

Antifungal Metabolites of *Pseudomonas fluorescens* against *Pythium aphanidermatum*

S.R. Prabhukarthikeyan^{1,2*} and T. Raguchander¹

¹Department of Plant Pathology, Centre for Plant Protection Studies,
Tamil Nadu Agricultural University, Coimbatore, India.

²Crop Protection Division, ICAR - National Rice Research Institute, Cuttack, Odisha, India.

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The production of one or more antibiotics is the mechanism most commonly associated with the ability of fluorescent pseudomonads to act as antagonistic agents against plant pathogenic fungi. *Pseudomonas fluorescens* strains were tested for antifungal activity against *Pythium aphanidermatum* that is known to attack turmeric crops. *P. fluorescens* produce antibiotics such as phenazine 1- carboxylic acid, 2, 4-diacetylphloroglucinol, pyoluteorin and pyrrolnitrin. Production of antibiotic compounds were assayed by Thin Layer Chromatography (TLC). The antifungal compounds of DAPG, phenazine and pyoluteorin were identified on TLC at R_f value of 0.88, 0.57 and 0.05 respectively. All the antibiotics extracted from different strains of *P. fluorescens* were tested for their antifungal activity against *P. aphanidermatum*. Among the strains, the antibiotics extracted from strain FP7 effectively inhibited the mycelial growth of *P. aphanidermatum* in agar well diffusion assay.

Keywords: *Pseudomonas fluorescens*, Thin Layer Chromatography, antifungal compounds.

In recent years, biological control of plant diseases is accepted as a durable and eco-friendly alternative for agrochemicals. Soil has enormous untapped potential antagonistic microbes i.e. fluorescent pseudomonads, *Bacillus* and *Trichoderma* spp. which show antagonistic effects against soil-borne plant pathogenic organisms. Among these, the role of Plant Growth Promoting Rhizobacteria (PGPR) viz., *Pseudomonas fluorescens* (Migula) in managing the pathogen in crop plants are well reported^{1,2,3}. Root associated fluorescent pseudomonads produce a wide range of secondary metabolites which are inhibitory to a number of serious plant pathogens. 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, 2, 4 diacetylphloroglucinol, hydrogen cyanide,

kanosamine, pyocyanin and viscosinamide. Among these metabolites DAPG has received a particular attention because of its broad spectrum activity. Numerous studies have demonstrated the determinative role of DAPG production in the suppression of a variety of soil borne diseases by fluorescent pseudomonads⁴.

Phenazines are heterocyclic nitrogen-containing secondary metabolites synthesized by *P. fluorescens* and a few other bacterial genera⁵. Biocontrol by phenazines is connected with their ability to undergo oxidation-reduction transformations thus causing the accumulation of toxic superoxide radicals in the target cells^{5,6,7}. In addition to inhibiting fungal pathogens, phenazine play an essential role in microbial competition in rhizosphere including survival and competence⁸. In addition to that, Pyoluteorin (PLT) is an aromatic polyketide antibiotics consisting of a resorcinol ring derived through polyketide biosynthesis. PLT is produced by several *Pseudomonas* sp. which suppress the plant diseases caused by plant pathogenic fungi⁹. PLT mainly inhibits the

* To whom all correspondence should be addressed.
E-mail: prabhukarthipat@gmail.com

oomycetous fungi including *Pythium ultimum* and it is strongly active, when applied to seeds¹⁰.

In this investigation the attempts were made to identify the antifungal compounds in *P. fluorescens* and testing their activity against turmeric rhizome rot pathogen, *P. aphanidermatum*.

MATERIALS AND METHODS

Bacterial isolates and Pathogen

Two *Pseudomonas fluorescens* strains (PF1 and FP7 isolated from rhizosphere soil of blackgram and rice, respectively) used in this study were obtained from a culture collection, Department of Plant Pathology, Tamil Nadu Agricultural University, India. Besides, the other bacterial strains namely, TPF17, TPF53 and TPF54 were isolated from turmeric rhizosphere. The bacterial strains were identified as *P. fluorescens* and bankitted in the NCBI Genbank, which are bearing the accession number viz., KP887812, KR818037 and KP714263, respectively. All the strains were maintained in King's Medium B (KMB)¹¹ at 4 °C for further use. The turmeric rhizome rot pathogen, *P. aphanidermatum* was isolated from infected rhizome of turmeric. The pathogen was identified based on morphological characters as described by Middleton¹².

Detection of antibiotics by Thin Layer Chromatography (TLC)

Extraction of crude antibiotics

The different bacterial strains were grown on King's B broth and crude antibiotics were extracted with ethyl acetate and the dried sample was dissolved with 1.5 ml methanol. Sample of 5il was spotted onto silica gel plate (Merck, Silica gel 60 F254, Germany). The plates were developed with Isopropanol: Ammonia: Water (8:1:1) and visualized by short wave length (254 nm). For specific antibiotics the *Rf* values was calculated.

Extraction of 2, 4 diacetylphloroglucinol (2, 4 DAPG)

The strains of antagonistic bacteria were grown separately in 20 ml of pigment production broth (peptone, 20 g; glycerol, 20 ml; NaCl, 5 g; KNO₃, 1 g; distilled water, 1 l; pH 7.2) for four days on a rotary shaker at 30°C. The fermentation broth was centrifuged at 3500 rpm for five minutes in a tabletop centrifuge and the supernatant was collected. It was acidified to pH 2.0 with 1N HCl

and then extracted with an equal volume of ethyl acetate. The ethyl acetate extract was reduced to dryness *in vacuo*. The residues were dissolved in methanol and kept at 4°C until use for TLC. For the detection of an antibiotic, 2, 4- DAPG, a volume of 5 il of sample was spotted on to the aluminium coated sheets with silica gel. Separation was performed with acetonitrile/ methanol/water (1:1:1) as a solvent system and visualized by short wave length (245 nm) and diazotized sulphanilic acid. *Rf* value for the spot confirming 2,4-DAPG was calculated¹³.

Extractions of Phenazine (Phenazine 1-carboxylic acid)

For Phenazine the extraction was done by acidifying the cultures with an equal volume of benzene (Phenazine in the benzene layer) and then extraction of the benzene phase with 5% NaHCO₃. PCA was recovered from the bicarbonate layer. The bicarbonate fraction was extracted once with benzene to recover the phenazine. The pigment was air dried, dissolved in methanol and purified by TLC on silica gel with a 250- μ m layer thickness. The solvent system containing isopropanol/ ammonia/ water (8:1:1). Plates were viewed under UV light at 254nm and sprayed with diazotized sulfanilic acid (DSA)¹³.

Extraction of Pyoluteorin

The cultures from different *P. fluorescens* were grown on King's B broth at 27°C for 3 days were centrifuged at 14,000 g for 20 min at 4°C and 20 ml of the supernatants were extracted with an equal volume of ethyl acetate for 2 h by using a rotary shaker. The ethyl acetate extracts were dried in a vacuum at 35°C and were dissolved in 1.5 ml of 65% methanol. A volume of 5 il was applied to aluminum coated sheets with silica gel. Separation was performed with the solvent solution system containing chloroform acetone (9:1 v/v). The TLC plates were sprayed with dinitro salicylic acid¹³.

Effect of antibiotics on the growth of *P. aphanidermatum*

The agar well diffusion assay, as reported by Tagg and McGiven¹⁴ and modified by Islam *et al*¹⁵ was used to determine the antagonistic activity of antibiotic compounds. The PDA medium (20 ml) was poured into each sterile Petri plate, followed by placement of mycelial disc (5 mm in diameter) of the tested pathogen at the center of the plates. A well (7 mm in diameter) was made by punching

the agar with a sterile cork borer on the corner of the plate in four places with equal distance. Then purified antibiotic compounds from different strains of *P. fluorescens* were poured into the wells at the rate of 100 ml per well separately and incubated for 72 h at 28±2°C. The inhibitory activity of each concentration was expressed as the percent growth inhibition, compared to the control (solvent only used in the wells), according to the following formula:

Growth inhibition (%) = (DC – DT)/DC × 100.
where, DC, diameter of fungal colony in control; and DT, diameter of fungal colony with treatment¹⁶.

RESULTS AND DISCUSSION

The antimicrobial activity of *P. fluorescens* had reported against several fungi. The antibiotics such as crude antibiotics, 2,4-DAPG, phenazine and pyoluteorin by different strains of *P. fluorescens* were tested against *P. aphanidermatum* in this study.

The results revealed that crude antibiotics extracted from *P. fluorescens* strain FP7 recorded 40 mm of mycelial growth which accounted for 55.55 per cent inhibition of mycelial growth. It was followed by *P. fluorescens* strain Pf1 which accounted 52.22 per cent inhibition (Table 1). Similar to this results, crude antibiotic compounds completely inhibited the growth in all rice pathogens at 5%¹⁷. Besides, *P. fluorescens* was shown to effectively inhibit the growth of *R. solani* and *M. grisea* by agar plate method¹³. Jayaswal *et al*¹⁸ had shown such inhibitory effect of individual metabolites from TLC against corn fungal pathogens.

In crude antibiotics assay, all the *P. fluorescens* strains significantly produced various antibiotics (Fig. 1). The presence of DAPG was detected by spraying dinitro salicylic acid (DNS) on the TLC plate with R_f value of 0.88, phenazine with the R_f value of 0.57 in the isolates *viz.*, Pf1, FP7, TPF17, TPF53 and TPF54 respectively. Pyoluteorin was found to be expressed in all the

Table 1. Effect of antibiotics from *P. fluorescens* on the growth of *P. aphanidermatum*

Antibiotics	Strains	Mycelial growth of pathogen* (mm)	Per cent inhibition
Crude antibiotics	Pf1	43.0 ^{ab}	52.22 (46.27) ^{ab}
	FP7	40.0 ^a	55.55 (48.19) ^a
	TPF17	45.0 ^b	50.00 (45.00) ^b
	TPF53	44.0 ^{ab}	51.11 (45.64) ^{ab}
	TPF54	46.0 ^b	48.88 (44.36) ^b
	Control	90.0 ^c	0.00 (0.00) ^c
2,4 diacetyl phloroglucinol	Pf1	45.0 ^b	50.00 (45.00) ^b
	FP7	39.0 ^a	56.66 (48.83) ^a
	TPF17	52.0 ^d	42.22 (40.52) ^d
	TPF53	49.0 ^c	45.55 (42.45) ^c
	TPF54	54.0 ^d	40.00 (39.23) ^d
	Control	90.0 ^c	0.00 (0.00) ^c
Phenazine	Pf1	48.0 ^b	46.66 (43.08) ^b
	FP7	44.0 ^a	51.11 (45.64) ^a
	TPF17	55.0 ^d	38.88 (38.57) ^d
	TPF53	51.0 ^c	43.33 (41.17) ^c
	TPF54	53.0 ^{cd}	41.11 (39.88) ^{cd}
	Control	90.0 ^c	0.00 (0.00) ^c
Pyoluteorin	Pf1	59.0 ^b	34.44 (35.93) ^b
	FP7	55.0 ^a	38.88 (38.57) ^a
	TPF17	59.0 ^b	34.44 (35.93) ^b
	TPF53	64.0 ^c	28.88 (32.50) ^c
	TPF54	60.0 ^b	33.33 (35.26) ^b
	Control	90.0 ^d	0.00 (0.00) ^d

*Values are mean of four replications.

Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at P ≤ 0.05. The data in the parentheses are arcsine transformed values.

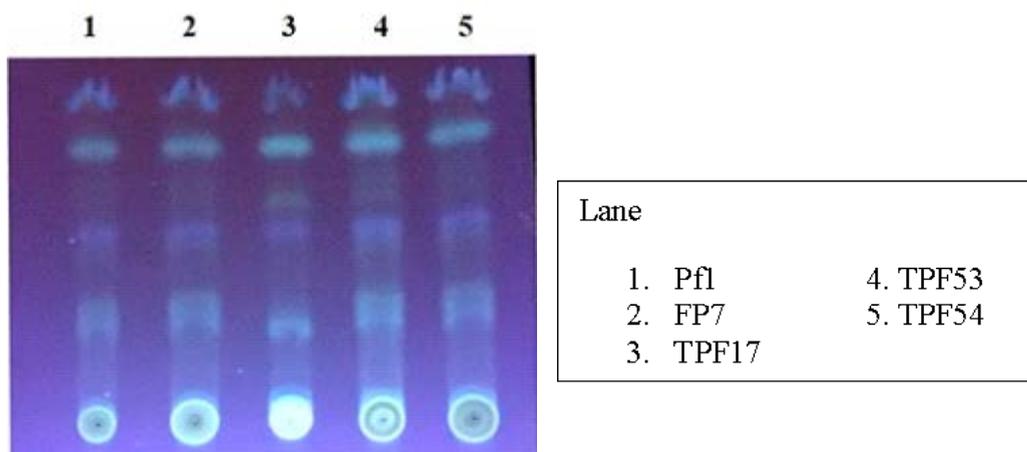


Fig. 1. Detection of crude antibiotics from *P. fluorescens* by Thin Layer Chromatography

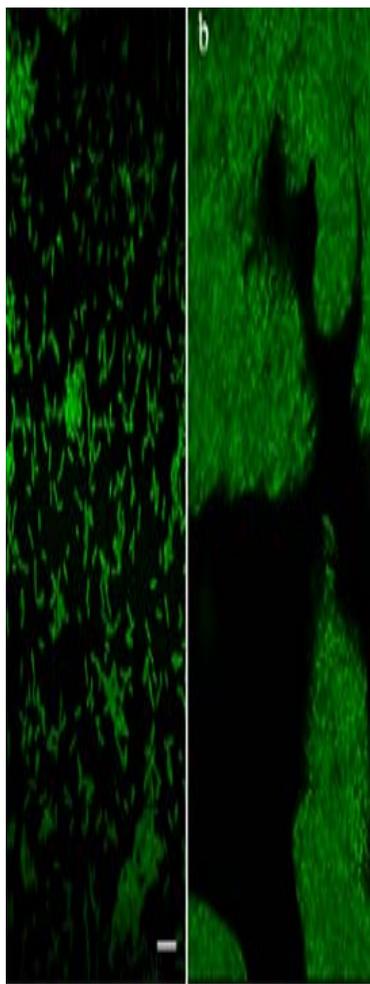


Fig. 2. Detection of different antibiotics from *P. fluorescens* strains

five strains, with R_f value of 0.05 (Fig. 2). These results has been supported by the following reports such as^{19,20}.

The polyketide antibiotic DAPG is a phenolic molecule synthesized by the condensation of three molecules of acetyl coenzymeA with one molecule of malonyl coenzymeA to produce the precursor monoacetylphloroglucinol, which is subsequently transacetylated to generate PHL utilizing a CHS-type enzyme²¹. Biosynthetic locus of DAPG is highly conserved. It comprises the biosynthetic genes *phlACBD*²². Rovera *et al*²³ had reported 2,4- DAPG produced by *Pseudomonas aurantica* and their antifungal activity against *Macrophomina phaseolina*. In our study, DAPG was eluted from the crude metabolites of *P. fluorescens* strains Pfl, FP7, TPF17, TPF53, TPF54 and tested for their antifungal action against *P. aphanidermatum*. Among the strains, 2, 4 DAPG isolated from FP7 recorded 56.66 per cent inhibition of mycelial growth of *P. aphanidermatum* over untreated control. It was followed by Pfl which recorded 50 per cent inhibition over control. In case of other strains of *P. fluorescens* such as TPF17, TPF53 and TPF54 accounted to 42.22, 45.55 and 40.00 per cent inhibition respectively (Table 1). This results further confirmed with Youn-Sig Kwak *et al*²⁴ who found that wheat take-all disease caused by *G. graminis* var. *tritici* was sensitive to 2,4-DAPG producing *P. fluorescens*.

Phenazine production is critical for the biocontrol properties of certain agriculturally important root-colonizing pseudomonads that protect food crops from attack by pathogenic

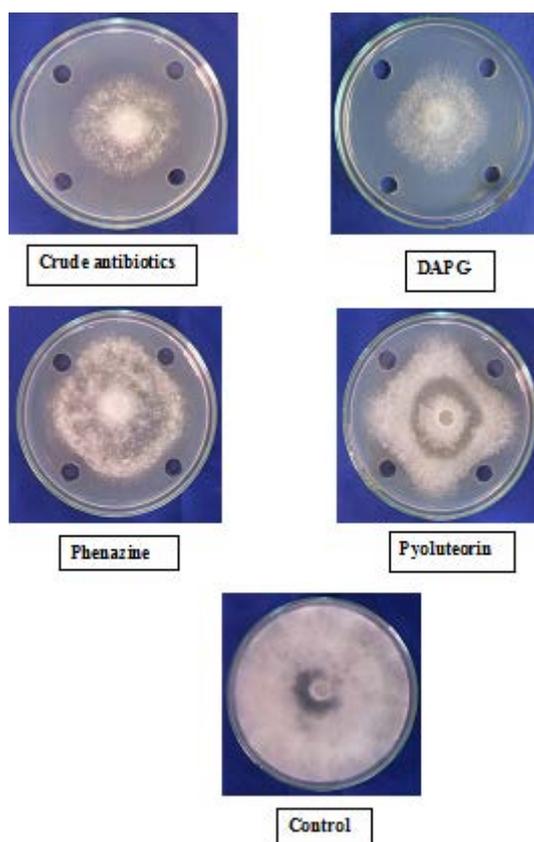


Fig. 3. Antagonistic action of different antibiotics of *P. fluorescens* (FP7) against *P. aphanidermatum*

fungi^{25,26}. The mechanisms of action of phenazines are assumed that they diffuse across or insert into the membrane and act as reducing agent, resulting in the uncoupling of oxidative phosphorylation and the generation of toxic intracellular superoxide radicals and hydrogen peroxide which are harmful to the organisms. The phenazine isolated from *P. fluorescens* (FP7) recorded the least mycelial growth of 44 mm which recorded 51.11 per cent inhibition of mycelial growth over control. The phenazine isolated from *P. fluorescens* strain TPF17 was the least one in inhibiting the mycelial growth of *P. aphanidermatum* which recorded only 38.88 per cent inhibition (Table 1). The results are in line with the findings of Bakker *et al.*²⁷ that *P. putida* WCS 358r produced an antifungal compound phenazine -3- carboxylic acid (PCA) and 2, 4 DAPG which had both antifungal and antibacterial action. Pyoluteorin (Plt) is a phenolic polyketide with resorcinol ring. Plt has bactericidal, herbicidal and fungicidal properties. In the present study,

pyoluteorin from FP7 recorded 38.88 per cent inhibition over control. The antibiotic pyoluteorin from *P. fluorescens* Pfl, TPF17 and TPF54 were on par with each other in per cent inhibition of mycelial growth of the pathogen (Table 1). Overall, antibiotics extracted from *P. fluorescens* strain FP7 was found to be highly effective against *P. aphanidermatum* (Fig. 3). In conclusion, we have demonstrated the production of antifungal compounds by *P. fluorescens* is now recognized as an important feature in plant disease suppression.

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