Antifungal Metabolites of *Pseudomonas fluorescens* Isolated from Rhizosphere of Rice Crop

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Abstract

Thirty isolates of *Pseudomonas fluorescens* obtained from rice rhizosphere were tested for antifungal activity against *Magnaporthe grisea, Dreschelaria oryzae*, *Rhizoctonia solani* and *Sarocladium oryzae* that are known to attack rice plants. One *P. fluorescens* isolate (Pf 003) effectively inhibited the mycelial growth in all these fungi in dual culture tests (62–85%). The antifungal compounds were extracted with equal volume of ethyl acetate. The antifungal compounds from *P. fluorescens* at 5% completely inhibited the pathogens. The antifungal compounds were tentatively identified on thin layer chromatography (TLC) at R_f 0.22, 0.35, 0.42 and 0.51. These compounds were individually purified by column chromatography and re-tested for antifungal activity. One compound with R_f 0.35 on TLC completely inhibited the mycelial growth of all test fungi at 0.5 %. This compound showed melting point at 168-173 C. The proton nmr and C^{13} nmr confirmed its identity as 2, 4- diacetylphloroglucinol (DAPG). IR spectrum of this sample showed OH functional group at 3341 cm^{-1} and the molecular weight was estimated as M/z 210 by mass spectrometry, which agreed with the 2,4- DAPG composition of $C_{10}H_{10}0_5$.

Key words: Rice, *P. fluorescens*, 2, 4 - DAPG, fungal pathogens, NMR, IR and Mass spectroscopy

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The search for alternatives to chemical control of plant pathogens, such as biological control 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. Bacteria found in rice fields produced fluorescent and non-fluorescent pigments on King's B medium were shown to be antagonistic to *Rhizoctonia solani* that causes rice sheath blight (Mew and Rosales 1986) and also Bakanae disease of rice caused by *Fusarium moniliforme* (Rosales et al 1986). *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 inhibitory to a number of serious plant pathogens (Anjaiah et al 1998; Weller 1988; Copper and Higgins 1993; Vidhyasekaran and Muthamilan 1995). *Pseudomonas fluorescens* was reported to provide 79-82 % control of rice blast and sheath blight and thus increase grain yield in rice (Gnanamanickam et al 1998). The ability of *P. fluorescens* to suppress soilborne fungal pathogens depends on their ability to produce antibiotic metabolites such as pyoluteorin, pyrrolnitrin, phenazine 1- carboxylic acid and 2, 4-diacetylphloroglucinol (Georgakopoulos et al 1994; Maurhofer et al 1995; Sullivan and Gara 1992). One of these metabolites DAPG is a major factor in the biological control of a range of plant pathogens (Defago 1993). A few reports are available on the characterization of 2,4- DAPG and its antifunagal activity against plant pathogens (Thompson et al 1994). In this investigation attempts were made to identify and characterize antifungal compounds in *P. fluorescens* isolated from rhizosphere region of rice crop.

Materials and Methods

Isolation and bioassay of antifungal compounds. The *P. fluorescens* strains were isolated from 150 soil samples collected from rhizosphere region of rice using King's B medium (King et al 1954). Colonies that showed fluorescence at 365 nm were selected and further purified. *M. grisea* and *R. solani* were procured from Directorate of Rice Research, Rajendranagar, Hyderabad and *D. oryzae* and *S. oryzae* were obtained from IARI, New Delhi. The strains were tested for antifungal activity against these pathogens by dual culture technique (Rabindran and Vidyasekaran 1996). The most effective strain that showed the highest antifungal activity in dual culture was grown in King's

B broth (p^H 7.0 and dissolved oxygen 40%) in a fermentor (B braun) under controlled conditions (28 C) with stirring (120 rpm) for 96 h. The culture was centrifuged at 10,000 rpm for 20 min at 4 C to obtain cell-free filtrate (Tripathi and Johri 2002). The antifungal compounds were extracted from cell-free broth with equal volume of ethyl acetate and evaporated in a rotary evaporator at 45 C to ensure complete solvent removal. The dry residue thus obtained was dissolved in a minimum quantity of acetone for further studies. This crude antifungal compounds were tested for the antifungal activity against the four fungal pathogens by poisoned food technique (Nene and Thapliyal 1971) at 1to 5 % concentrations with only acetone as control.

Identification and purification of antifungal compounds. TLC was carried out with the crude extract on silica gel (TLC silica gel 60, 20 x 20, 0.5 mm, Merck & Co, Inc) with acetonitrile:methanol:water (1:1:1) solvent system (Rosales et al 1995). The crude extract (30 µl) 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. The compounds were purified by column chromatography. The glass column (50x2 cm) was packed with slurry of silica gel (60-120 mesh) preactivated at 120 C for 4 h and loaded with sample. Then the column was successively eluted with hexane-benzene (1:3) to collect 5 ml fractions at a flow rate of 1 ml/min (Samanta and Dutta 2004). The fractions were spotted individually on TLC and observed under UV to locate fluorescence samples showing similar compositions were mixed together, concentrated on rotoevaporator to dryness which was then dissolved in a minimum quantity of acetone. The individual compounds were tested for antifungal activity by poisoned food technique (Nene and Thapliyal 1971) at 0.1 to 0.5 % concentrations with only acetone as control.

Crystallization and characterization of antifungal compound. The effective antifungal compound was crystallized by dissolving in acetone (6 ml) and filtered to remove insoluble impurities. The sediment was again washed with acetone (2 ml) and the wash was added to acetone solution filtrate. The solution was left to stand over night at –20 C to dryness to collect the crystals. The melting point of crystallized metabolite was determined by subjecting a capillary tube filled at one end with dried powder to heat at 180 C. The crystallized antifungal component was re-suspended in a minimum quantity of acetone and analyzed by nuclear magnetic resonance spectroscopy (NMR 400 MHz), Fourier transform infrared spectroscopy (FTIR, model 460 and Jasco) and Electron ionization mass spectroscopy (EIMS) to identify the chemical structure of antifungal compound.

Results and Discussion

Antifungal compounds on rice fungal pathogens. Thirty strains of *P. fluorescens* were isolated from soil samples. Among the 30 isolates, one isolate Pf 003 was found to effectively inhibit (62 to 85%) the mycelial growth of all fungal pathogens tested (Table 1).

Table 1. Inhibition (%) of mycelial growth in dual culture between *P. fluorescens* and rice fungal pathogens

Strains	*Mg*	*Do*	*Rs*	*So*
Pf 001	42	0	42	4
Pf 002	17	53	15	13
Pf 003	62	72	85	67
Pf 004	21	55	27	0
Pf 005	38	45	34	25
Pf 006	34	0	20	33
Pf 007	42	13	33	0
Pf 008	30	29	3	13
Pf 009	18	36	13	18
Pf 010	20	50	11	7
Pf 011	27	15	18	28
Pf 012	24	13	3	44
Pf 013	20	0	20	53
Pf 014	22	28	3	46
Pf 015	14	33	13	0
Pf 016	11	53	3	20
Pf 017	13	38	11	4
Pf 018	6	13	13	0
Pf 019	3	0	17	25
Pf 020	7	23	3	0
Pf 021	2	44	17	12
Pf 022	17	46	7	50
Pf 023	13	46	18	13
Pf 024	1	13	11	0
Pf 025	2	18	13	24
Pf 026	6	15	3	18
Pf 027	18	11	11	33
Pf 028	3	8	3	4
Pf 029	13	0	17	24
Pf 030	11	13	14	28

Mg= Magnaporthe grisea, Do= Dreschelaria oryzae, Rs = Rhizoctonia solani, So = Sarocladium oryzae

The crude compounds completely inhibited the growth in all pathogens at 5%. In dual cultures with rhizospheric bacteria, soil borne pathogens *viz., R. solani, Macrophomina phaseolina, Sclerotiana sclerotiorum* and *Sclerotium rolfsii* (Samantha and Dutta 2004) were reported to be inhibited. The antimicrobial activity of *P. fluorescens* had reported against numerous fungi (Sivamani and Gnanamanickam 1988; Khan and Zaidi 2002). The powder formulation of *P. fluorescens* had effectively inhibited the rice blast

disease in glass house and field conditions (Vidyasekaran et al 1997). *P. fluorescens* was shown to effectively inhibit *R. solani* and *P. oryzae* by agar plate method (Rosales et al 1995). The peat-based formulation of *P. fluorescens* had effectively inhibited the rice sheath blight in *in vitro* and glasshouse (Rabindran and Vidyasekaran 1996). Fluorescent pseudomonads have been used to suppress *R. solani* on rice (Mew and Rosales 1984). Sakthivel and Gnanamanickam (1986a) had reported that the *P. fluorescens* was antagonistic to *Sarocladium oryzae* and that bacterisation of rice with *P. fluorescens* substantially reduced sheath infection (Sakthivel and Gnanamanickam 1986b). However in none of these studies, the metabolites were isolated and characterized.

Identification of antifungal compounds. TLC of the metabolites produced by *P. fluorescens* showed four antifungal compounds at R_f 0.22, 0.35, 0.42 and 0.51. These antifungal components were purified individually using column chromatography. The antifungal compound with R_f 0.35 on TLC completely inhibited the mycelial growth of all fungal pathogens at 0.5 % while another antifungal compound at R_f 0.22 showed 71– 89 % inhibition (Table 2). In recent years, fluorescent pseudomonads have drawn worldwide attention as they produce secondary metabolites such as siderophore, antibiotics, volatile compounds HCN, enzymes and phytohormones. Although isolates of *P. fluorescens* could be obtained from the rhizosphere of different rice, antagonistic potential of these isolates appeared to vary in great deal (Castric and Castric 1983; Hagedron et al 1989). Jayaswal et al (1990) had shown such inhibitory effect of individual metabolites from TLC against corn fungal pathogens. Rovera et al (2000) had reported the similar R_f 0.35 for 2,4- DAPG produced by *P. aurantica* and their antifungal activity against *Macrophomina phaseolina*.

Table 2. Bioefficacy of individual antifungal compounds against rice fungal pathogens

Antifungal compounds & Conc. (%)	*M.grisea*		*D.oryzae*		*R.solani*		*S.oryzae*	
	MG (mm)	INH (%)	MG (mm)	INH (%)	MG (mm)	INH (%)	MG (mm)	INH (%)
Antifungal compound 1 (R_f-0.22)								
0.1	32	64	26	71	28	69	42	53
0.2	30	66	22	75	25	72	34	62
0.3	29	68	15	83	22	75	30	66
0.4	27	70	12	87	19	78	26	71
0.5	26	71	10	89	15	83	20	78
Antifungal compound 2 (R_f-0.35)								
0.1	12	86	20	78	14	84	35	61
0.2	9	90	16	82	12	86	26	71
0.3	8	91	8	91	9	90	18	80
0.4	6	93	5	95	8	91	10	89
0.5	0	100	0	100	0	100	0	100
Antifungal compound 3 (R_f-0.42)								
0.1	46	49	55	40	33	63	49	45
0.2	43	52	48	46	30	66	42	53
0.3	39	56	36	60	26	71	37	59
0.4	35	61	30	67	24	73	28	69
0.5	32	64	25	72	20	78	22	75
Antifungal compound 4 (R_f-0.51)								
0.1	56	38	64	30	62	31	68	24
0.2	52	42	55	40	60	33	59	34
0.3	46	49	52	42	59	34	42	53
0.4	39	56	46	49	58	35	34	62
0.5	35	61	32	64	56	38	28	69
Control	90	0	90	0	90	0	90	0
CD ($P \leq 0.5$)	6.8	-	4.2	-	6.2	-	3.8	-
CV (%)	4.0	-	2.6	-	4.8	-	1.6	-

MG= Mycelial growth, INH= inhibition over control

Characterization of antifungal compound. The melting point of the antifungal compound showed 168-173 C. This was matched with the earlier report of Strunz et al (1978). The H^1NMR (400 $MH_{Z,}$ acetone) spectrum showed peaks at δ5.85, δ 5.0, and δ 2.55. The C^{13} NMR spectrum showed peaks at δ 204.5, δ 172.2, δ 169.1, δ 104.3, δ 95.6 and δ 31.7. The FTIR spectrum showed the presence of different bands corresponding to the following functional groups present in the molecular structure; a broad absorption at 3341 cm^{-1} corresponding to a bounded OH stretching; two sharp bands at 2921 and 2857 cm^{-1} due to the stretching of methyl group attached to a benzene ring; the stretching of the carbonyl group at 1742.5 cm^{-1} and a sharp band at 1073.7 cm^{-1} due to the bending of a phenyl-methyl ether group (Fig 1).

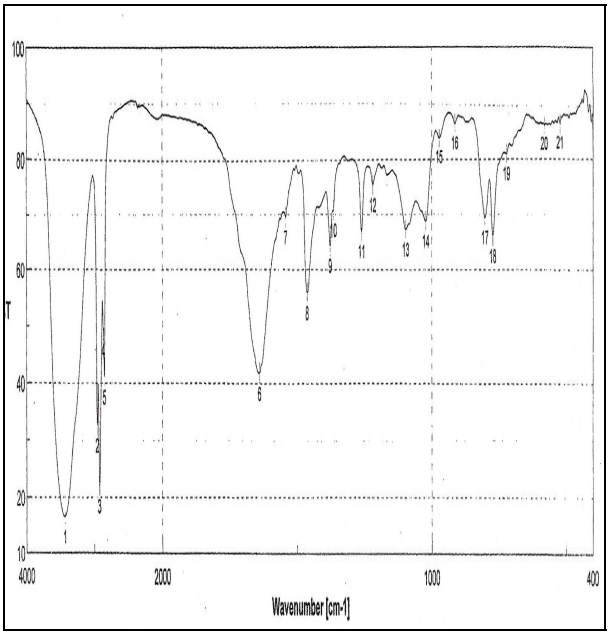

Figure 1. IR Spectrum of 2, 4- DAPG produced by *P. fluorescens*

The molecular weight was estimated at M/z 210 by mass spectroscopy (Fig 2), which agreed with the composition $C_{10}H_{10}O_{5.}$ Based on these spectral analysis the antifungal compound was identified as 2,4 diacetyl phloroglucinol. These spectra of DAPG were matched with earlier reports with *P. fluorescens* isolated in Canada (Thompson et al 1994). Antibiotic production by fluorescent pseudomonads in wheat rhizosphere was recognized as an important feature in plant disease suppression by some strains (Thomashow et al 1990). We have demonstrated the production of antifungal compound DAPG by *P. fluorescens* isolated from rice. This antifungal compound can be effectively used against rice fungal pathogens.

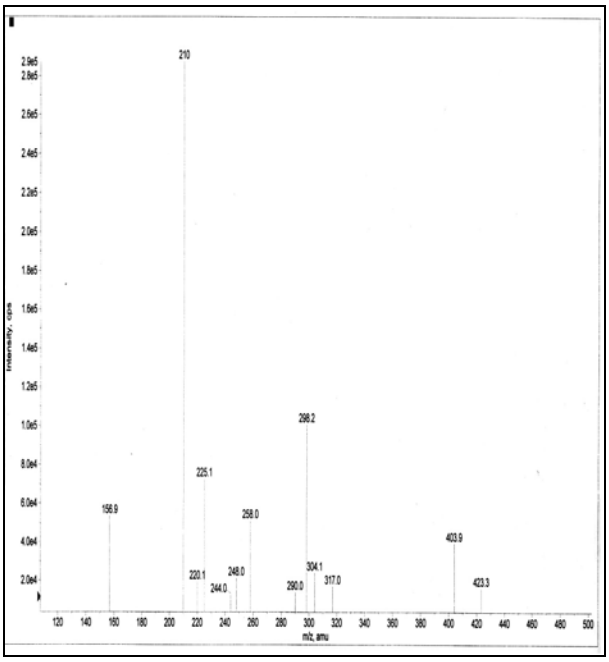

Figure 2. Mass spectrum of 2, 4- DAPG produced by *P. fluorescens*

P. fluorescens culture broth or its formulations can be used to control the fungal diseases of rice an ecofriendly option.

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