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Study of Antimicrobial Compounds of *Bacillus subtilis* (PSB5) and its Interaction with Fungicides against *Fusarium oxysporum* f. sp. *gerberae*

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Abstract

Objectives: To study the antifungal activity of *Bacillus* spp. against *Fusarium oxysporum* f. sp. *gerberae* (FOG) and studies of interaction between *Bacillus subtilis* (PSB5) and commercially active fungicides against FOG. **Methods:** Extraction of crude antibiotics (PSB5, BSD5, BmTNAU1) was done by continuous precipitation and acidification to pH 2.0 using solvent ethyl acetate and further subjected to GC/MS analysis (PSB5). Bioassay of these extract against the pathogen (FOG) was done by Agar Diffusion method. The control employed was ethyl acetate alone in the media. Further the compatibility test was done by Poisoned food technique. **Findings:** The crude antibiotic extracts (PSB5, BSD5, BmTNAU1) were highly effective against the FOG probably due to the presence of antimicrobial compounds in strain PSB5 detected through GC/MS. Moreover, the test of compatibility of PSB5 with fungicides revealed the growth promotion of the bacteria at lower dosages of fungicides alone. The most effective fungicide, tebuconazole 250 EC(Score) against FOG showed compatibility at moderate dosage (250 ppm) with the highly efficient strain of bacteria, PSB5. **Application:** In our present study, we tried to emphasize on the efficacy of antifungal activity of *Bacillus* spp. and compatibility interactions of antagonistic bacteria and fungicides which could be utilized for further field experiments against FOG.

Keywords: Antimicrobial Compounds, *Bacillus*, Fungicides, *Fusarium*, *Gerbera*

1. Introduction

Gerbera jamesonii Bolus ex Hook is a flourishing cut flower crop grown intensively under protected cultivation. According to the global trends in floriculture, *Gerbera* occupies the fourth place among cut flowers[1]. In India, many soil borne diseases has been recorded[2], among which *Fusarium* wilt also causes devastating losses in *Gerbera*.

Manifestation of biological control by *Bacillus* spp. against various soil borne plant pathogens has been observed from several years. The production of metabolites with antimicrobial activity is one determinant of their ability to control plant diseases[3]. T**he volatile compound of** *B. subtilis* **was investigated by GC-MS analysis and its intense inhibitory effect against pathogenic** fungi, including *Ascochyta citrullina, A. solani* and *A. brassicae*[4].

Moreover, chemical fungicides do have the importance for their very quick action against the plant pathogens. The antagonistic bacterium, *Bacillus subtilis* strain BSF4 was compatible with the fungicides like azoxystrobin, penconazole, fosetyl aluminium and sulphur[5]. Hence, in order to study the complex interaction between the *Bacillus* spp. and chemical fungicides, compatibility test was undertaken to design the combined management module of the two in future studies.

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2. Materials and Methods

2.1 Isolation of Pathogen (FOG)

Pathogen was consistently isolated from the varieties Bellwater white and Donavan yellow. The infected roots were cut into 1cm bits and surface sterilized with 0.1% mercuric chloride ($HgCl_2$) solution for 30 seconds and washed thrice in the series of sterile distilled water, then transferred to sterilized Petri plates containing potato dextrose agar (PDA) medium amended with 1000 ppm of streptomycin sulphate and were incubated at room temperature (27±2°C) for 5 days. After emergence of fungal growth, the pathogen was pure cultured by single hyphal tip technique[6].

2.2 Identification of Vascular Wilt Pathogen

The phenotypic characteristics like colony features, growth rate, pigmentation, microconidia, macroconidia and chlamydospore production were observed and evaluated[7] using a light microscope (Labomed – IVU 5100) and photographed using a Labomed camera model LX400 with an image analyser - pixelpro programme.

2.3 Establishment of Pathogenicity

Potting mixture comprising of laterite soil, sand and compost were mixed in the ratio of 3:1:1 and steam sterilized at 120 lb pressure for 1hr on alternate days. The sterilized potting mixture was filled in to the plastic pots @ 5kg/ pot. The vascular wilt pathogen *F. oxysporum* multiplied in potato dextrose broth, consisting of 10^7 conidia/ml was inoculated @ 1% to the soil weight. Then, *Gerbera* (var, Bellwater white) plants were planted and an uninoculated control was also maintained. After symptom development, re-isolation was done and compared with the original culture for confirmation of the pathogen identity.

2.4 Collection of Bacterial Antagonists

Among various bacterial isolates, three most effective stains were collected from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore and were taken for the further experimentation (Table 1).

2.5 Extraction of Crude Antibiotics

The crude antibiotics were extracted by using ethyl acetate as solvent[8]. The isolates of antagonistic bacteria (PSB5, BSD5, BmTNAU1) were grown at 28±2° C for 5 days in Pigment Production (PP) broth containing peptone-20 g/l, glycerol-20 g/l, NaCl-5 g/l, K NO_3-1g/l, pH-7.2 and 1000ml distilled water. After incubation they were centrifuged at 10000 rpm for 10 -15 min and the supernatant was adjusted to pH 2.0 with concentrated HCl. 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 l M NaOH to adjust the pH 7.0. The resultant suspension was extracted twice with ethyl acetate. The ethyl acetate phase was transferred into the vacuum flask evaporator maintained at 60°C with 80rpm till the ethyl acetate fraction gets evaporated. The crude antibiotics was re-suspended in 1ml of methanol – chloroform mixture (1:1) and used for further bioassay and GC/MS analysis.

2.6 Bioassay of Crude Antibiotics against FOG

This bioassay was performed by Agar diffusion method[9]. Spore suspension with conidial load 10^6 cfu/ ml was prepared by pouring 15ml of sterile water into the 10 days old culture of FOG and scrapped with sterile scalpel. Each sterile plate was poured with 1 ml of the spore suspension and 15ml of PDA medium. After solidification, a sterilized cork borer of 5mm dia. was used to punch the

Table 1. List of the bacterial antagonists used in the study

S.No.	Isolates	Name of the species	Source of isolates	Location	Accession number of the isolate in NCBI
1	PSB5	B. subtilis	Gerbera	Ooty	KJ817861
2	BSD5	*Ochrobactrum* spp.	Carnation	Nilgiris	JX036527
3	BmTNAU1	B. megaterium	Carnation	Nilgiris	KC540802

medium around the plate of 1cm away from the periphery making 4 wells. Extracted crude antibiotics from *Bacillus* spp. (BSD5, PSB5, BmTNAU1) were poured into 4wells at the rate of 20, 40, 60, 100 µl per plate and incubated for 72 h at 28±2˚C. The control was added with organic solvent (ethyl acetate) alone in a separate plate. The zone of inhibition was measured by placing the fully grown plates on to the graph sheets (in mm^2).

2.7 GC-MS Analysis of Crude Antibiotics

The crude antibiotics of the effective *Bacillus* spp. isolate PSB5 was analyzed for the detection of active bio-molecules responsible for the suppression of FOG through GC-MS (GC Clarus 500 Perkin Elmer). Volatile components were identified by GC-MS using a column Elite-5MS (100% Dimethyl poly siloxane), 30 x 0.25mm x 0.25µm df equipped with GC Clarus 500 Perkin Elmer. Electron impact (EI) mass scan (m/z) was recorded in the 45-450 AMU 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.

2.8 Compatibility of *Bacillus subtilis* (PSB5) with Fungicides

Compatibility of bacterial antagonist, *Bacillus subtilis* (PSB5) with fungicides (carbendazim 50% WP, difenoconazole 25% EC, azoxystrobin 25% SC, fosetyl Al 80% WP, kresoxim methyl 44.3% SC, tebuconazole 250 EC, tebuconazole 50%+trifloxystrobin 25% WG, propineb 70 WP and propioconazole 25% EC) which were commonly used against *Fusarium* wilt was tested by Poisoned food technique[10]. The minimum inhibitory concentrations (50ppm, 100ppm, 250ppm, 500ppm, 1000ppm, 1500ppm, 2000ppm) of systemic and contact fungicides were amended in 100 ml of Nutrient Agar medium and incubated at room temperature (28±2°C). Three replications were maintained for each treatment @ 10 plates per replication. Growth of antagonist was compared with control plates (medium without fungicides) for their compatibility with fungicide.

3. Results and Discussion

3.1 Symptomatology

Wilt symptoms were observed in seedlings and in older plants. The symptoms were yellowing of the leaves and subsequently spread to entire plant. Affected leaves droop down and finally wilted. Wilting of the entire plant occurred within 3 to 4 weeks after infection. Examination of the infected plants showed the presence of black discolouration in collar areas and brownish discolouration in petioles. Similarly, yellowing of leaves, stunting, wilting and death of the infected *Gerbera* plants in patches were made by many authors[11,12].

3.2 Morphological Characterization

The mycelium of the fungal culture on PDA medium was initially white and later turned light pink to dark

Table 2. Bioassay of Crude Antibiotics of *Bacillus* spp. against FOG

S.No	Crude antibiotics of *Bacillus* spp.	Efficacy of *Bacillus* crude antibiotics against *F. oxysporum* f. sp. *gerberae*			
		Area of inhibition (mm^2)*			
		20(µl)	40(µl)	60(µl)	100(µl)
1	*Orchrobactrum* spp. (BSD5)	155.00[a] (12.43)	171.00[a] (13.05)	192.00[a] (13.83)	219.00[a] (14.77)
2	*Bacillus subtilis* (PSB5)	142.00[b] (11.93)	158.00[b] (12.58)	179.00[b] (13.39)	206.00[b] (14.37)
3	*Bacillus megaterium* (BmTNAU1)	130.00[c] (11.42)	146.00[c] (12.10)	167.00[c] (12.94)	194.00[c] (13.94)
4	Ethyl acetate	0.00[d] (0.71)	0.00[d] (0.71)	0.00[d] (0.71)	0.00[d] (0.71)
5	Untreated control	0.00[d] (0.71)	0.00[d] (0.71)	0.00[d] (0.71)	0.00[d] (0.71)

*Values are mean of four replications

Means followed by a common letter are not significantly different at 5% level by DMRT

Values in parentheses are square root transformed values

pink in different isolates. Macroconidia was sparse, and fusoid, 2-3 septate and measured 16.0-29.0 x 2.5-4.2 µm. Microconidia were abundant, hyaline, continuous, ovoid and measured 3.8-8.5 x 2.0-3.5 µm. Chlamydospores were hyaline and spherical, measured 4.0 – 7.5 µm in diameter. Based on these phenotypic characters, the pathogen was confirmed as *Fusarium oxysporum* f. sp. *gerberae* (**KJ570974**). The morphological characters were similar with the descriptions made by Booth in *Fusarium* wilt of Carnation[13].

3.3 Pathogenicity

Inoculation of *F. o.* f. sp. *gerberae* (FOG) in to the healthy *Gerbera* seedlings of var. Bellwater white (30 days old) expressed the typical symptoms of wilt of *Gerbera* after 15 days of inoculation. Infected plants showed typical stunting of the plants and yellowing of leaves with brown to black streaks noticed in the crown portion and petioles of the plant. No symptoms were observed in un-inoculated control plants. Garibaldi and Minuto recorded the similar pathogenicity results[12].

3.4 Bioassay of Crude Antibiotics of *Bacillus* spp. against FOG

The crude antibiotics extracted in ethyl acetate from the effective bacterial isolates *viz*., *Ochrobactrum* sp. isolate BSD5, *B. subtilis* isolate PSB5 and *B. megaterium* isolate BmTNAU1 were tested against FOG2. Their effectiveness were in the order of BSD5, PSB5 and BmTNAU1 having the area of inhibition 219 mm^2, 206 mm^2 and 194 mm^2 at 100 µl concentration. The control with only ethyl acetate was not effective against FOG (Table 2).

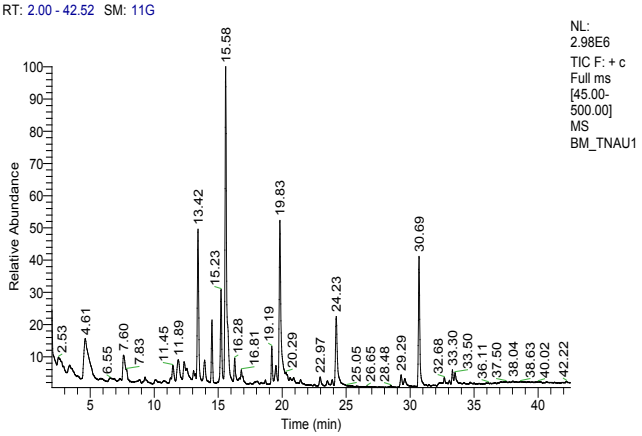

Figure 1. Total Ions Chromatogram (TIC) of antimicrobial compounds identified from B. *subtilis* isolate PSB5 through GC/MS.

The crude lipopeptides extracted from cell-free culture broth of *B. amyloliquefaciens* strain TF28 exhibited a wide-spectrum of antifungal activity and it strongly inhibited the growth and spore germination of *F. moniliforme*[14]. The influence of crude antimicrobial compounds

Table 3. Antimicrobial compounds identified from *B. subtilis* isolate PSB5 through GC/MS

S.No.	RT	Name of the compound	Molecular formula	MW	Peak Area %
1.	2.53	2(3H)-Furanone, dihydro-4,4-dimethyl	$C_6H_{10}O_2$	114	1.17
2.	3.40	Benzeneacetic acid, hexyl ester	$C_{14}H_{20}O_2$	220	1.28
3.	4.60	Benzenepropanoic acid, silver salt	$C_9H_9AgO_2$	256	4.60
4.	6.55	3-Phenylpropionic acid, tridec-2-ynyl ester	$C_{22}H_{32}O_2$	328	0.46
5.	6.88	9-Octadecenoic acid, (2-phenyl-1,3-dioxalan-4-yl)methyl ester	$C_{28}H_{44}O_4$	444	0.11
6.	7.60	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	206	3.24
7.	9.29	1-Hexadecanol, 2-methyl	$C_{17}H_{36}O$	256	0.37
8.	10.08	2,5-Piperazinedione, 3-methyl-6-(1-methylethyl)	$C_8H_{14}N_2O_2$	170	0.28
9.	11.45	3-methyl-1,4-Diazabicyclo[4,3-O]nonan-2,5-dione, N-acetyl	$C_{10}H_{14}N_2O_3$	210	1.57
10.	11.89	(3S,6S)-3-Butyl-6-methyl piperazine-2,5-dione	$C_9H_{16}N_2O_2$	184	2.40
11.	16.28	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	1.45
12.	14.51	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl)ester	$C_{16}H_{22}O_4$	278	3.56
13.	18.69	Heneicosane,11-(1-ethylpropyl)	$C_{26}H_{54}$	366	0.16
14.	33.07	Heptanoic acid, docosyl ester	$C_{29}H_{58}O_2$	438	0.14
15.	33.50	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	C21H42O4	358	0.86

extract of *B. subtilis* C9 in controlling growth of *R. solani* was studied under *in vitro* condition[15].

3.5 Identification of Antimicrobial Compounds produced by PSB5 through GC/MS

The crude antibiotic extract of the effective strain *B. subtilis,* PSB5 was analyzed through GC/MS to detect the antimicrobial compounds. The compounds associated were identified as aliphatic hydrocarbons, terpenes, phenols (Table 3, Figure 1).

Likewise, antifungal activity of Benzeneacetic acid against *Botrytis cinerea*, *Glomerella cingulate*, *Phytophthora drechsleri*, *Penicillium citrinum*, *Penicillium digitatum* and *Fusarium oxysporum* was reported in the crude antibiotic extract of *Lactobacillus plantarum*[16]. Antibacterial and insecticidal activity of Benzenepropanoic acid was reported in the essential oils of *Acasia* modesta[17]. Antifungal and antibacterial activity against the pathogens *Pectobacterium caratovora* and *Aspergillus niger* was reported in fatty acid methyl esters from air dried wood, bark and leaves of *Brachichyton diversifolius*[18]. Similarly, antibacterial activity against *Xanthomonas axonopodis* pv. *citri* and *Xanthomonas campestris* pv. *malvalearum* was recorded in white crystalline solid from red algae *Portieria hornemannii*[19]. Moreover, antimicrobial protein was extracted from *Bacillus amyloliquefaciens* MBL27 recently[20].

3.6 Compatibility of *Bacillus subtilis* PSB5 with Fungicides

Among all the tested fungicides, Kresoxim methyl 44.3% SC was compatible with PSB5 at all the tested concentrations. The strain PSB5 showed the growth tolerance against carbendazim 50% WP, difenoconazole 25% EC, azoxystrobin 25% SC, fosetyl Al 80% WP and tebuconazole 250 EC (effective against FOG)[21] under moderate dosages. Tebuconazole 50%+trifloxystrobin 25% WG (effective against FOG) [21], propineb 70 WP and propioconazole 25% EC were in-compatible with PSB5 (Table 4).

Three *Bacillus* spp. strains (MB1, MB2 and MB3) showed growth tolerance against carbendazim, and then followed by hexaconazole which were effective against grey blight of Tea[22].

4. Conclusion

Fusarium wilt of *Gerbera* is a frequently occurring soil borne disease under protected cultivation and its control is difficult to achieve which generally relies on the integration of several control methods. Therefore, a study was undertaken to understand the antimicrobial capacity of bacterial antagonists by extracting crude antibiotics and GC/MS analysis of crude antibiotic extract of the effective *Bacillus subtilis* isolate PSB5. Also the strain was subjected to the compatibility test with various fungicidal agents so

Table 4. Compatibility test of *Bacillus subtilis* strain PSB5 with fungicides

S. No.	Fungicides	Lower dosages		Moderate dosages		Recommended dosages		Higher dosages
		50 ppm	100 ppm	250 ppm	500 ppm	1000 ppm	1500 ppm	2000 ppm
1.	Tebuconazole 250 EC	++	++	++	-	-	-	-
2.	Propioconazole 25% EC	-	-	-	-	-	-	-
3.	Azoxystrobin 25% SC	+++	+++	+++	++	-	-	-
4.	Propineb 70 WP	-	-	-	-	-	-	-
5.	Difenoconazole 25% EC	+++	+++	++	-	-	-	-
6.	Tebuconazole 50% + Trifloxystrobin 25% WG	-	-	-	-	-	-	-
7.	Kresoxim-Methyl 44.3% SC	+++	+++	+++	+++	++	++	++
8.	Fosetyl aluminium 80% WP	+++	+++	+++	++	-	-	-
9.	Carbendazim 50% WP	+++	+++	++	++	-	-	-
10.	Control	+++	+++	+++	+++	+++	+++	+++

:No growth; ++ : Medium growth ; +++ :High growth

as to deliver both fungicide and biocontrol agents simultaneously, which would control the pathogen.

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