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Antifungal metabolites produced by *Pseudomonas fluorescens* against *Fusarium oxysporum* f.sp. cepae

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ABSTRACT

Antifungal metabolites were isolated from *Pseudomonas fluorescens* and tested against *Fusarium oxysporum* f.sp. *cepae* causing basal rot of onion. Phenazine and 2,4 DAPG produced by the *Pseudomonas* isolate (Pf 12) were recorded 67.03 and 77.34 per cent inhibition of mycelial growth over control respectively. *P. fluorescens* isolates were strongly produced hydroxymate and Carboxymate type of siderophore at various level. Antifungal metabolites produced by *Pseudomonas* isolate (Pf 12) was recorded maximum inhibition of basal rot pathogen in *in vitro* condition.

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INTRODUCTION

Onion (*Allium cepa* var *aggregatum* G.Don) is one of the important crops grown in India. Basal rot is the most destructive disease of onion and causes yield losses in all growing areas of the world (Coskuntuna and Ozer, 2008) and which causes severe loss in the productivity both in field and in storage condition. The disease occurs in all stages of growth of the crop.

Pseudomonas suppress soil-borne fungal pathogens by producing antifungal metabolites such as pyoluteorin, pyrrolnitrin, phenazines, and 2,4-diacetyl phloroglucinol. In addition, psuedomonads can indirectly suppress fungal pathogens by scavenging iron in the rhizosphere environment through the release of siderophores. Antibiotics are low-molecular weight compounds, secondary metabolites produced by micro-organisms at low concentrations. They are deleterious to the growth or metabolic activities of other micro-organisms (Thomashow *et al*., 1997). Phenazines are pigments which exhibit broad-spectrum antibiotic activity against fungi and bacteria. Structurally simple phenazines such a phenazine-1-carboxylic acid (PCA) and its hydroxy and carboxamide derivatives are produced by beneficial strains of *Pseudomonas* on the roots of plants, where they have a critical role in suppressing fungal pathogens. The antibiotic 2,4 diacetylphloroglucinol (2,4 DAPG) produced by several strains of *P. fluorescens*, not only have activity against a wide range of plant pathogenic fungi but also have antibacterial, antihelminthic and phytotoxic properties (Keel *et al.,* 1992; Thomashow and Weller, 1996). Now-a-days antibiotic producing micro-organisms are received considerable attention.

Hence, the present investigation was conducted to study the antibiotic activity of metabolites produced by *P. fluorescens* against *Fusarium oxysporum* f.sp. *cepae*.

MATERIAL AND METHODS

Isolation of *Pseudomonas* isolates from the onion rhizosphere region :

Antagonistic bacteria were isolated from the rhizosphere soil collected from various onion growing areas of Tamil Nadu. The plants were pulled out gently with intact roots and the excess soil adhering on roots was removed gently. Ten gram of rhizosphere soil was transferred to 250 ml Erlenmeyer flask containing 100ml of sterile distilled water. After thorough shaking, the antagonist in the suspension was isolated by serial dilution plate method. From the final dilutions of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}, one ml of each aliquot was pipetted out, poured in sterilized Petri dish containing King's B medium (King *et al.*, 1954) and they were gently rotated clockwise and anti clockwise for uniform distribution and incubated at room temperature (28±2^{0}C) for 24 hours. Colonies with characteristics of *Pseudomonas* spp. were isolated individually and purified by streak plate method (Rangaswami, 1993) on King's B medium. The pure cultures were maintained on respective agar slants at 4^{0}C.

Isolation of Phenazine and 2, 4 diacetylpholoroglucinol (2, 4 DAPG) from *Pseudomonas* isolates :

The antagonistic bacterial isolates were grown in King's B (KB) medium at 30^0 C on a rotary shaker. The cells were collected by centrifugation at 3500 rpm for seven minutes. The pellets were resuspended in pigment production broth and then incubated on a rotary shaker for four days at 30^{0}C. The antibiotic phenazine - 1-carboxylic acid (PCA) was isolated as per the procedure described by Rosales *et al*. (1995).

The antibiotic was separated in to respective fractions after acidifying the culture filtrate with 1N HCl to pH 2.0 and then extracting the culture filtrate with an equal volume of benzene. Then the benzene phase was extracted with 5 per cent $NaHCO_3$. Phenazine was recovered from the bicarbonate layer while oxychlororaphine remained in the benzene layer. The bicarbonate fraction was extracted once again with benzene to recover phenazine from bicarbonate fraction. The antibiotic was air dried and dissolved in methanol. For the isolation of 2,4 DAPG the above procedure was followed except use of benzene, ethyl acetate was used. The ethyl acetate extracts were reduced *in vaccuo*. The residues were dissolved in methanol and used for further studies (Rosales *et al*., 1995).

Effect of phenazine and 2, 4 DAPG on the growth of *Fusarium oxysporum* f.sp. *cepae* :

The antibiotic phenazine and 2, 4 DAPG were assayed for its antifungal action by filter paper disc assay (Lam and Ng, 2001). Three filter paper discs were placed on solidified PDA in Petri plates. The antibiotic extracted was pipetted and placed on filter at the rate of 150 µl. Five mm mycelial agar disc of pathogen was placed at the centre of the plate and incubated at 28 ± 2^{o}C. The surface area of inhibition was recorded by tracing the area of inhibition in a tracing paper and plotting them on a graph sheet and the per cent inhibition was calculated.

Estimation of Siderophore produced by antagonistic bacteria :

Siderophore production of *Pseudomonas* isolates was estimated by the method described by Reeves *et al*. (1983). King's B was prepared for *Pseudomonas* isolates and 100 ml of broth was poured in to 250 ml conical flask. After sterilization, one ml of standard inoculum of bacterial strains was inoculated into each flask and incubated at room temperature 28±2°C for seven days. Seven days after incubation, the broth culture was centrifuged at 10,000 rpm for 20 minutes and the supernatant was used for the estimation of catecholate type and salicylate type of siderophore.

The pH of supernatant was adjusted to 2.0 with 1N HCl and equal quantity of ethyl acetate was added in a separating funnel, mixed well and ethyl acetate fraction was collected. This process was repeated three times to bring the entire quantity of siderophore from the supernatant. The ethyl acetate fractions were pooled, air-dried and dissolved in 5 ml of ethanol (50 %). Five ml of ethyl acetate fraction was mixed with 5 ml of Hathway's reagent. The absorbance for dihydroxy phenol was read at 560 nm. Sodium salicylate acid was used as standard for the estimation of salicylate type of siderophore. The quantity of siderophore synthesized was expressed as µg ml^{-1} of culture filtrate.

To measure catechol type of siderophore, 5 ml of

ethyl acetate fraction was reacted with 5 ml of Hathway's reagent and absorbance was determined at 700 nm with 2, 3 Dihydroxybenzoic acid as standard. The quantity of siderophore synthesized was expressed as µg ml^{-1} of culture filtrate.

Detection of Hydroxymate and Carboxylate type of siderophores :

It was examined by tetrazolium salt test. Instant appearance of a deep red colour by addition of siderophore sample to tetrazolium salt under alkaline conditions for the presence of hydroxymate type of siderophore (Snow, 1984). Carboxylate type was detected by Vogel's chemical test where the disappearance of pink colour on addition of phenolphthalein to siderophores sample under alkaline condition for the presence of carboxylate type of siderophore (Vogel, 1987).

Estimation of antagonistic *Pseudomonas* isolates for the production of Hydrogen cyanide (HCN) :

Qualitative assay :

HCN production of bacterial biocontrol agents was tested qualitatively according to the method of Miller and Higgins (1970). The antagonistic bacteria were streaked on tryptic soy agar (TSA). Sterile filter paper saturated with picric acid solution (2.5 g of picric acid; 12.5 g of Na_2CO_3; 1000 ml of distilled water) was placed in the upper lid of the Petri plate. The dishes were sealed with parafilm and incubated at 28 ± 2°C for 48 h. A change of colour of the filter paper from yellow to light brown, brown or reddish-brown was recorded each isolate.

Quantitative assay :

Antagonistic bacteria were grown in tryptic soy broth (TSB). Uniform strips of filter paper (10 x 0.5 cm^2) were soaked in alkaline picrate solution and placed inside the conical flask in a hanging position. After incubation at 28 ± 2°C for 48 h the sodium picrate in the filter paper was reduced to a reddish compound in proportion to the amount of hydrocyanic acid evolved. The colour was eluted by placing the filter paper in a test tube containing 10 ml of distilled water and its absorbance was read at 625 nm (Sadasivam and Manickam, 1992).

RESULTS AND DISCUSSION

The findings of the present study as well as relevant discussion have been presented under the following heads:

Efficacy of Phenazine and 2, 4 diacetyl phloroglucinol produced by *P. fluorescens isolates* against *Fusarium oxysporum* f. sp. *cepae in vitro* :

Antibiotics produced by different PGPR have a broad-spectrum activity (Thomashow *et al*., 1997). Phenazine produced by Pf 12 was recorded maximum inhibition of mycelial growth (2.12 cm) followed by Pf 27 which recorded 2.96 cm mycelial growth (Table 1). 2, 4 DAPG produced by Pf 12 was recorded maximum inhibition of mycelial growth (2.03 cm) followed by Pf 27 which recorded 2.19 cm mycelial growth (Table 2). The results were in line with the findings of Bakker *et al*. (2002) revealed that *P. putida* WCS 358r produced phenazine-3-carboxylic acid (PCA) and 2, 4 DAPG which has antifungal and antibacterial action. Slininger

Table 1 : Efficacy of phenazine produced by effective *P. fluorescens* against *Fusarium oxysporum* f. sp. *cepae in vitro*

Sr. No.	Isolates	Mycelial growth (cm)*	Per cent reduction over control
1.	Pf 5	5.58	37.86
2.	Pf 12	2.12	67.03
3.	Pf 23	4.74	47.21
4.	Pf 27	2.96	65.25
5.	Pf 30	3.53	60.46
6.	Pf 32	5.38	40.08
7.	Pf 37	3.92	45.21
8.	Pf 42	5.85	34.85
9.	Pf 46	4.72	47.43
10.	Pf 51	5.83	35.07
11.	Control	8.98	-
C.D. (P=0.05)		0.46	

* Mean of three replications

and Shea – Wilbur (1995) observed that Pf strain 2-79 produced phenazine 1-carboxylic acid (PCA) suppressed take all disease of wheat. Raaijmakers *et al.* (1997) focused the role of phenazine (Phz) and 2,4-diacetylphloroglucinol (PHL) produced by Pseudomonas spp. in soils that are naturally suppressive to take-all of wheat caused by *G. graminis var. tritici*. Fakhouri *et al*. (2001) found that the antifungal substance produced by fluorescent pseudomonas (Pyuluteorin and Phenazine derivatives) caused a collapse of the hyphae of *F.oxysporum* f.sp *lycopersici.*

Kavitha (2004) reported that *P. chlororaphis* isolate PA 24 and *B. subtilis* isolate CBE 4 produced 2,4 DAPG and phenazine which were inhibitory to the growth of the *P. aphanidermatum* in turmeric and to the other soil borne pathogens, *viz*., *M. phaseolina*, *F. oxysporum* f. sp. *cubense* and *Sclerotium rolfsii*. Kamalakannan (2004) reported that phenazine and 2, 4 DAPG antibiotics were inhibitory to mycelial growth of *M. phaseolina* and *R. solani* under *in vitro* condition. Malathi (2015) reported that Pf 12 and Pf 27 of the *Pseudomonas* isolates were found to be the most effective in inhibiting the growth of *F. oxysporum* f. sp.*cepae.* This might be due to the production of antibiotics, volatile compounds and lytic enzymes.

Siderophore production by effective *P. fluorescens* isolates *in vitro :*

Siderophore play a vital role in the suppression of plant pathogens by creating competition for iron. In the present study, all the bacterial strains produced siderophores in Chromeazurol S (CAS) plate assay

Table 2 : Efficacy of 2, 4 diacetyl phloroglucinol produced by effective *P. fluorescens* against *Fusarium oxysporum* f. sp. *cepae in vitro*

Sr. No.	Isolates	Mycelial growth (cm)*	Per cent reduction over control
1.	Pf 5	4.57	48.99
2.	Pf 12	2.03	77.34
3.	Pf 23	3.74	58.25
4.	Pf 27	2.19	75.55
5.	Pf 30	4.26	52.45
6.	Pf 32	5.38	39.95
7.	Pf 37	3.92	56.25
8.	Pf 42	5.85	34.70
9.	Pf 46	4.62	48.43
10.	Pf 51	5.73	36.04
11.	Control	8.96	-
	C.D.(P=0.05)	0.42	

* Mean of three replications

Table 3 : Siderophore Production by effective bacterial antagonists

Antibiotic of the antagonistic bacteria	Colour of the siderophores pigment	Area of production (mm^2)**	Nature of siderophore		Quantitative assay	
			Hydroxymate type	Carboxymate type	Salicylate type (µg/ ml)	Catecholate type (µg/ ml)
Pf 5	Yellow	53.45	+	-	64.25	19.47
Pf 12	Greenish yellow	87.38	+++	-	134.16	27.94
Pf 23	Yellow	64 .21	++	-	89.41	20.02
Pf 27	Yellow	85 .32	+++	-	126.27	27.45
Pf 30	Yellow	80.65	+++	-	93.39	23.19
Pf 32	Greenish yellow	65.43	+	-	74.28	17.45
Pf 37	Yellow	67.32	++	-	55.16	12.45
Pf 42	Yellow	59.31	+	-	79.41	18.45
Pf 46	Yellow	81.34	++	-	66.27	15.32
Pf 51	Yellow	54.32	+	-	91.29	20.54

*Mean of three replications

+: Appearance of light red colour, ++: Appearance of moderate red colour

+++ : Appearance of deep red colour; -: Disappearance of pink colour,

Table 4 : Hydrogen cyanide production by effective *P. fluorescens*

Isolates	Qualitative assay	Quantitative assay (O.D value)
Pf 5	++	0.264
Pf 12	+++	0.457
Pf 23	+	0.241
Pf 27	+++	0.397
Pf 30	++	0.273
Pf 32	+	0.154
Pf 37	++	0.257
Pf 42	+	0.141
Pf 46	+	0.096
Pf 51	+	0.154
Control	-	0.0
C.D.(P=0.05)		0.023

* Mean of three replications
Change of colour from yellow to light brown (+)- slight HCN production; Brown (++): moderate HCN production; Reddish-brown (+++): strong HCN production (-) No production of HCN.

method. In this study the pseudomonas isolates produced either hydroxamate type or carboxylate nature of siderophore. *P. fluorescens* isolate Pf 12 and Pf 27 strongly produced hydroxymate type of siderophore (Table 3). Kloepper *et al*. (1988) documented the production of fluorescent siderophore by *P. fluorescens* which attributed to its antagonistic action. Leeman *et al*. (1996) documented that siderophore could induce systemic resistance against *Fusarium* wilt of radish. The hydroxymate type of siderophore was ferribactin produced by *P. fluorescens* (Linget *et al*., 1992).

Similar results obtained by Lim *et al*. (1999) showed that siderophore production *P. fluorescens* GL 20 inhibited spore germination and hyphal growth of *F. solani in vitro* and reduced the disease incidence with enhanced plant growth. Meena *et al*. (2001) studied the effect of siderophore on the growth of *M. phaseolina* under *in vitro* condition.

HCN Production by effective *P. fluorescens* isolates:

Hydrogen cyanide (HCN), a volatile inhibitor produced by *P. fluorescens* is known to inhibit the electron transport disrupting the energy supply leading to cell death (Knowels, 1976). In the present study, production of HCN was very strong in Pf 12 and Pf 27 isolates (Table 4). HCN produced by *P. fluorescens* was the major contribute for the suppression of black root rot of tobacco caused by *T. basicola* (Ahl *et al*., 1986). Meena *et al*. (2001) compared the HCN production by several strains of *P. fluorescens* and their efficacy in controlling root rot of groundnut caused by *M. phaseolina*. Mondal *et al*. (1998) suggested that *P. flourescens* (Rb-26) produced maximum HCN production which in turn showed stronger growth inhibition of *X. axonopodis* pv. *malvacearum*. Role of HCN in disease suppression was demonstrated by several scientists in various crops (Voisard *et al*., 1989; Defago *et al*., 1990).

Pseudomonas isolate (Pf 12) produce extremely effective broad spectrum antifungal molecules against various phytopathogens, thus acting as efficient biocontrol agents. They shows potential bioinoculants for agricultural system to enhance the productivity moreover which is highly specific, ecofriendly and cost-effective. The present investigation revealed that the potentiality of the biocontrol agents as an alternative to the chemical for controlling the onion basal rot incidence.

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