluorescens has been extensively studied as pathogen's natural enemies as biocontrol agents, which is less destructive and environmental friendly than chemical treatments (Hillel, 2005). The *Pseudomonas* isolates from rhizosphere of apple and pear in our study were found to be effective against fungi (Table 2) *i.e. Fusarium oxysporium* and *Alternaria solani* indicating that antagonistic metabolites may be broadspectrum in nature like the antibiotics. This is in agreement with that of Pandey *et al*. (2006) who reported that in Petridish assay by the *Pseudomonas* bacterium tested positive for inhibition of the growth of two phytopathogenic fungi. It caused transparent clearing in case of *Alternaria alternata*, and it suppressed the process of conidiation in *Fusarium oxysporium*.

Proteolytic activity :

Inhibitory effect against various types of

Table 2: Antifungal activity of fluorescent *Pseudomonas* isolates from the rhizosphere of apple and pear against different indicator test fungi

Plant	*Pseudomonas* isolates	Antifungal activity(% inhibition) Indicator test fungi	
		Fusarium sp.	*Alternaria* sp.
Apple	An-1-Naga	33.3(35.24)	-
	An-2-Naga	-	21.1(4.70)
	An-3-Naga	28(31.49)	-
	An-4-Naga	28(31.49)	-
	An-1-kho	-	-
	An-2-kho	-	25(5.09)
	An-3-kho	32(34.44)	-
	An-4-kho	-	-
	An-1-bagh	-	26.9(5.28)
	An-2-bagh	-	23.07(4.90)
	An-3-bagh	-	-
	An-4-bagh	30.6(33.58)	-
	An-1-panch	-	-
	An-2-panch	33.3(35.24)	-
	An-3-panch	-	-
	An-4-panch	30.6(33.58)	-
	An-1-nali	-	28.8(5.45)
	An-2-nali	29.3(33.77)	-
	An-3-nali	-	-
	Ar-1-Kho	-	-
	Ar-2-Bagh	-	-
	Ar-3-Nali	-	-
	$CD_{0.05}$	1.17(.752)	.248(.024)
Pear	Pn-1-panch	-	-
	Pn-2-panch	30.6(33.58)	-
	Pn-3-panch	-	-
	Pn-1-kho	-	28.8(5.45)
	Pn-2-kho	-	-
	Pr-1-panch	-	-
	Pr-2-panch	-	-
	Pr-3-kho	-	-
	$CD_{0.05}$	.061(.038)	.061(.0056)

Antifungal activity expressed in terms of mm diameter of growth inhibition of mycelia *i.e.* Per cent growth inhibition (% I) = $\frac{C - T}{C} \times 100$, where T= Growth of mycelia in treatment, C = growth of mycelia in control *of Fusarium, Alternaria*

*Values in paranthesis are arc sin transformed values

microorganisms may also be due to the lytic or cell wall degrading enzymes produced by the microorganisms (Kaur *et al*., 1989). Chaiharn (2008) reported the production of cell wall degrading enzymes such as proteolytic enzyme by phosphate-solubilizing bacteria. The screening of isolates for protease production on skim milk agar plate according to Cattelan *et al*. (1999) showed that *Pseudomonas* isolates were positive for the proteolytic activities. In present study, maximum proteolytic activity (Table 3) was produced by Pr-2-panch in terms of mm diameter *i.e.*18 mm and maximum by An-1-naga and Pn-2-khoh *i.e*. 27 mm of clear zone produced around the well. In case of quantitative assay, proteolytic activity ranged between 1.9 to 1.5 mg/ml. The values were significantly different from each other.

HCN and ammonia :

HCN and ammonia are released as product of secondary metabolism by several microorganisms and affects sensitive organisms by inhibiting the synthesis of ATP-mediated cytochrome oxidases and is a potential and environmentally compatible mechanism for biological control of diseases as well as weeds (Heydari *et al.*, 2008). It has been proposed that

Table 3: Proteolytic activity of fluorescent *Pseudomonas* isolates from the rhizosphere of apple and pear

Plant	*Pseudomonas* isolates	Proteolytic activity	
		Plate assay (mm dia)*	Quantitative assay** units/ml
Apple	An-1-Naga	27	4.1
	An-2-Naga	23	4.2
	An-3-Naga	26	3.8
	An-4-Naga	20	1.9
	An-1-kho	24	3.7
	An-2-kho	26	3.5
	An-3-kho	22	5.2
	An-4-kho	24	2.6
	An-1-bagh	22	4.7
	An-2-bagh	20	4.6
	An-3-bagh	24	5.4
	An-4-bagh	24	5.0
	An-1-panch	26	4.8
	An-2-panch	24	4.3
	An-3-panch	22	2.7
	An-4-panch	22	5.5
	An-1-nali	0	2.0
	An-2-nali	24	3.5
	An-3-nali	24	3.3
	Ar-1-Kho	24	4.8
	Ar-2-Bagh	20	4.0
	Ar-3-Nali	21	3.5
$CD_{0.05}$		1.02	0. 37
Pear	Pn-1-panch	20	4.7
	Pn-2-panch	24	5.0
	Pn-3-panch	22	4.2
	Pn-1-kho	24	3.5
	Pn-2-kho	27	4.5
	Pr-1-panch	20	2.0
	Pr-2-panch	18	3.3
	Pr-3-kho	19	3.0
$CD_{0.05}$		1.49	0.24

* Proteolytic activity expressed in terms of mm diameters of clear zone produced around the well (mm diameter) on skim milk agar plates at 37°C for 48 h

** Proteolytic activity expressed in terms of mg/ml of casein degradation as calibrated from the standard curve (10-100 µg/ml). One unit of proteolytic activ expressed as the enzyme required to solubilize 1mg of casein in Tris-HCl buffer (0.05M , pH 7) at 37ºC within 5 minutes.

HCN may induce plant defence mechanisms (Tilak *et al*., 1999). Some biocontrol PGPB produce a wide range of low molecular weight metabolites with antifungal potential. The best known is hydrogen cyanide (HCN), to which the producing bacterium, usually a *Pseudomonas*, is resistant (Hillel, 2005).

The HCN production is found to be a common trait of *Pseudomonas* (88.89 %) and *Bacillus* (50%) in the rhizospheric soil and plant root nodules and is a biocontrol metabolite in *Pseudomonas* species (Ahmad *et al.*, 2008).

Ahmad *et al*. (2008) reported that the plant growth promoting rhizobacteria were found to produce ammonia. Chaiharn *et al*. (2008) also reported the production of ammonia by phosphate-solubilizing microorganisms. More than 64 per cent of the isolates produced ammonia. In present study, all the strains of *Pseudomonas* isolates produce ammonia (Table 4) in liquid culture (peptone water) as observed from the change of colour from light yellow to brown. All the isolates of apple and pear were found to be statistically significant. In our study, almost all the strains of *Pseudomonas* isolates from the rhizosphere of apple and pear showed production of HCN (Table 4) *in vitro* as evaluated from the colour change of pre-dipped picric acid paper strips from yellow to orange brown.

Table 4: Production of HCN and ammonia by isolates of fluorescent *Pseudomonas* from the rhizosphere of apple and pear

Plant	*Pseudomonas* isolates	HCN*	Ammonia **
		Change of color (yellow to br own)	
Apple	An-1-Naga	++	+++
	An-2-Naga	++	+
	An-3-Naga	+++	++
	An-4-Naga	+	++
	An-1-kho	+++	+++
	An-2-kho	++	+
	An-3-kho	++	++
	An-4-kho	++	++
	An-1-bagh	++	++
	An-2-bagh	+++	++
	An-3-bagh	++	++
	An-4-bagh	++	+
	An-1-panch	++	++
	An-2-panch	+	+
	An-3-panch	++	++
	An-4-panch	-	+
	An-1-nali	++	+++
	An-2-nali	-	++
	An-3-nali	+++	++
	Ar-1-Kho	++	+++
	Ar-2-Bagh	+	++
	Ar-3-Nali	+++	+
Pear	Pn-1-panch	++	++
	Pn-2-panch	+++	+++
	Pn-3-panch	++	++
	Pn-1-kho	+	+
	Pn-2-kho	++	+++
	Pr-1-panch	++	++
	Pr-2-panch	++	++
	Pr-3-kho	++	++

\- Indicates yellow; ++ indicates light brown; +++ indicates dark brown

* HCN production on king's media expressed in terms of change of color of paper strip already dipped in picric acid from deep yellow to orange brown

** Ammonia production expressed in terms of change of color of culture broth from faint yellow to deep brown at 30ºC for 4days

Conclusion :

Research into the mechanisms of plant growth promotion by PGPB have provided a greater understanding of the multiple facets of disease suppression by these biocontrol agents. Still, most of the focus has been on free-living rhizobacterial strains, especially to *Pseudomonas* and *Bacillus*. Identifying different mechanisms of action facilitate the combination of strains, bacteria with bacteria or bacteria with fungi, to hit pathogens with a broader spectrum of microbial weapons (Kilic-Ekici and Yuen, 2004). Along this same line, biotechnology can be applied to further improve strains that have prized qualities (e.g., formulation ease, stability, or otherwise exceptionally suited to plant colonization) by creating transgenic strains that combine multiple mechanisms of action (Huang *et al*., 2004).

The use of plant growth promoting rhizobacterial agents will probably be one of the most significant strategies for disease management and sustainable agriculture/horticulture in the near future. Therefore, based on the findings of present study, selected isolates can be a potential bioprotector against soil borne fungal disease of apple and pear.

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