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ANTIFUNGAL ACTIVITY OF SECONDARY METABOLITES PRODUCED BY *PSEUDOMONAS FLUORESCENS*

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ABSTRACT
Thirty isolates of *Pseudomonas fluorescens* obtained from citrus rhizosphere were tested for antifungal activity against *Phytophthora* spp. *P. fluorescens* isolate Pf_{20} was found efficient in inhibiting the mycelial growth upto 38.88%. The antifungal compounds were extracted with equal volume of ethyl acetate and were tentatively identified on thin layer chromatography (TLC) at R_f 0.28. The antifungal compounds extracted from *P. fluorescens* at 5% were found inhibitory to the growth of *Rhizoctonia solani* (42.79%), *Phytophthora parasitica* (28.57%), *P. palmivora* (25.98%) and *Fusarium solani* (20.45%). In case of HPTLC analysis the characteristic colour and fluorescent band after derivatization with anisaldehyde reagent proved the presence of secondary metabolites in crude extract.

INTRODUCTION

The search for alternatives to chemical control of plant pathogens has gained momentum in the recent years due to the emergence of fungicide resistance in pathogens besides increased health concerns for the producer and the consumer.

Currently studies pertaining to the use of botanicals in management of pathogens and related diseases are highly focused (Koche, 2013; Toppo, 2013; Mathad, 2013; Mathad, 2013; Mahapatra, 2013; Bisht, 2013).

Pseudomonas spp. have been studied mainly because of their widespread distribution in soil, their ability to colonize the rhizosphere of host plants and ability to produce a wide range of compounds inhibiting number of serious plant pathogens (Anjaiah *et al.,* 1998, Copper and Higgins, 1993, Vidhysekaran and Muthamilan, 1995 and Weller, 1998). Fluorescent Pseudmonas, particularly *Pseudomonas putida* and *Pseudomonas fluorescens*, which are commonly isolated from the plant rhizosphere, have been shown to protect plants from fungal infection. Two factors have been cited as essential for biocontrol; colonization of the rhizosphere and production of antibiotics.

The ability of *P. fluorescens* to suppress soil borne fungal pathogens depends on their ability to produce antibiotic metabolites such as pyoluteorin, pyrrolnitrin, phenazine 1-carboxylic acid and 2, 4-diacetyl phloroglucinol (Georgakopoulos, 1994, Maurhofer *et al.,* 1995, Sullivan and Gara 1992). Certain fluorescent Pseudomonads from soil have been shown to promote plant growth by inhibiting bacteria and fungi that are deleterious to plant (Schroth and Hancock 1982). The production of antibiotic substances by some strains has been recognized as a major factor in the suppression of many root pathogens.

Antibiotic production by *P. fluorescens* is now recognized as an important feature in plant disease suppression by some strain. In this investigation attempts were made to isolate antifungal compounds from *P. fluorescens* and to see their efficacy to inhibit soil pathogens in rhizosphere of citrus.

MATERIALS AND METHODS

Isolation and bioassay of antifungal compound

Soil samples were collected from rhizosphere of citrus in Vidarbha region. 30 isolates were obtained through serial dilution using King's B medium (King *et al.,* 1954). Colonies that showed fluorescens were selected and further purified. The strains were tested for antifungal activity against *Phytophthora* spp. by dual culture technique (Rabindran and Vidhyasekaran, 1996). The most effective strain (Pf_{20}) that showed the highest inhibition was grown in King's B broth (pH 7.0) under controlled conditions (28ºC) with stirring at 120 rpm for 96h. The culture was centrifuged at 10,000 rpm for 20min at 4ºC to obtain cell free filtrate (Tripathi and Johri 2002). The antifungal compounds were extracted from cell-free broth with equal volume of ethyl acetate, methanol, chloroform and benzene and evaporated in a rotary evaporator at 45ºC to ensure complete solvent removal. The dry residue thus obtained was dissolved in minimum quantity of DMSO

Table 1: Efficacy of *P. fluorescens* isolates against *Phytophthora parasitica* by dual culture technique

Isolates	Radial mycelial growth (mm)	% growth inhibition
Pf_1	70.20	22.00
Pf_2	90.00	00
Pf_3	90.00	00
Pf_4	80.50	10.55
Pf_5	88.60	01.55
Pf_6	77.80	13.55
Pf_7	90.00	00
Pf_8	90.00	00
Pf_9	73.50	18.33
Pf_{10}	82.30	08.55
Pf_{11}	90.00	00
Pf_{12}	88.00	02.22
Pf_{13}	90.00	00
Pf_{14}	80.50	10.55
Pf_{15}	73.10	18.77
Pf_{16}	81.70	09.22
Pf_{17}	88.10	02.11
Pf_{18}	85.80	04.66
Pf_{19}	84.00	06.66
Pf_{20}	55.00	38.88
Pf_{21}	90.00	00
Pf_{22}	85.80	04.66
Pf_{23}	87.50	02.77
Pf_{24}	90.00	00
Pf_{25}	90.00	00
Pf_{26}	70.50	07.05
Pf_{27}	88.80	01.33
Pf_{28}	90.00	00
Pf_{29}	90.00	00
Pf_{30}	81.30	09.60
Control	90.00	
'F' Test	Sig	
S.E. (M) ±	1.99	
CD (P = 0.01)	7.66	

Table 2: Screening of antifungal activity of crude extract @ 5% concentration

Sr.No.	Microorganisms	Growth inhibition %
1	*Rhizoctonia solani*	42.79
2	*Phytophthora parasitica*	28.57
3	*P. palmivora*	25.98
4	*Fusarium solani*	20.45

for further studies. Antifungal activity of crude extract was tested against fungal pathogens by poisoned food technique@ 5% (Nene and Thapliyal 1971).

Identification and purification of antifungal compounds

TLC was carried out with the crude extract on silica gel with acetonitrile: methanol: water (1:1:1) solvent system (Rossales *et al.,* 1995). The crude extract (10μL) was spotted on TLC plates and the solvent front was allowed to run up to 16 cm. Plates were then dried and observed under UV to identify spots. After cut into portions (1 by 2.5cm), these portions were scrapped into micro centrifuge tubes. Each fraction was concentrated by evaporating off the ethyl acetate, methanol, chloroform and benzene and the compounds were purified by HPTLC.

The developed plate was visualized under day light, UV at 254nm and UVat366nm, by using a photodocumentation system (Camag).The densitogram of the developed plate was taken using Scanner 3 (Camag) and the retention factor (R_f) for each track was noted. Anisaldehyde -sulphuric acid was used as a visualizing agent for derivatization of plates.

RESULTS AND DISCUSSION

Antifungal compounds on fungal pathogens

Thirty strains of *P. fluorescens* were isolated from soil samples. Among the 30 isolates the most efficient isolate was Pf_{20} which inhibited mycelial growth of *Phytophthora* spp. up to 38.88 per cent (Table 1).

Secondary metabolites extracted from *P. fluorescens* were evaluated for antimicrobial activity @ 5% and per cent growth inhibition was calculated. The crude compound inhibited the growth of all pathogens at 5%. Maximum growth inhibition was recorded in *Rhizoctonia solani* (42.79%) followed by *Phytophthora parasitica* (28.57%) and *P. palmivora* (25.98%). Least inhibition was observed in *Fusarium solani* (20.45%) (Table 2). *P. fluorescens* was found effective against numerous fungi (16, 8). *P. fluorescens* was found to inhibit *R. solani* on agar plate method (Rossales *et al.,* 1995). Fluorescent Pseudomonads produce secondary metabolites with antibiotic activities and suppressed many soil borne diseases (Thomashow and Weller 1996). In recent years, fluorescent Pseudomonads have drawn worldwide attention as they produce secondary metabolites such as siderophore, antibiotics, volatile compounds HCN, enzymes and phytoharmones (Reddy *et al.,* 2007).

The compound purified by thin layer chromatography showed one antifungal compound at R_f 0.28 using ethyl acetate as a extraction solvent which showed antifungal activity against pathogens that were inhibited by Pf_{20} isolates. This indicated that the antagonistic property of isolates Pf_{20} is due to this compound. These antifungal compounds were dissolved in different organic solvents (DMSO, Ethyl acetate and Methanol) and subjected to high performance thin layer chromatography for characterization. Results are presented in Table 3. HPTLC analysis of crude metabolite extracts indicated 3, 2 and 0 spots at 254nm, 366nm and after derivatization of plates of sample DMSO which was extracted with ethyl acetate. Whereas, maximum number of (3) spots obtained in the Ethyl acetate-B and Methanol-4 sample which was extracted with ethyl acetate and chloroform, respectively at 366 nm and in DMSO which was extracted with ethyl acetate at 254nm. DMSO- A indicates 1, 2 and 0 spots at 254nm, 366nm and after derivatization of plates. DMSO- B indicated 1, 0 and 1 at 254nm, 366nm after derivatization of plates. No spots was observed in sample Methanol-5 extracted with benzene. Thus analysis of crude extract was done for the characterization and comparison patterns of bands. The characteristic colour and fluorescent band obtained after derivatization with anisaldehyde reagent proved the presence of secondary metabolites in crude extract.

The active substance was partially characterized by HPTLC and noted the R_f values. The R_f values of the active fractions are different from the R_f values reported for other antibiotic-producing strains of *Pseudomonas* (Gurusiddaiah *et al.,* 1986,

Table 3: HPTLC analysis of crude metabolite extracts

S. N.	Sample	Extraction solvent	No. of spots detected 254 nm	366 nm	After derivatization
1	DMSO	Ethyl acetate	3	2	-
2	DMSO-A	Ethyl acetate	1	2	-
3	DMSO-B	Ethyl acetate	1	-	1
4	Ethyl acetate-A	Ethyl acetate	1	1	1
5	Ethyl acetate-B	Ethyl acetate	1	3	1
6	Methonol-1	Methanol	1	2	1
7	Methonol-2	Methanol	1	1	1
8	Methonol-3	Chloroform	2	2	1
9	Methonol-4	Chloroform	2	3	-
10	Methonol-5	Benzene	-	-	-

Table 4: High performance thin layer chromatographic migration of the antifungal compound produced by Pf_{20} isolates

Sr.No.	Sample	Extraction solvent	R_f values At 254 nm			At 366 nm			After derivatization
1	DMSO	Ethyl acetate	0.30	0.44	0.54	0.28	0.30	-	-
2	DMSO-A	Ethyl acetate	0.25	-	-	0.40	0.70	-	-
3	DMSO-B	Ethyl acetate	0.33	-	-	-	-	-	0.22
4	Ethyl acetate-A	Ethyl acetate	0.35	-	-	0.39	-	-	0.40
5	Ethyl acetate-B	Ethyl acetate	0.34	-	-	0.31	0.44	0.60	0.40
6	Methanol-1	Methanol	0.36	-	-	0.38	0.71	-	0.39
7	Methanol-2	Methanol	0.37	-	-	0.72	-	-	0.30
8	Methanol-3	Chloroform	0.40	0.66	-	0.42	0.60	-	0.22
9	Methanol-4	Chloroform	0.38	0.51	-	0.31	0.46	0.51	-
10	Methanol-5	Benzene	-	-	-	-	-	-	-

Howell and Stipanovic 1979 and Howell and Stipanovic 1980) (Table 4).

In conclusion, antibiotic production by fluorescent Pseudomonads is now recognized as an important feature in plant disease suppression by some strains. However due to the scarcity of nutrients in most soils, antibiotic production is generally restricted. Thus the results suggest that production of secondary metabolites in citrus rhizosphere is yet another benefit that reduces the severity of these diseases.

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