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Isolation and Identification of Antifungal Compounds from *Bacillus subtilis* Inhibiting the Growth of *Fusarium incarnatum* (Desm.) Sacc. incitant of Crossandra Wilt

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ABSTRACT: *Antagonistic microorganisms* Bacillus *spp against* Fusarium incarnatum *were isolated and their antifungal activities were investigated. Thirty* Bacillus subtilis *isolates were isolated from various soil samples and all the isolates were found to antagonize the pathogen with varying degree ranging from 37.59% to 74.96%. Crude culture filtrates from nine effective* Bacillus *spp were extracted and tested for their efficacy against test pathogen* F.incarnatum *Among them, isolate Bs-10 found to be most effective isolate with mycelia inhibition of 47.6 per cent and based on dual culture and antibiotic assay Bs-10 selected as an antagonistic microorganism with potential for use in further studies. Crude antibiotics were analyzed for noval metabolites through GC-MS analysis revealed the presence of antifungal volatile compounds from isolate Bs-10. The promising volatile compounds observed through GC-MS, included dedeconal, trideconol, tetradecanoic acid, pentadecinoic acid, octodeconoic acid, 9-octodeconoic acid, hexadeconoic acid, 1-2-benzenedicarboxylic acid, n-pyrrolidine, ipconazole, heptadeconoic acid, Docosane, nonacosane, and cyclohexane. These compounds may play an important role in the inhibition of mycelial growth and reducing disease level. Studies are under way to understand this phenomenon under glass house and field conditions.*

Key words: Antagonistic microorganisms, Antimicrobial compounds, Bacillus subtilis*, Crossandra,* Fusarium incarnatum*, Secondary metabolites.*

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 problem 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. 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 (1). 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 *Bacillus* spp. has been reported to produce several antibiotics.

Non-pathogenic plant growth promoting rhizobacteria, *Bacillus* spp., form endospores that gives tolerate to extreme pH, temperature, and osmotic conditions, therefore, they offer several advantages over other organisms. *Bacillus* spp. was found to colonize the root surface, increase plant growth, and cause lysis of fungal mycelia (2). They are considered as safe biological agents and their potential is considered high (3). The antagonistic effects are mainly through production of antifungal antibiotics, which play a major role in biological control of plant pathogens. More than seventy

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different antibiotics are produced by *B.subtilis* and some of these metabolites show antimicrobial activity against a number of phytopathogenic micro-organisms (4). *B.subtilis* produce large number of non ribosomal antibiotics and exhibit potent antibacterial or antifungal activity, as represented by surfactin, the iturin, mycobacillins, mycosubtilins and fegycin (5) and compounds that induce plant resistance mechanism. Volatile metabolites from *Bacillus* spp. have been reported to inhibit mycelia growth of *Fusarium oxysporum*, the incitant of *Fusarium* wilt of onion (6). Therefore, volatile substances producing bacteria can be used as biocontrol agents for protection fungal plant diseases. The present study was aimed at isolation of potential antagonistic Bacillus spp. and also extraction and identification of antibiotics and volatile compounds produced.

MATERIALS AND METHODS

All the laboratory experiments were conducted 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 2014.

ISOLATION OF ANTAGONISTS

Antagonistic bacteria Bacillus spp. were isolated from the rhizosphere soil collected from different crops growing areas of Tamil Nadu. The plants were pulled out gently with intact roots and the excess soil adhering on roots was removed gently. One gram of soil sample is mixed with 9ml of sterilized nutrient broth in test tube and kept on boiling water bath at 80^{o}C for 10 minutes. Then it is kept for incubation at room temperature for 24-48 hrs. From this serial dilutions are prepared up to 10^{-6} dilution. Dilution 10^{-5} and 10^{-6} are plated in nutrient agar and incubated for 48 hrs. Colonies observed were identified.

Dual culture studies

The bacterial isolates were tested for their inhibitory effect on 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 and a mycelial disc (9 mm diameter) of 5 day old *F.incarnatum culture* was placed on the opposite side of the Petri dish perpendicular to the bacterial streak. The plates were incubated at room temperature (28 $\pm$ 2^{o}C) for 8 days. After eight days of incubation, the pathogen growth and inhibition zone were measured and expressed in cm.

EXTRACTION OF ANTIFUNGAL COMPOUNDS

The crude antibiotics of *Bacillus* spp. were extracted as per the protocol described (7) with some modifications. Bacterial cells were cultivated in Nutrient broth (NB) and incubated at 28°C for 3 days. The supernatant was collected at 72 h (stationary phase) by centrifugation at 8,000 rpm for 30 min. Then supernatant was adjusted to acidic pH 2.0 by adding with concentrated HCl and the mixture was stirred at 100 rpm in an orbital shaker for 8hrs. Antifungal compounds in supernatant or culture broth were extracted by adding the equal volume of solvent ethyl acetate and shaken vigorously for 1-2h. Culture broth was extracted twice with ethyl acetate solvent for complete extraction. The solvent fraction that contained antifungal compounds were combined and concentrated by evaporation in the rotary flash evaporator maintained at 60°C, at 80rpm. The concentrated crude extract of the extracellular antifungal compounds were then dissolved in 1 ml methanol: chloroform mixture (1:1) for *in vitro* antifungal activity assay and GC/MS analysis.

IN VITRO ANTIBIOTIC ASSAY AGAINST *F.INCARNATUM*

A nine mm mycelial disc of the Crossandra pathogen *F.incarnatum* was placed on the centre of the petri plate 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 *B.subtilis* was dropped over the sterile filter paper discs. The plates were incubated at room temperature (28C$\pm$ 2^{o}C) and the plates were scored when the mycelium grew over the control disc. Control was maintained with the sterile distilled water instead of crude extract.

GC-MS ANALYSIS OF CRUDE ANTIBIOTICS

Detection of active bio-molecules present in the crude antibiotics of *B. subtilis(BS-10)* responsible for the suppression of *F.incarnatum* were 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 0.25ìm 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 3ìl. The column temperature was maintained initially at 110°C at the rate of 10°C/min-No hold followed by increases up to 280°C at the rate of 5°C /min and 9 min (hold). The injector temperature was 250°C and

this temperature was held constant for 36 min. The electron impact energy was 70eV, Julet line temperature was set at 2000°C and the source temperature was set at 200°C. 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 (8).

RESULTS AND DISCUSSION

Thirty isolates of *Bacillus subtilis,* 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, Bs-10 significantly exerted highest (74.96) per cent reduction of mycelial growth followed by Bs-11 and Local tnau strain recorded 69.73 per cent reduction of mycelial growth. The lowest per cent reduction of mycelial growth was observed in isolate Bs-18 (37.59 %) (Table 1, Plate.1).

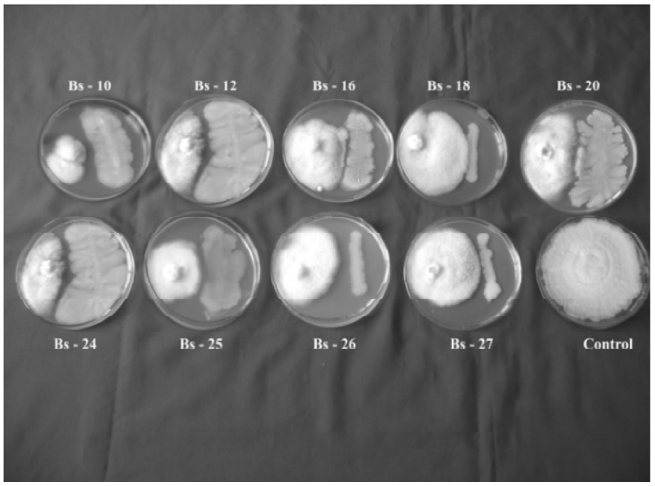

Plate 1. Antifungal activity of different *Bacillus subtilis* isolates on *F.incarnatum*.

Table 1
Efficacy of different isolates of *Bacillus subtilis* against *F.incarnatum* associated with Crossandra wilt *in vitro*

S.No	*Isolates*	*Place of collection*	*District*	*Crop*	*Mycelial growth (cm)**	*Per cent reduction over control*
1	Bs-1	Ottumpatti	Dindugal	Crossandra	4.20	52.91
2	Bs-2	Trichy	Trichy	Banana	5.10	42.83
3	Bs-3	Ottanchathram	Dindugal	Banana	4.70	47.31
4	Bs-4	Puduchukkapuram	Dindugal	Crossandra	4.41	50.67
5	Bs-5	Annur	Coimbatore	Brinjal	4.23	52.54
6	Bs-6	Mettupatti	Theni	Jasmin	4.20	41.70
7	Bs-7	Nelakottai	Dindugal	Crossandra	4.83	45.81
8	Bs-8	Periyakulam	Theni	Groundnut	4.53	49.18
9	Bs-9	Udamalpet	Coimbatore	Jasmin	5.23	41.33
10	Bs-10	Sempatti	Dindugal	Crossandra	2.23	74.96
11	Bs-11	Usilampatti	Madurai	Jasmin	2.70	69.73
12	Bs-12	Palamedu	Madurai	Groundnut	3.17	64.50
13	Bs-13	Vengumpur	Erode	Jasmin	4.10	54.04
14	Bs-14	Kudumudi	Erode	Onion	4.13	53.66
15	Bs-15	Sekkanurani	Erode	Onion	5.07	43.20
16	Bs-16	Gobichettipalayam	Erode	Jasmin	4.73	46.94
17	Bs-17	Sathyamangalam	Erode	Banana	5.20	41.70
18	Bs-18	Pedhappampatti	Coimbatore	Groundnut	5.57	37.59
19	Bs-19	Palani	Dindugal	Jasmin	5.30	40.58
20	Bs-20	Bodinakanurya	Theni	Crossandra	4.13	53.66
21	Bs-21	Thirumangalam	Madurai	Jasmin	5.83	34.60
22	Bs-22	Melur	Madurai	Crossandra	5.17	42.08
23	Bs-23	Pugalur	Karur	Chrysanthemum	4.23	52.54
24	Bs-24	Sathiram	Karur	Chrysanthemum	3.20	64.13
25	Bs-25	Kottampatti	Madurai	Crossandra	2.80	68.61
26	Bs-26	Mallur	Salem	Chrysanthemum	3.40	61.88
27	Bs-27	Pallipatti	Karur	Crossandra	5.20	41.70
28	Bs-28	Navalurkottapatti	Trichy	Chrysanthemum	4.20	52.91
29	Bs-29	Tharamangalam	Salem	Jasmin	5.83	34.60
30	Bs-30	Thiruvallur	Thiruvallur	Crossandra	3.80	57.40
31	Control	Control			8.92	
		CD(P=0.05)			0.255	

ANTIFUNGAL COMPOUNDS AND PLATE ASSAY

The crude antibiotics extracted from nine *Bacillus* spp were tested for their antifungal action against *F.incarnatum*. The results revealed that, crude antibiotics extracted from Bs-10, recorded 4.66 cm of mycelial growth, which accounting for 47.6 percent inhibition of mycelial growth over control followed by Bs-11 which recorded 42.7 per cent reduction of mycelial growth. The other isolates were less effective against the pathogen (Table 2). Bacillus spp. from rhizosphere of tea plants also the produced both volatile and diffusible antifungal compounds(9). Antifungal compounds like bacillomycin, fengycin, iturin A and surfactin are key factors of antagonism in *B.subtilis* against *Podoshaera fusca* (10).

Table 2
Efficacy of crude antibiotics eluted from antagonistic bacteria (*Bacillus spp*) found effective against *F.incarnatum* (F-ISO-4) *in vitro*

S.No.	*Isolates*	*Mycelial growth (cm)**	*Per cent reduction over control (%)*
1	Bs-1	5.95	33.2
2	Bs-5	6.04	32.1
3	Bs-10	4.66	47.6
4	Bs-11	5.10	42.7
5	Bs-12	5.37	39.7
6	Bs-13	5.62	36.9
7	Bs-20	5.70	36.0
8	Bs-24	5.31	40.3
9	Bs-25	5.21	41.5
10	Bs-30	5.42	39.1
11	Control	8.92	-
	CD (P=0.05)	**0.11**	
	SE(m)±	**0.038**	

* Mean of three replications

GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

The crude antibiotics and extracellular antifungal compounds from *B. subtilis(Bs-10)* were analyzed through GC/MS to detect the novel compounds and secondary metabolites responsible for antifungal action. Fifteen prominent peaks with retention time of 12.17, 17.42, 18.40, 20.21, 21.17, 21.55, 24.86, 30.29, 31.33, 32.94 and 36.68 min were selected based on relative abundance of the peaks (Fig.1). The peaks with retention time 12.17 corresponds to I-Dodecanol with 1.29% of peak area; and I-Trideconol with 1.4% of peak area; 17.42 min pertaining to Tetradecanoic acid with 2.1% of peak area; 18.40min corresponds to the Pentadeconoic acid with 1.9% of peak area and Octodecanoic acid with 1.8% of peak area; 20.21min corresponds to 1,2-Benzenedicarboxylic acid,bis(2-methylpropyl)ester(CAS) with 2.18% of peak area; 21.17min corresponds N-pyrrolidine with 1.29% of peak area; 21.55 min corresponds to the Hexadeconoic acid with 1.36% of peak area; 24.86min corresponds to 9-Octodeconoic acid, methyl ester with 1.34% of peak area; 30.29min corresponds Ipconazole with 1.22% of peak area; 31.33min corresponds Heptadecanoic acid,10-methyl ester(CAS)with 5.06% of peak area; 32.94min corresponds to the Docosanewith 1.2% of peak, Nonacosane with 0.95% of peak area and Dodecane,2,6,11-trimethyl with peak area of 1.3% and 36.68min corresponds Cyclohexane,1,4-dimethyl-2-octodecyl with 1.65% of peak area. Biological activity and chemical structure of phytocompound were identified (Table).

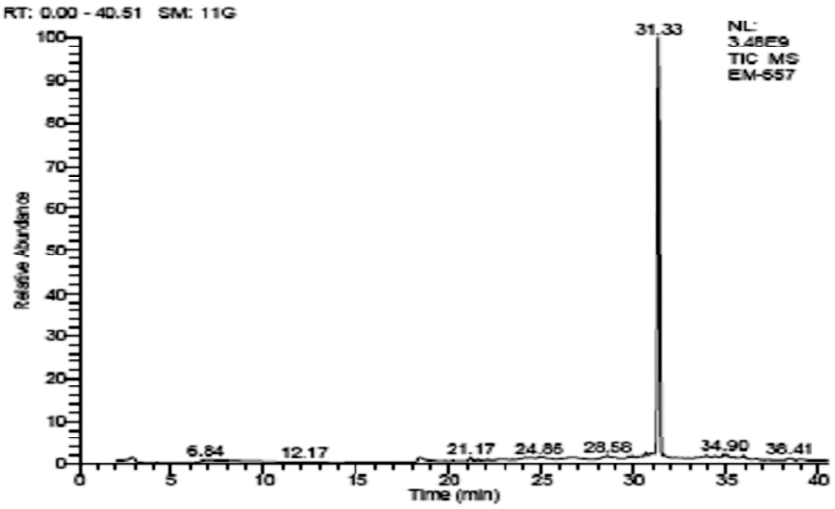

Figure 1. The gas chromatodram of antimicrobial compounds identified from B.subtilis (Bs-10) through,GC/MS,

Compounds like Hexadeconoic acid methyl ester, Pentadecanoic acid,2-hydroxyl-1-ethyl ester, belonging to fatty acid with antibacterial and antifungal activity(11). Reports available on antimicrobial activity of 1,2-Benzenedicarboxylic acid, dibutyl este (12). The detailed list of different antimicrobial compounds from *Bacillus subtilis* with their antimicrobial activity were furnished in Table 3. So the present study shows that presence of these kind of compounds in *B.subtilis* strain Bs-10, providing the strong antibiotic activity with broad antimicrobial spectrum.

Table 3
Identification of antimicrobial compounds from *B.subtilis* strain Bs-10 trough GC/MS

Retenion time	*Compound name*	*Molecular formula*	*Molecular weight*	*Probability*	*Activity*	*References*
12.17	I-Dodecanol	$C_{12}H_{26}O$	186	16.54	Antibacterial	(13)
12.17	I-Trideconol	$C_{13}H_{28}O$	200	11.04	Antibacterial	(13)
17.42	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	48.97	Antimicrobial	(14)
18.40	Pentadeconoic acid	$C_{15}H_{30}O_2$	242	8.85	Antifungal, Antibacterial	(11)
18.40	Octodecanoic acid	$C_{18}H_{36}O_2$	284	40.24		(15)
20.21	1,2-Benzenedicarbo-xylic acid,bis(2-methyl-propyl) ester (CAS)	$C_{16}H_{22}O_4$	278	16.80	Antifungal, Antibacterial	(12)
21.17	N-pyrrolidine	$C_{17}H_{19}NS$	269	74.08	Antibacterial	(16)
21.55	Hexadeconoic acid	$C_{17}H_{34}O_2$	270	40.29	Antifungal, Antibacterial	(17)
24.86	9-Octodeconoic acid, methyl ester	$C_{18}H_{36}O_2$	296	10.05	Antimicrobial	
30.29	Ipconazole	$C_{18}H_{24}CIN_3O$	333	12.26	Antifungal	(18)
31.33	Heptadecanoic acid, 10-methyl ester(CAS)	$C_{19}H_{38}O_2$	298	12.29		(19)
32.94	Docosane	$C_{22}H_{46}$	310	13.33	Antibacterial	(20)
32.94	Nonacosane	$C_{29}H_{60}$	408	7.27	Antibacterial	(20)
32.94	Dodecane,2,6,11-trimethyl	$C_{15}H_{32}$	212	13.33	Antifungal, Antibacterial	(21)
36.68	Cyclohexane	$C_{26}H_{52}$	364	17.97	Antibacterial	(22)

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