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ISOLATION AND IDENTIFICATION OF ANTIFUNGAL COMPOUNDS FROM *PSEDOMONAS FLUORESCENS* INHIBITING THE GROWTH OF *FUSARIUM INCARNATUM* (DESM.)SACC. INCITANT OF CROSSANDRA WILT

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ABSTRACT
An experiment was conducted to identify the most efficient isolate of *Psedomonas fluorescens* and also to identify the secondary metabolites involved in antagonistic mechanism against *Fusarium incarnatum* causing wilt in crossandra. Thirty isolates of *P. fluorescens* were isolated from various soil samples and all the isolates along with TNAU isolate were found to antagonize the pathogen with varying degree ranging from 27.50 to 74.8 per cent. Crude culture filtrates from nine most efficient *Psedomonas* isolates in dual culture were extracted and tested for their efficacy against test pathogen *F. incarnatum*. Among them, isolate Pf-18 was found to be most effective isolate with mycelia growth inhibition of 65.4 per cent. Based on dual culture and antibiotic assay, metabolites produced by isolate Pf-18 were used for further metabolite identification through GC-MS analysis. Fourteen prominent peaks with retention time of 5.04, 8.91, 10.59, 11.45, 12.16, 15.01, 20.21, 21.74, 24.86, 29.70, 32.94, 35.93 and 39.54 min are corresponds to 9-Azabicyclo (6.1.0) non-8-ene:, Butanoic acid, 3-methyl:, 3-Decenoic acid, methyl ester:, 6-(2-Thienyl)-3-(2-pyridyl)-1,2,4-triazine 4-oxide:, Oxacyclododecan-2-one:, Trideconol:, Tetradecanoic acid:, 1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester(CAS):, Pyrrolo (1,2-a) pyrazine-1,4-dione:, 9-Octodecanoicacid:, I-Heptadecanol:, Nonacosane:, Phthalic acid, decyldec-2-yl-easter:, and Cyclohexanepropanoic acid, 2-oxo-methyl ester were observed under GC-MS analysis with different peaks indicating their role in antagonistic mechanism.

INTRODUCTION

Crossandra (Fire cracker) is an important commercial flower, mainly grown in India, Tropical Africa and Madagascar. Crossandra (*Crossandra infundibuliformis*) is affected by various fungal, bacterial, viral and nematode diseases. Among the various fungal diseases, wilt disease caused by *Fusarium spp.* is one of the major problems in crossandra production and limits the crop cultivation. The overuse of chemical pesticides for disease management has caused soil pollution and harmful effects on human beings. So presently biological control of soil borne diseases has been attracting attention. Soil-borne bacteria that are antagonistic to plant pathogens could make a substantial contribution to prevention of plant diseases and therefore represent an alternative to the use of chemical pesticides in agriculture (Walsh *et al.,* 2001). Due to their role in plant health and soil fertility, they have been used as a model environment in biological control of soil-borne plant pathogens. Among the different genera of bacteria, *Bacillus* spp., *Pseudomonas* spp., and *Streptomyces* spp. are widely used as biocontrol agents and reported to produce several antibiotics. In recent years, florescent Pseudomonas have drawn the attention worldwide owing the ability of production of secondary metabolites and these metabolites have been implicated in reduction of plant pathogenic fungi and harmful rhizobacteria with simultaneous induction of growth of plants and are metabolically and fuctionally more diverse(Choudhary *et al.,* 2009). The *Pseudomonas fluorescens* is a gram-negative,rod-shaped and non-pathogenic bacterium that is known to inhibit primarily the soil, plant and water (Peix *et al.,* 2009). It derives its name from its ability to produce fluorescent pigments under iron-limiting conditions (Baysse *et al.,* 2003). Bactriak biocontrol agents are very effective in plant diseases even at field conditions (*Harit kumar et al.,* 2015).

Keeping it in view, an attempt has been made to isolation of potential antagonistic *Pseudomonas* spp. having lethal effect on the phytopathogenic fungi *F. incarnatum* and also extraction and identification of secondary metabolites produced by *Pseudomonas* spp which are believed to be associated with biocontrol mechanism.

MATERIALS AND METHODS

All the laboratory experiments were carried at the Department of Plant Pathology, Agricultural College and Research Institute, TNAU, Madurai and GC-MS studies were carried at South Indian Textile Research Association, Coimbatore during 2013 to 2015.

Isolation of antagonists

Antagonistic bacteria *Pseudomonas* spp. were isolated from

the rhizosphere soil collected from the different crops grow in various places 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 collected from the different crops was transferred to 250 ml Erlenmeyer flask containing 100ml of sterile distilled water separately. After thorough shaking, the antagonist present 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 pipettedout and poured into sterilized Petridishes 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 ($28 \pm 2^{æ\%}$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.

Dual culture studies

The above isolates *Pseudomonas* spp. were tested for their inhibitory effect on the growth of *F. incarnatum* by following the dual culture technique. The bacterial isolates were streaked on one side of the Petri dish (1 cm away from the edge of the plate) on PDA medium separatly and a mycelial disc (9 mm diameter) of 5 days old *F. incarnatum* culture was placed on the opposite side of the each Petri dish perpendicular to the bacterial streak. The plates were incubated at room temperature (28 + 2ºC) for 8 days. After eight days of incubation, the pathogen growth in each petridishes measured separatly and expressed in cm.

Extraction of antifungal compounds

The crude antibiotics of the selected nine isolates of *P. fluorescens* were grown in pigment production broth (PP) (peptone-20g/l, glycerol-20g/l, NaCl-5g/l, KNO_3-1g/l, pH-7.2 and 1000ml distilled water) separately and incubated in shaker at 28±2º C for 5 days. The supernatant of each isolate was collected at 5 days old culture (stationary phase) by centrifugation at 8,000 rpm for 30 min. Then supernatant was adjusted to pH 2.0 by the addition of concentrated HCl and the mixture was stirred at 100 rpm in an orbital shaker for 8hrs. After shaking, the precipitate was collected by centrifugation,

re-suspended in 1ml of lM NaOH to adjust the pH 7.0. The resultant suspension of each isolate was extracted twice with ethyl acetate. The ethyl acetate phase was transferred into the vacuum flash evaporator maintained at 60ºC, at 80rpm, till the ethyl acetate fraction gets evaporated. The crude antibiotic of each isolate of *P. fluorescens* was re-suspended in 1ml of methanol – chloroform mixture (1:1) separately and used for further bioassay and GC/MS analysis (Senthilkumar *et al.*, 2011).

In vitro* antibiotic assay against *F. incarnatum

A nine mm mycelial disc of the *F. incarnatum* was placed at the centre of each petri plate containing PDA medium and sterile What man no 40 filter paper disc with six mm diameter were placed 1cm away from the edge at four sides centering around the fungal disc. Ten microliters of crude extract of each isolates of *P. fluorescens* was dropped over the sterile filter paper discs separately. Control was maintained with the sterile distilled water instead of crude extract. The plates were incubated at room temperature (28C+ 2ºC) and the plates were scored when the mycelium grew over the control disc.

GC-MS analysis of crude antibiotics

Detection of active bio-molecules present in the crude antibiotics of *P. fluorescens (Pf-18)* responsible for the suppression of *F. incarnatum* was carried out through GC-MS (GC Clarus 500 Perkin Elmer). Volatile compounds were identified by GC/MS using a coloumn Elite-5MS (100% Dimethyl poly siloxane), 30 x 0.25 mm x 0.25ì m df equipped with GC clarus 500 Perkin Elmer. The turbo mass-gold-perkin-Elmer detector was used. The carrier gas flow rate was 1 ml per min, split 10:1, and injected volumes were 3ì l. The column temperature was maintained initially at 110ºC at the rate of 10ºC/min - No hold followed by increases up to 280ºC at the rate of 5ºC /min and 9 min (hold). The injector temperature was 250ºC and this temperature was held constant for 36 min. The electron impact energy was 70eV, Julet line temperature was set at 20006°C and the source temperature was set at 200ºC. Electron impact (EI) mass scan (m/z) was recorded in the 45-450 a MU range. Using computer searches on the NIST Ver.2005 MS data library and comparing the spectrum obtained through GC/MS the compounds present in the crude sample were identified (Senthilkumar *et al.,* 2011).

RESULTS AND DISCUSSION

Thirty isolates of *P. fluorescens* were isolated from the rhizosphere regions of different crops grown in different parts of Tamil Nadu. Among the thirty isolates tested for their antagonistic activity against *F. incarnatum* by dual culture technique, Pf-18 and TNAU isolate were found to record significantly highest reduction of mycelial growth (74.7 and 74.8% respectively) followed by Pf 12 which recorded 74.2 per cent reduction of mycelial growth over control. The lowest per cent reduction of mycelial growth was recorded in the isolate Pf 30 (27.5 %) over control (Table 1). It might be due to the production of antibiotics, volatile compounds and lytic enzymes.

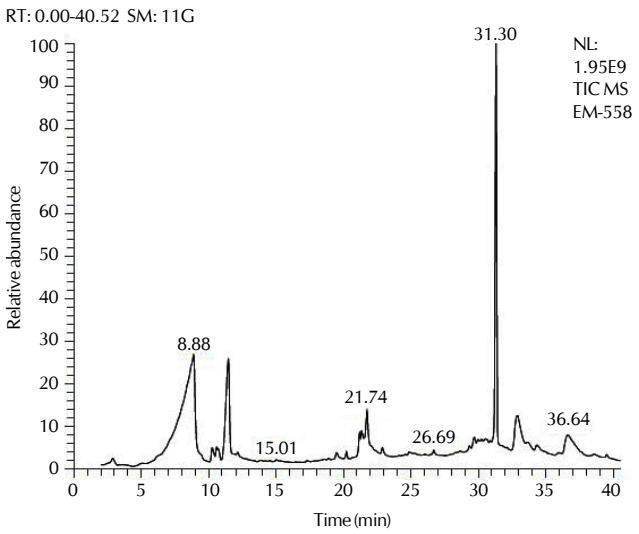

Figure 1: The gas chromatogram of antimicrobial compounds produced by *P. fluorescens* (Pf- 18) through GC/MS

Table 1: Efficacy of different isolates of *Pseudomonas* spp against *F. incarnatum* causing Crossandra wilt *in vitro*

S.No	Isolates	Place of collection	Crop	Mycelial growth (cm)* at 10 DAI	Growth reduction over control (%)
1	Pf-1	Ottumpatty	Crossandra	3.12	64.8
2	Pf-2	Trichy	Banana	4.02	54.6
3	Pf-3	Ottanchathram	Banana	4.61	47.9
4	Pf-4	Puduchukkapuram	Crossandra	4.81	45.7
5	Pf-5	Annur	Brinjal	5.13	42.0
6	Pf—6	Mettupatty	Jasmine	4.23	52.2
7	Pf-7	Nelakottai	Crossandra	4.20	52.5
8	Pf-8	Periyakulam	Groundnut	4.34	51.0
9	Pf-9	Udamalpet	Jasmine	2.42	72.7
10	Pf-10	Sempatty	Crossandra	5.03	43.1
11	Pf-11	Usilampatti	Jasmine	4.70	46.9
12	Pf-12	Palamedu	Groundnut	2.28	74.2
13	Pf-13	Vengumpur	Jasmine	2.51	71.6
14	Pf-14	Kudumudi	Onion	2.44	72.4
15	Pf-15	Sekkanurani	Onion	4.30	51.4
16	Pf-16	Gobichettipalayam	Jasmine	4.07	54.0
17	Pf-17	Sathyamangalam	Banana	4.47	49.5
18	Pf-18	Pedhappampatti	Crossandra	2.24	74.7
19	Pf-19	Palani	Crossandra	3.50	60.5
20	Pf-20	Bodinayakanur	Crossandra	3.48	60.7
21	Pf-21	Thirumangalam	Jasmine	4.90	44.6
22	Pf-22	Melur	Crossandra	3.37	61.9
23	Pf-23	Pugalur	Chrysanthemum	3.42	61.4
24	Pf-24	Sathiram	Chrysanthemum	5.10	42.4
25	Pf-25	Kottampattu	Crossandra	4.80	45.8
26	Pf-26	Mallur	Chrysanthemum	5.17	41.6
27	Pf-27	Pallipatti	Crossandra	5.85	33.9
28	Pf-28	Navalurkottapatti	Chrysanthemum	5.23	40.9
29	Pf-29	Tharamangalam	Jasmine	5.95	32.7
30	Pf-30	Thiruvallur	Crossandra	6.41	27.5
31	TNAU isolate	Coimbatore		2.23	74.8
32	Control	Control		8.85	-
CD(P = 0.05)		0.22	-		

*Mean of three replications DAI - Days after incubation.

Table 2: Efficacy of crude antibiotics produced by the selected isolates of *P. fluorescens* against *F. incarnatum in vitro*

S.No.	Isolates	Mycelial growth (cm) 10 DAI*	Growth reduction overControl (%)
1	Pf -1	4.60	48.3
2	Pf -12	3.43	61.4
3	Pf -13	3.93	55.9
4	Pf -14	3.84	56.8
5	Pf -18	3.08	65.4
6	Pf -19	5.61	36.9
7	Pf -20	5.32	40.2
8	Pf -22	4.95	44.3
9	Pf -23	5.17	41.9
10	Control	8.9	-
CD (P = 0.05)	0.04	-	
SE(m) ±	0.01	-	

* Mean of three replications ; DAI- Days after incubation

The isolates of *P. fluorescens* showed inhibitory action against chickpea wilt pathogen *F. o.* f. sp. *ciceris* (Vidhyasekaran and Muthamilan, 1995). Rao *et al*. (1999) observed that under *in vitro* condition the growth of *F. o.* f. sp. *lini,* the wilt pathogen in lentil was inhibited strongly by *P. fluorescens*. Thangavelu *et al*. (2001) screened 11 isolates of *P. fluorescens*, among these Pf10 was found to be the most effective in inhibiting the mycelial growth of *F. o.* f. sp. *cubense*. Salah Eddin Khabbaz (2006) reported that *P. fluorescens* isolates Pf 32, Pf 93 were found to be most effective in inhibiting the growth of *M. phaseolina*. Madhumiha pandey and Sobitasimon (2015) also reported the mycelia inhibition of *Fusarium oxysporum sp. cieri* by *P. fluorescens* in chickpea.

Effect of antifungal compounds

The crude antibiotics of nine isolates of *P. fluorescens* were tested for their antifungal activity against *F. incarnatum*. The crude antibiotics isolated from Pf-18 was found to record maximum (65.4%) reduction of mycelial growth followed by Pf-12 which recorded 61.4 per cent reduction of mycelial growth over control. The other isolates were found to be less effective against the pathogen (Table 2). In the present study, crude antibiotics produced by the isolates of *P. fluorescens* were inhibitory to the growth of *F. incarnatum in vitro*. Thomashaw *et al*. (1997). reported the production of antibiotics by PGPR strains with broad-spectrum activity. Michereff *et al*. (1994) indicated that *P. fluorescens*, *P. marginalis* and *B. subtilis* produced antibiotics that were inhibitory to *Colletotrichum graminicolum*.

Kavitha (2004) reported that crude antibiotics of *B. subtilis* and *P. fluorescens* inhibited the growth of *P. aphanidermatum* in turmeric. The crude antibiotics produced by *P. fluorescens*

Table 3: Identification of antimicrobial compounds produced by *P. fluorescens* (Pf-18) through GC/MS

Retention time	Compound name	Molecular formula	Molecular weight	Biological activity
5.04	9-Azabicyclo(6.1.0)non-8-ene(CAS)	$C_8H_{13}N$	123	Antibacterial
8.91	Butanoic acid,3-methyl	$C_5H_{10}O_2$	102	Antimicrobial
10.59	3-Decenoic acid, methyl ester	$C_{11}H_{20}O_2$	184	Antimicrobial
11.45	6-(2-Thienyl)-3-(2-pyridyl)-1,2,4-triazine 4-oxide	$C_{12}H_8N_4OS$	256	Antibacterial
12.16	Oxacyclododecan-2-one	$C_{11}H_{20}O_2$	184	Antibacterial
15.01	Trideconol	$C_{13}H_{26}O$	198	Antibacterial
15.01	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	Antimicrobial
20.21	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl)ester(CAS)	$C_{16}H_{22}O_4$	278	Antifungal, Antibacterial
21.74	Pyrrolo(1,2-a)pyrazine-1,4-dione	$C_{11}H_{18}N_2$	210	Antibacterial
24.86	9-Octodecanoic acid	$C_{18}H_{36}O_2$	284	Antimicrobial
29.70	I-Heptadecanol	$C_{17}H_{36}O$	256	Antifungal, Nematicidal
32.94	Nonacosane	$C_{29}H_{60}$	408	Antibacterial
35.93	Phthalic acid,decyl dec-2-yl-easter	$C_{28}H_{46}O_4$	446	Antiviral
39.54	Cyclohexanepropanoic acid, 2-oxo-methyl ester	$C_{10}H_{14}O_3$	184	Antimicrobial,Antiviral

(CHA0) suppressed damping off disease in cucumber (Maurhofer *et al*., 1992). Indumathi, (2012) also reported that crude antibiotic from *P. aeruginosa* (P1) showed maximum inhibition of mycelial growth of *F. oxysporum* f.sp. *dianthi*. In addition, *B. amyloliquefaciens* and *P. fluorescens* were regarded as non-pathogenic bacterial species. Thus, the use of *Pseudomonas* as a biocontrol agent may be an environmentally safe way to suppress this plant disease.

Many strains of *Pseudomonas* have been found to produce broad spectrum antibiotics *viz*., Phenazine, Pyrrolnitrin, Pyoverdine, 2,4-diacetylphloroglucinol (Gardener *et al*., 2000), lytic enzymes such as chitinases and β-1,3-glucanases thus degrade fungal chitin (Velazhahan *et al*., 1999), production of siderophore (Loper, 1988), production of HCN (Ahl *et al*., 1986) and induced systemic resistance (Van Peer *et al*., 1991).

Gas chromatography and mass spectrometry

The crude antibiotic and extracellular antifungal compounds from *P. fluorescens* (Pf-18) were analyzed through GC/MS and yielded fourteen prominent peaks with retention time of 5.04, 8.91, 10.59, 11.45, 12.16, 15.01, 20.21, 21.74, 24.86, 29.70, 32.94, 35.93 and 39.54 min (Fig. 1, Table 3). The peaks with reaction time 5.04 corresponds to 9-Azabicyclo (6.1.0) non-8-ene (CAS) with 1.26% of peak area, 8.91 min represent to the Butanoic acid, 3-methyl with 3.25% of peak area, 10.59 min pertaining to 3-Decenoic acid, methyl ester with 2.15% of peak area, 11.45 min corresponds to 6-(2-Thienyl)-3-(2-pyridyl)-1,2,4-triazine 4-oxide with 4.35% of peak area, 12.16 min corresponds to Oxacyclododecan-2-one with 1.29% of peak area, 15.01min corresponds Trideconol with 1.23% of peak area and Tetradecanoic acid with peak area of 1.25 per cent. 20.21 min corresponds to the 1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester(CAS) with 1.75% of peak area, 21.74 min corresponds to Pyrrolo (1,2-a) pyrazine-1,4-dionewith 6.25% of peak area, 24.86 min corresponds 9-Octodecanoic acid with 3.22% of peak area, 29.70 min corresponds to the I-Heptadecanol with 2.67% of peak area, 32.94 min. corresponds to Nonacosane with 1.67% of peak area, 35.93 min corresponds Phthalic acid, decyldec-2-yl-easter with 1.66% of peak area and 39.54 min corresponds Cyclohexanepropanoic acid, 2-oxo-methyl ester with 1.66% of peak area. Biological activity and chemical structure of phytocompounds were identified. *B.subtilis* and *P.fluorescens* are important biocontrol agents that have shown strong antifungal activity against a number of plant pathogens. The antibiotics produced from them were generally assumed to be responsible for the antimicrobial activities (Kim *et al*., 2010; Wang *et al*., 2010). In the present investigation,*P. fluorescens* produced secondary metaboloites which have inhibitory effect on mycelial growth, sporulation, lysis and electrolytic leakage of ions from mycelium responsible for the control of *F. incarnatum*. These bacteria are capable of producing a wide variety of secondary metabolites that are diverse in structure and function. The production of antimicrobial metabolites determines the ability to control plant diseases .The biological activity of compounds were identified. Antibacterial activity of I-Trideconal against *Staphylococcus aureus* was reported by Naokotogash *et al*. (2007) and 1,2-benzene dicarboxylic acid-dis(2-methylpropyl) ester, with antifungal and antibacterial activity (Vimalavadya and Kadavula, (2013)). Takia *et al*. (2012) reported the antimicrobial property of Tetradeconoic acid. Gershon *et al*. (2013) observed the antifungal activity of Octodecanoic acid against *Myrothecium verrucaria*. Radhika *et al*. (2007) reported the antibacterial activity of N-pyrrolidine. Amutha and kottai, (2014) reported the antimicrobial activity of 9-Octodecanoic acid, methyl ester. Kumar *et al*. (2009) reported the role of Hepatadecanoic acid from *B. subtilis* in biocontrol. Demirci *et al*. (2000) reported that nonacosane had antifungal activity against *F. solani* and *G. fujikuroi*. Triterpene based compound namely 1,2-benzenedicarboxylic acid, diisooctyl ester functioned as antioxidant, antibacterial, lipoxygenase inhibitor and pesticidal activity (Senthilkumar *et al*., 2011). William *et al*. (1988) observed the antibacterial activity of Cyclohexane.

Hence, production of these compounds by bacterial bio control agents may responsible for the antimicrobial action and served as a potent inhibitor of fungal pathogens, which could be the reason for the suppression of *F. incarnatum* causing wilt of crossandra under *in vitro*.

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