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Investigations on Antifungal Activity of *Bacillus* spp. against *Fusarium oxysporum* f. sp. *gerberae* (FOG) Causing Wilt of *Gerbera* Under Protected Cultivation

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A survey was conducted to assess the incidence of wilt of *Gerbera* caused by *Fusarium oxysporum* f. sp. *gerberae* (FOG) in Nilgiris and Salem districts of Tamil Nadu during 2013-2014. The wilt incidence ranged from 10.11% to 19.81% in nine varieties Donavan, Valletta, Alamo, Bellwater, Avimaria, Blessing, Atletico, Farida and Dalma. 26 isolates were screened for the management of disease, of which, *Ochrobactrum* spp. (BSD5), *B. subtilis* (PSB5) and *B. megaterium* (BmTNAU1) isolates showed highest antagonistic activity against FOG. 9 isolates of *Bacillus* spp. were studied for the presence of biosynthetic genes of antimicrobial peptides (AMPs). The effective biocontrol agents were applied through root dipping and soil drenching @ 5ml/L either individually or in consortia. Seedling dip and soil drenching @ 0.5% with PSB5+BSD5+BmTNAU1 resulted in reduced disease incidence, enhanced plant height, root length and flower yield of *Gerbera*.

Keywords: GC-MS, *Fusarium,* Formulations, *Gerbera, Bacillus* spp.

African Daisy, *Gerbera jamesonii* Bolus ex Hook is one of the most important commercially grown cut flower crops under protected cultivation and has great export potential. The wilt incidence in *Gerbera* varies depending upon the variety, monocropping and on the population density of the pathogen. Von Arx described a foot rot and wilt disease of *Gerbera* in the Netherlands, caused by *Fusarium oxysporum* Schelchtend[1]. Gordon distinguished this pathogen as *Fusarium oxysporum* f. sp. *gerberae*[2].

Biocontrol has gained considerable attention and appears to be promising as a viable supplement or alternative to chemical control[3]. *Bacillus* spp. are widely used in biological control of plant pathogens because of their safety, widespread distribution in diverse habitats, remarkable ability to survive under adverse conditions and ability to produce growth promoting substances[4,5,6,7]. *Fusarium* wilt of chickpea was effectively reduced by various antagonistic *Bacillus* strains (Rb29, Rb6, Rb12, Rb4 and Rb 15). The mycelial growth was inhibited up to 71.11% under *in vitro* and *in vivo* conditions[8].

B. subtilis, devoted 4– 5% of its genome to antibiotic synthesis and have the potential to produce more than two dozen structurally diverse AMPs which include cyclic lipopeptides such as fengycin, iturin, bacillomycin and surfactin[9]. These compounds are characterized by broad spectrum action and hence, the *Bacillus* spp. has played both the role of crop protection and crop improvement[9]. As a seed treatment, *Bacillus* spp. increased the yields of carrots by 48 per cent, oats by 33 per cent[10] and peanuts up to 37 per cent [11].

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MATERIALS AND METHODS

Survey for the occurrence of *Gerbera* wilt

Survey was conducted in different varieties of *Gerbera* for the incidence of wilt disease in Ooty, Nilgiris district and Yercaud, Salem district in Tamil Nadu, India. Totally 24 commercially cultivated varieties were surveyed in poly house under protected condition. The per cent disease incidence was recorded as per the formula given by Wheeler[12].

Per cent disease incidence = No. of infected plants/ Total no. of observed plants x 100

Isolation of *Gerbera* wilt pathogen

Five pathogenic isolates of *Fusarium oxysporum* f. sp. *gerberae* namely FOG1, FOG2, FOG3, FOG4 and FOG5 were isolated from 5 commercial cultivars Donavan (yellow), Bellwater (white), Dalma (cream), Farida (cream) and Avimaria (white) respectively. The pathogen was isolated from infected root portion. Infected root tissues were surface sterlized with 0.1% mercuric chloride ($HgCl_2$) for 30sec, plated on potato dextrose agar (PDA) medium, amended with 100 µg/ml of streptomycin sulphate and incubated at 20 ± 2°C for 7 days. After emergence of fungal growth, the pathogen was pure cultured by single hyphal tip technique[13].

Establishment of pathogenicity

Steam sterilized potting mixture of laterite soil, sand and compost (3:1:1) was filled in to the plastic pots @ 5kg/pot. The vascular wilt pathogenic isolates were multiplied in potato dextrose broth, consisting of 10^7 conidia/ ml and were inoculated @ 1% to the soil weight. Later the *Gerbera* (v. Bellwater white) plants were dipped in conidial suspension and planted. Similarly, uninoculated control was also maintained.

Morphological characterization of *Fusarium*

The morphological characteristics of the fungi were studied viz. colony features, growth rate, pigmentation, microconidia, macroconidia and chlamydospore production from slide cultures and *in situ* observation on PDA by using the description of Burgess *et al.* [14,15] and Leslie and Summerell [16].

Molecular characterization of *Fusarium*

Isolation of genomic DNA

The genomic DNA was extracted from the frozen mycelium of *Fusarium* grown at room temperature (28 ± 2°C) on potato dextrose broth (PDB) for 10days using Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Knapp and Chandlee[17] with slight modifications [18].

Identification of *Fusarium oxysporum* by PCR

To confirm the pathogen as *Fusarium oxysporum* species, 16S rDNA intervening sequence specific ITS FU F (5' CAACTCCCAAACCCCTGTGA 3') and ITS FU R (5' GCGACGATTACCAGTAACGA 3') primers were used to get an amplicon of 389 bp size[19]. Amplification was conducted with a total reaction volume of 25µl in Eppendorf Master Cycler. The PCR settings used were as follows: a hold of 2min at 95°C, 30 cycles of 1min at 94°C, 30 sec at 54°C and 1min at 72°C and a final extension of 10min at 72°C[20].The PCR reaction was performed in Thermal Cycler (Eppendorf Master Cycler, Germany) with ready to use Taq DNA Polymerase 2x master mix comprising of Tris-HCl pH 8.5, $(NH_4)_2SO_4$, 3mM $MgCl_2$, 0.4mM dNTPs, 0.2units/µl Ampliqon Taq DNA polymerase and inert red dye. The PCR products were resolved on 2 per cent agarose at 50 Volts for 45 minutes using 1X TAE buffer stained with ethidium bromide (0.5µg/ml) and were photographed**.**

DNA sequencing, sequence alignment and phylogenetic analysis

The amplified genomic DNA of FOG was partially sequenced by Sanger dideoxy sequencing method at Excelris genomics, Ahmedabad, India using ABI 3730 x I Genetic Analyzer (Applied Biosystems, USA). The sequences of *Fusarium* obtained were submitted in the NCBI GenBank database, New York, USA and were aligned by CLUSTAL W and edition was performed by using the Bio-Edit Program V 7.0.5 [22]. Phylogenetic analysis was conducted by MEGA4 software [23] and a maximum parsimony tree was constructed by Neighbour-Joining method with bootstrap value more than 500 of 1000 replications[24].

Isolation of antagonistic *Bacillus* spp

Nine bacterial antagonists were isolated from the rhizosphere soil samples of *Gerbera* plants using Nutrient Agar medium by serial dilution technique. The colonies of *Bacillus* spp. were identified according to the description given by Bergey's manual of Systematic Bacteriology[25] and by molecular identification. In addition to these 9 isolates of *Bacillus* spp., the 17 cultures available

in the Department of Plant Pathology, TNAU were also used for the screening (Table-1).

Screening of antagonistic bacteria under *in vitro* condition

All the isolates of *Bacillus* spp. were tested using PDA medium for their antifungal activity against *Fusarium oxysporum* f. sp. *gerberae* (FOG2) by dual culture technique [26] and Percent inhibition was calculated using the formula[26]: PI = C – T/C X100 Where, PI is Percent Inhibition; C is the growth of test pathogen (mm) in the absence of the antagonist; T is the growth of test pathogen (mm) in the presence of the antagonist.

Molecular characterization of *Bacillus* spp

Isolation of genomic DNA

The genomic DNA from 9 antagonistic isolates of *Bacillus* spp. was extracted by Cetyl Trimethyl Ammonium Bromide (CTAB) method[17] with slight modifications [18] and were confirmed through 16S rDNA intervening sequence specific BCF1 (5'CGGGAGGCAGCAGTAGGGAAT 3') and BCR2 (5' CTCCCCAGGCGGAGTGCTTT 3') primers [27] to get an amplicon of 546bp size. The PCR settings used were as follows: a hold of 2min at 95°C, 40 cycles of 1min at 95°C, 1min at 57°C and 1min at 72°C and a final elongation of 5min at 72°C. The PCR products were resolved on 2 per cent agarose at 50 Volts stained with ethidium bromide (0.5µg/ml) and were photographed. The *Bacillus* spp. DNA was partially sequenced, submitted in NCBI (USA) using BLAST program [28] sequences were compared, scored with the CLUSTAL W program and phylogenetic tree was built by using MEGA4 software [23].

Molecular detection of antibiotic genes of *Bacillus spp*

Six antibiotic genes *Itu C*, *ItuD*, *SrfA*, *SfP* (4' Phosphopantetheinyl transferase gene), *BamC* and *FenD* were studied in nine isolates of *Bacillus*

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	PSB1	*B. subtilis*	*Gerbera*	Ooty	KJ817857
2	PSB2	*B. amyloliquefaciens*	*Gerbera*	Ooty	KJ817858
3	PSB3	*B. methylotrophicus*	*Gerbera*	Ooty	KJ817859
4	PSB4	*B. subtilis*	*Gerbera*	Ooty	KJ817860
5	PSB5	*B. subtilis*	*Gerbera*	Ooty	KJ817861
6	PSB6	*B. amyloliquefaciens*	*Gerbera*	Ooty	KJ817862
7	PSB7	*B. subtilis*	*Gerbera*	Ooty	KJ817863
8	PSB8	*B. tequilensis*	*Gerbera*	Ooty	KJ817864
9	PSB9	*Peanibacillus kribbensis*	*Gerbera*	Ooty	KJ817865
10	BSD1	*B. licheniformis*	Carnation	Nilgiris	JX036524
11	BSD2	*B. methylotrophicus*	Carnation	Nilgiris	JX036525
12	BSD3	*B. subtilis*	Carnation	Nilgiris	JX036526
13	BSD5	*Ochrobactrum* spp.	Carnation	Nilgiris	JX036527
14	BmTNAU1	*B. megaterium*	Carnation	Nilgiris	KC540802
15	BSC1	*B. subtilis*	Cotton	Coimbatore	JX036516
16	BSC2	*B. tequilensis*	Cotton	Coimbatore	JX036517
17	BSC3	*B. subtilis*	Cotton	Coimbatore	JX036518
18	BSC4	*B. pumilis*	Cotton	Coimbatore	JX036519
19	BSC5	*B. cereus*	Cotton	Coimbatore	JX036520
20	BSC6	*B. amyloliquefaciens*	Cotton	Coimbatore	JX036521
21	BSC7	*B. amyloliquefaciens*	Cotton	Coimbatore	JX036522
22	BSC11	*B. cereus*	Cotton	Coimbatore	JX036523
23	BS2	*B. subtilis*	Cotton	TNAU,Coimbatore	JF926691
24	BacnTNAU1	*B. amyloliquefaciens*	Sorghum	TNAU,Coimbatore	KC540833
25	BacnTNAU2	*B. amyloliquefaciens*	Cotton	TNAU,Coimbatore	KC540834
26	BCcnTNAU1	*B. cereus*	Cotton	TNAU,Coimbatore	KC540835

spp. isolated from the rhizosphere region of the host *Gerbera*. The primers used for amplification and PCR conditions were followed as given by Chung *et al.*[29] The 20 µl PCR mixture contained approximately 50·g of total DNA, 5 mM each dNTPs, 20 pmol of each forward and reverse primer and 0.5U of *Taq* DNA polymerase (Table 2). The PCR products were resolved on two per cent

Table 2. Molecular detection of antibiotic genes *Bacillus* spp.

S. No.	Antibiotic gene	Antibiotic	Forward Primer	Reverse Primer	Gene size	PCR conditions
1.	*ItuC*	Iturin C	ITUC1F (5' CCCCCTCGGTCAA GTGAATA 3')	ITUC1R (5' TTGGTTAAGCCCT GATGCTC 3')	594 bp	94°C for 3min 40 cycles consisting of 94°C for 1min 60°C for 1min 72°C for 1min 72°C for 10 min
2.	*ItuD*	Iturin D	ITUD1F (5' GATGCGATCTCCT TGGATGT 3')	ITUD1R (5' ATCGTCATGTGCT GCTTGAG 3')	482 bp	95°C for 15min 40 cycles consisting of 95°C for 1min 57°C for 1min 72°C for 1.5min 72°C for 7 min
3.	*SrfA*	Surfactin A	SRFAF1 (5' AGAGCACATTGA GCGTTACAAA 3')	SRFAR1 (5' CAGCATCTCGTTC AACTTTCAC 3')	626 bp	95°C for 15min 40 cycles consisting of 95°C for 1min 60°C for 1min 72°C for 1.5min 72°C for 7min
4.	*SfP*	Surfactin P	SFPF1 (5' TTGGTTAAGCCCT GATGCTC 3')	SFPR1 (5' TTATAAAAGCTCT TCGTACG 3')	675 bp	95°C for 15min 40 cycles consisting of 95°C for 1min 56°C for 1min 72°C for 1.5min 72°C for 7min
5.	*BamC*	Bacillomycin C	BACC1F (5' GAAGGACACGGC AGAGAGTC 3')	BACC1R (5' CGCTGATGACTGT TCATGCT 3')	957 bp	95°C for 15 min 40 cycles consisting of 95°C for 1min 60°C for 1 min 72°C for 1.5min 72°C for 7 min
6.	*FenD*	Fengycin D	FEND1F (5' TTTGGCAGCAGG AGAAGTTT 3')	FEND1R (5' GCTGTCCGTTCTG CTTTTTC 3')	964 bp	94°C for 3 min 40 cycles consisting of 94°C for 1min 60°C for 1 min 72°C for 1 min 72°C for 10 min

PCR conditions are given in the order: Initial Denaturation, Denaturation, Annealing, Primer Extension, Final Extension

agarose gel at 50 Volt, stained with ethidium bromide (0.5µgml^{-1}), and photographed.

Development of liquid formulation of Bacterial antagonists

The bacterial *viz*., *Bacillus megaterium*-BmTNAU1, *Bacillus subtilis*-PSB5 and

Table 3. Biocontrol treatment schedule for *Fusarium* wilt management

Treatment	Treatment details
T_1	Root dipping of seedlings with *B. megaterium* isolate BmTNAU1 (10^9cfu/ml) 5ml/litre during planting + Soil drenching with BmTNAU1 at 5 ml/litre at 15 days interval.
T_2	Root dipping of seedlings with *B. subtilis* isolate PSB5 (10^9cfu/ml) 5ml/litre during planting + Soil drenching with isolate PSB5 at 5 ml/litre at 15 days interval.
T_3	Root dipping of seedlings with *Ochrobactrum* spp. isolate BSD5 (10^9cfu/ml) 5ml/litre during planting + Soil drenching with isolate BSD5 at 5ml/litre at 15 days interval.
T_4	Root dipping of seedlings with BSD5+PSB5 (10^9cfu/ml) 5ml/litre during planting + Soil drenching with BSD5+PSB5 at 5ml/litre at 15 days interval.
T_5	Root dipping of seedlings with BSD5+PSB5+BmTNAU1 (10^9cfu/ml) 5ml/litre during planting + Soil drenching with BSD5+PSB5+BmTNAU1 at 5ml/litre at 15 days interval.
T_6	Root dipping of the seedlings with carbendazim (1g/litre) during planting + Soil drenching with carbendazim at 1g/litre at 15 days interval.
T_7	Untreated control

Table 4. Survey for the occurrence of *Fusarium* wilt in commercial *Gerbera* varieties

S.No	Variety	Colour of flower	Wilt incidence (%)	
			Ooty	Yercaud
1	Valletta	Yellow	16.12	11.32
2	Donavan	Yellow	19.81	15.14
3	Palmbeach	Yellow	15.47	-
4	Alamo	Yellow	14.23	17.85
5	Mammut	Yellow	-	12.74
6	Estoria	Red	9.54	4.12
7	Kaisur	Red	0.00	-
8	Expression	Red	5.89	9.30
9	Sangreena	Red	-	0.00
10	Ruby Red	Red	7.21	7.45
11	Avimaria	White	16.15	18.72
12	Bellwater	White	19.74	15.51
13	Blessing	White	13.18	19.24
14	Aqua	Pink	8.87	-
15	Esmara	Pink	7.40	0.00
16	Elegance	Pink	-	17.25
17	Atletico	Orange	16.11	10.11
18	Five burster	Orange	12.40	7.51
19	Carrera	Orange	9.45	0.00
20	Goliath	Orange	0.00	-
21	Farida	Cream	10.23	15.15
22	Dalma	Cream	14.42	13.26
23	Snow flake	Cream	16.23	-
24	Winter queen	Cream	-	9.87
Mean	11.62	10.76		

: Not grown

Ochrobactrum spp.- BSD5 were cultured on NB kept in an orbital shaker at 150rpm at room temperature (28±2°C) for 48hr and were mixed with 1% glycerol (10ml), tween 20 (10ml) and poly vinyl pyrrolidone – 40000 ml. wt (10g) each separately[30]. Finally standardized to 10^9cfu/ml and was stored at 5°C for further study.

Seedling dip and soil drenching with liquid formulation of *Bacillus* spp. against wilt of *Gerbera* under protected cultivation

Field experiment was conducted during 2013-2014 in *Gerbera* (var. Donavan yellow) fields located at Spic Agro Biotech centre, Ooty, to assess the efficacy of liquid formulation of *Bacillus* spp. (10^9 cfu/ ml) @ 5ml/litre against wilt under protected condition (polyhouse). Thirty days old plants of *Gerbera* were used and the experiment was laid out with 7 treatments and 3 replications in RBD. The bed size of each replication was $5m^2$ with 30 × 30 cm spacing (Table 3).

Statistical analysis

All the experiments were statistically analyzed independently. The treatment means were compared by Duncan's Multiple Range-Test (DMRT) [31]. The package used for analysis was IRRISTAT version 92-1 developed by the International Rice Research Institute, Biometrics unit, The Philippines.

RESULTS AND DISCUSSION

Survey for the occurrence of *Gerbera* wilt

Survey results revealed that, the mean wilt incidence in commercially cultivated *Gerbera*

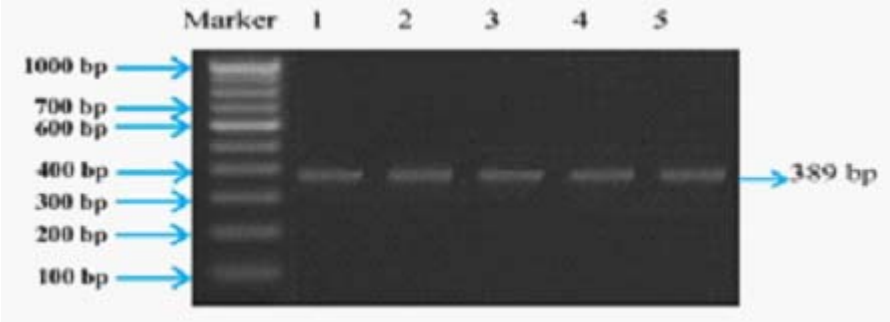

Fig. 1. PCR amplification of ITS region of *Fusarium oxysporum* f.sp. *gerberae*

(Lane 1- *Fusarium oxysporum* f.sp. *gerberae* isolate FOG1; Lane 2- FOG2; Lane 3-FOG3; Lane 4; FOG4 and Lane 5: FOG 5)

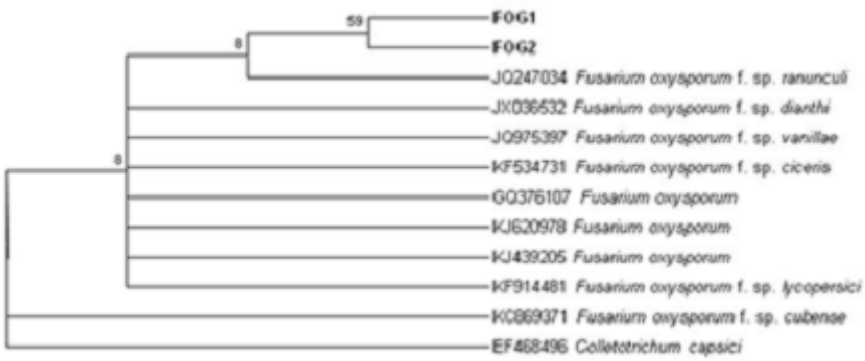

Fig. 2. Phylogenetic tree generated from a Neighbour-Joining (NJ) analysis of 18S r-DNA sequence of *Fusarium oxysporum* using MEGA4 software

varieties at Ooty and Yercaud was 11.62% and 10.76% respectively. In Ooty, maximum wilt incidence of 19.81% was recorded in var. Donavan yellow. In Yercaud, it was 19.24% in var. Blessing white. The practice of monocropping in polyhouses more than 3 years might be resulted in the susceptibility of various *Gerbera* varieties to the root borne disease (Table-4).

Symptomatology of *Fusarium* wilt

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 2 weeks after infection. Examination of the infected plants showed the presence of black discolouration in collar areas and brownish discolouration in petioles. Similar observations of yellowing of leaves, stunting, wilting and death of the infected *Gerbera* plants in patches were seen by Garibaldi *et al.* [32] and Garibaldi & Minuto[33].

Morphological characterization of the pathogen

All the five isolates (FOG1, FOG2, FOG3, FOG4 and FOG5) of the pathogen were examined for their phenotypic characters based on which it was confirmed as *Fusarium oxysporum*. The mycelium of the fungal culture on PDA medium was initially white and later turned light pink to dark pink. 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.

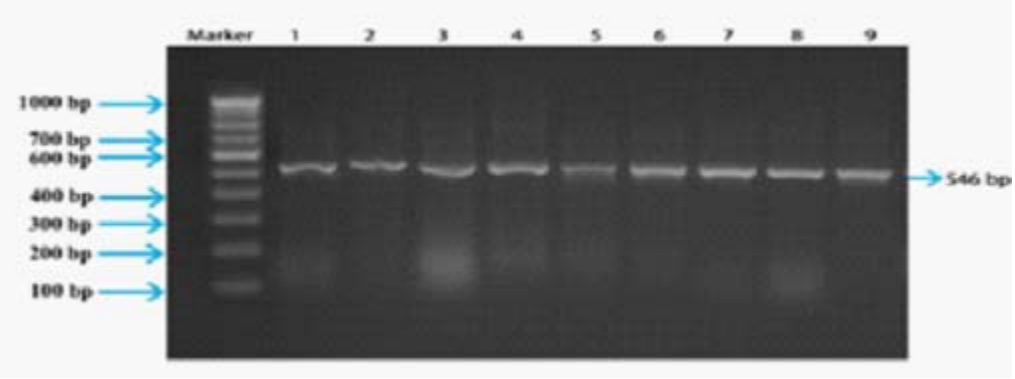

Fig. 3. PCR amplification of ITS region of *Bacillus* spp.

(Lane1-*Bacillus subtilis* isolate PSB1; Lane2-*Bacillus amyloliquefaciens* PSB2; Lane3- *Bacillus methylotrophicus* PSB3; Lane4- *Bacillus subtilis* PSB4; Lane5- *Bacillus subtilis* PSB5; Lane6- *Bacillus amyloliquefaciens* PSB6; Lane7- *Bacillus subtilis* PSB7; Lane8- *Bacillus tequilensis* PSB8; Lane9- *Paenibacillus kribbensis* isolate PSB9).

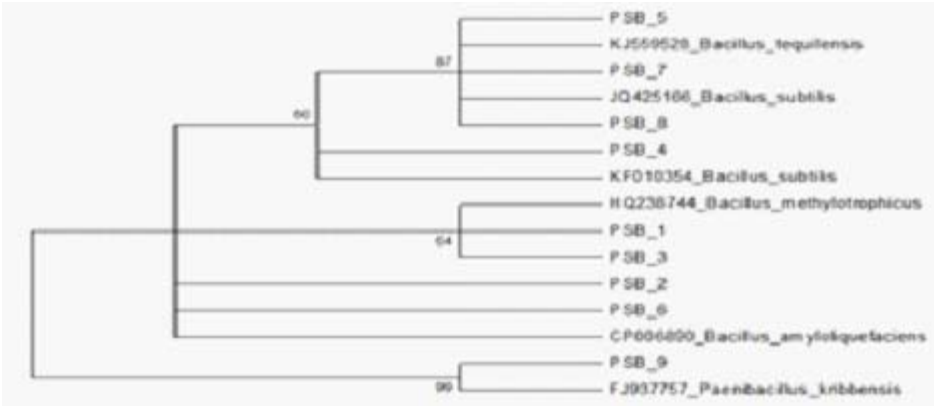

Fig. 4. Phylogenetic tree generated from a Neighbour-Joining (NJ) analysis of 16S rDNA sequence of *Bacillus* spp. using MEGA4 software

Chlamydospores were hyaline and spherical, measured 4.0 – 7.5 µm in diameter. These results were according to the description made by Mc Culloch in Gladiolus *Fusarium* wilt[34] and Booth in *Fusarium* wilt of Carnation[35] and Rajesh Kumar *et al.* in Carnation *Fusarium* wilt[36].

Pathogenicity

Inoculation of five isolates of *F. oxysporum* f. sp. *gerberae* (FOG1 to FOG5) in to the healthy *Gerbera* seedlings of 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. Among the 5 isolates tested FOG2 followed by FOG1 showed severe wilting symptoms, hence FOG2 was chosen for further studies.

Garibaldi and Minuto proved pathogenicity of *Fusarium oxysporum* by dipping healthy roots of *Gerbera* (cv. Jaska) in conidial suspension(5×10^7 conidia per ml), wilt symptoms and vascular discoloration in the roots, crown and veins developed within 30 days on each inoculated plant[33].

Molecular characterization of FOG

PCR was performed for the DNA of all the 5 pathogenic isolates (FOG1 to FOG5) of the

Table 5. Antifungal activity of antagonistic bacteria against *F. oxysporum* f. sp. *gerberae* (FOG2) under *in vitro*

S.No.	Isolates	Mycelial growth (mm)*	Per cent Inhibition over control
1	*B. megaterium*-BmTNAU1	40.00^{b}	55.55^{b} (48.18)
2	*B. subtilis* – BS2	63.00^{m}	29.99^{m} (33.20)
3	*B. amyloliquefaciens*- BaCnTNAU1	51.00^{f}	43.33^{f} (41.16)
4	*B. amyloliquefaciens* -BaCnTNAU2	52.00^{fg}	42.22^{fg} (40.52)
5	*B. cereus* – BCcnTNAU1	54.00^{hi}	39.99^{hi} (39.22)
6	*B. subtilis* –BSC-1	62.00^{lm}	31.11^{lm} (33.89)
7	*B. tequilensis* -BSC 2	90.00^{p}	-
8	*B. subtilis* -BSC 3	71.00^{o}	21.11^{o} (27.34)
9	*B.pumilis*-BSC-4	61.00^{l}	32.22^{l} (34.58)
10	*B. cereus*- BSC 5	55.00^{i}	38.88^{i} (38.57)
11	*B. amyloliquefaciens*-BSC 6	61.00^{l}	32.22^{l}(34.58)
12	*B. amyloliquefaciens* -BSC7	57.00^{j}	36.66^{j} (37.26)
13	*B. cereus* -BSC11	57.00^{j}	36.66^{j} (37.26)
14	*B. licheniformis*-BSD1	41.00^{bc}	54.44^{bc}(47.54)
15	*B. methylotrophicus* -BSD2	54.00^{hi}	39.97^{hi} (39.21)
16	*B. subtilis* -BSD3	53.00^{gh}	41.11^{gh} (39.87)
17	*Ochrobactrum* spp., BSD5	35.00^{a}	61.11^{a} (51.42)
18	*B. subtilis*-PSB1	59.00^{k}	34.44^{k} (35.93)
19	*B.amyloliquefaciens*-PSB2	49.00^{e}	45.55^{e} (42.44)
20	*B.methylotrophicus*-PSB3	59.00^{k}	34.44^{k} (35.93)
21	*B.subtilis*-PSB4	62.00^{lm}	31.11^{lm}(33.89)
22	*B.subtilis*-PSB5	39.00^{b}	56.66^{b}(48.82)
23	*B.amyloliquefaciens*-PSB6	45.00^{c}	49.99^{c} (44.99)
24	*B. subtilis* -PSB7	58.00^{jk}	35.55^{jk}(36.59)
25	*B. tequilensis* -PSB8	47.00^{d}	47.77^{d} (43.72)
26	*Paenibacillus kribbensis*-PSB9	67.00^{n}	25.53^{n}(30.34)
27	Control	90.00^{p}	-

*Values are mean of three replications

In a column, means followed by a common letter is not significantly different at the 5% level by DMRT

Values in parentheses are arc sine transformed values

wilt pathogen using genus specific primers amplified a fragment of ~389 bp corresponding to the region of the 18S-23S rDNA intervening sequence (Fig 1).

The amplified genomic product was partially sequenced for the two isolates FOG1 & FOG2 and performed rDNA homology searches by using BLAST, NCBI (USA) for the isolates FOG1 (KJ570973) and FOG2 (KJ570974) which were 94 and 98 per cent homologous with the *F. oxysporum* respectively. Since the pathogen was isolated from *Gerbera*, the identified pathogen was referred as *Fusarium oxysporum* f. sp. *gerberae*.

Abd-salalem *et al.* designed ITS-Fu-f and ITS-Fu-r primers by comparing the aligned sequences of internal transcribed spacer regions (ITS) of a range of *Fusarium* species. The primers showed good specificity for the genus *Fusarium*, and approximately 389-bp product was amplified exclusively for *Fusarium oxysporum*[20].

Phylogenetic analysis

Phylogenetic analysis of 2 isolates (FOG1 & FOG2) and various formae specialis of *Fusarium oxysporum* based on 18S-rDNA sequences revealed the six different clusters formed in phylogenetic tree (Fig 2). The maximum sequence similarity ranging from 90 to 100 percent was recorded among the various isolates of *Fusarium oxysporum*.

Phylogenetic analysis was performed to study the relationship of saprophytic, endophytic and pathogenic strains of *Fusarium oxysporum* isolated from roots of *Acorus calamus* by Barik *et al.* using maximum parsimony method which were identified based on 18S rDNA sequence analysis[37].

Molecular characterization of *Bacillus* spp

PCR was performed to identify 9 isolates of *Bacillus* spp. isolated from the rhizosphere of *Gerbera* by using genus specific primers which were amplified at ~546 bp corresponding to 16S-23S rDNA intervening sequence region (Fig 3). The amplified genomic product was partially sequenced. The rDNA homology searches were performed by using BLAST, NCBI (USA) and resulted homology percentage from 98 to 100. The 16S rDNA gene sequence analysis is necessary mainly to detect some misidentification of *Bacillus* and related strains[38].

Phylogenetic analysis

Phylogenetic analysis of 9 isolates of

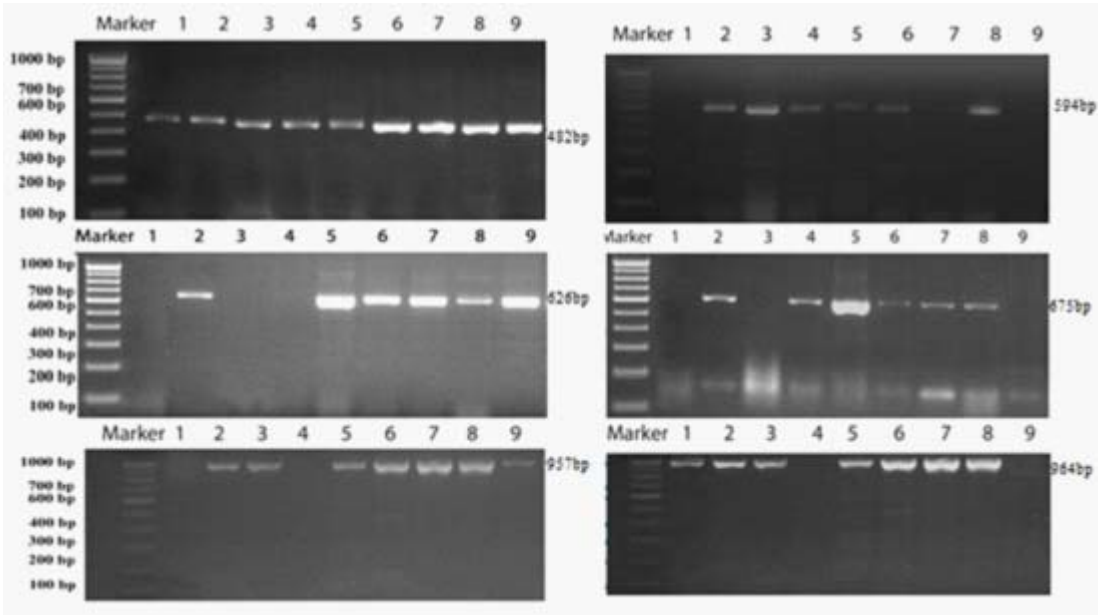

Fig. 5. PCR amplification of antibiotic biosynthetic genes (orderly *Itu D, Itu C, Srf A, Sf P, Bam C* and *Fen D)* of *Bacillus* spp.

(Lane1-*Bacillus subtilis* isolate PSB1; Lane2-*Bacillus amyloliquefaciens* PSB2; Lane3- *Bacillus methylotrophicus* PSB3; Lane4- *Bacillus subtilis* PSB4; Lane5- *Bacillus subtilis* PSB5; Lane6- *Bacillus amyloliquefaciens* PSB6; Lane7- *Bacillus subtilis* PSB7; Lane8- *Bacillus tequilensis* PSB8; Lane9- *Paenibacillus kribbensis* isolate PSB9)

Bacillus spp. based on 16S-rDNA sequences revealed the six different clusters formed in phylogenetic tree (Fig 4). The maximum sequence similarity was ranging from 79 to 99 percent was recorded among the various isolates of *Bacillus* spp.

The 16S rRNA gene has been widely used as a reliable molecular marker for phylogeny

Table 6. Effect of biocontrol agents on wilt incidence, growth characters and flower yield of *Gerbera* under protected cultivation

S. No.	Treatments**	Wilt incidence*	Root length (cm) *	Plant height (cm) *	No. of flowers /m^2 *
T1	Root dipping and soil drenching of *B. megaterium* BmTNAU1@5ml/lt	8.16^{e}(20.31)	19.35^{e}	40.26^{e}	46.20^{e}
T2	Root dipping and soil drenching of *B. subtilis* PSB5@5ml/lt	7.66^{d}(25.19)	20.68^{d}	41.30^{d}	47.30^{d}
T3	Root dipping and soil drenching of *Ochrobactrum* sp. BSD5@5ml/lt	7.52^{d}(26.56)	21.66^{c}	42.40^{c}	49.10^{c}
T4	Root dipping and soil drenching of PSB5+BSD5@5ml/lt	5.17^{b}(49.51)	22.26^{b}	44.80^{b}	50.50^{b}
T5	Root dipping and soil drenching of PSB5+BSD5+BmTNAU1 @5ml/lt	4.20^{a}(58.98)	23.60^{a}	45.46^{a}	51.50^{a}
T6	Root dipping and soil drenching of carbendazim @ 1g/ml	6.33^{c}(38.18)	18.75^{f}	39.46^{f}	46.40^{e}
T7	Uninoculated control	10.24^{f}	18.30^{f}	37.53^{g}	40.10^{f}

*Values are mean of three replications
Means followed by a common letter are not significantly different at 5% level by DMRT
Data in the parenthesis are per cent reduction over control

Table 7. Effect of biocontrol agents on flowering in *Gerbera* under protected cultivation (wilt management trial)

S. No.	Treatments	Days taken for flower bud initiation*	Days taken for flower bud opening*	Blooming Period (days)*	Length of flower stalk(cm)*	Flower diameter (cm)*
T1	Root dipping and soil drenching of *B. megaterium* BmTNAU1@5ml/lt	82.00^{c}	113.00^{e}	25.00^{e}	34.30^{e}	8.30^{e}
T2	Root dipping and soil drenching of *B. subtilis* PSB5@5ml/lt	81.60^{b}	111.00^{d}	27.00^{d}	35.80^{d}	8.70^{d}
T3	Root dipping and soil drenching of *Ochrobactrum* sp. BSD5@5ml/lt	81.00^{b}	109.00^{c}	29.00^{c}	37.50^{c}	8.90^{c}
T4	Root dipping and soil drenching of PSB5+BSD5@5ml/lt	80.66^{a}	100.00^{b}	33.66^{b}	38.20^{b}	9.30^{b}
T5	Root dipping and soil drenching of PSB5+BSD5+BmTNAU1 @5ml/lt	80.00^{a}	98.00^{a}	35.00^{a}	39.40^{a}	9.50^{a}
T6	Root dipping and soil drenching of Carbendazim @1g/ml	83.33^{d}	117.00^{f}	23.00^{e}	33.33^{f}	7.80^{f}
T7	Uninoculated control	89.00^{e}	119.00^{g}	21.33^{f}	30.00^{g}	7.20^{g}

*Values are mean of three replications
Means followed by a common letter are not significantly different at 5% level by DMRT

identification. The 16S rRNA gene contains conserved region as unique sequence which is relative among species or different species[39]. Phylogenetic analysis of 22 isolates of *Bacillus* spp. based on 16S rDNA sequences were analysed by Wahyudi *et al.* which formed three major clades of *B. cereus, B. subtilis* and *B. fusiformis*[40].

In vitro testing of antifungal activity of *Bacillus* spp. against FOG

25 *Bacillus* spp. isolates and one *Ochrobactrum* sp. were screened for their antagonistic activity against FOG2. Among these, *Ochrobactrum* sp. (BSD5) inhibited FOG2 to an extent of 61.11 per cent over control and was followed by *B. subtilis* isolate PSB5 and *B. megaterium* isolate BmTNAU1 with per cent inhibition of 56.66 and 55.55 respectively (Table-5).

The maximum inhibition (51.16 %) by *B. subtilis* B28 isolate was recorded against chickpea wilt pathogen *F. o.* f. sp. *ciceris*[41]. 12 *Bacillus* spp. strains, of which three BSC5, BSC6 and BScnTNAU2 showed highest antagonistic activity against *F. o.* f. sp. *dianthi*[36].

Molecular detection of antibiotic biosynthetic genes of *Bacillus* spp

Nine isolates of *Bacillus* spp. (PSB1, PSB2, PSB3, PSB4, PSB5, PSB6, PSB7, PSB8 and PSB9) were studied through PCR for the presence of 6 antibiotic genes viz. *ItuD*, *Itu C*, *SrfA*, *SfP* (4' Phosphopantetheinyl transferase gene), *BamC* and *FenD* which amplified at ~482bp, ~594bp, ~626bp, ~675bp, ~957bp and ~964bp respectively. Six isolates viz. PSB2, PSB3, PSB4, PSB5, PSB6 and PSB8 showed positive reaction for Iturin C (*Itu C*). All the nine *Bacillus* isolates showed positive reaction for Iturin D (*Itu D*). 7 isolates of *Bacillus* spp. (PSB1, PSB2, PSB3, PSB5, PSB6, PSB7 and PSB8) showed positive reaction for both Bacillomycin C (*Bam C*) and Fengycin D (*Fen D*). 6 isolates viz. PSB2, PSB4, PSB5, PSB6, PSB7 and PSB8 showed positive reaction for Surfactin A (*Srf A*). Six isolates of *Bacillus* spp., *viz.* PSB2, PSB5, PSB6, PSB7, PSB8 and PSB9 showed positive reaction for Surfactin P (*Sf P*). Most effective strain *B. subtilis* (PSB5) against FOG got amplified for all the antibiotic biosynthetic genes tested (Fig 5).

Lipopeptide antibiotic genes of bacillomycin and fengycin were detected using specific primers in case of *Bacillus* spp. antagonistic to the pathogens of wheat and canola[42]. Several strains of *Bacillus,* has AMP biosynthetic genes *bmyB*, *fenD*, *ituC*, *srfAA*, and *srfAB* responsible for the suppression of plant pathogens[43, 44]. The antimicrobial peptides (AMPs) such as fengycin, iturin, bacillomycin, and surfactin with broad antimicrobial activity and intense surfactant activities in *Bacillus* spp. have played a vital role in the biocontrol of several plant diseases[29, 45].

Effect of *Bacillus* spp. on the management of *Fusarium* wilt and Plant growth promotion in polyhouse

Among all the treatments applied, combination of *Bacillus* spp. isolates (BSD5+ PSB5+ BmTNAU1) delivery through root dipping and soil drenching @ 5ml/litre recorded the lowest wilt incidence of 4.20% with highest mean flower yield of 51.50 numbers/m^2 and also increased the root length and plant height. It was found to differ significantly from the application of other *Bacillus* spp. isolates (Table-6).

Similarly, variation of duration of flowering (Days of bud opening- Days of first flush shedding) was recorded of 30 to 35 days in the plants inoculated with bacterial consortia as against the untreated control, which indicated the induction of earliness in flowering with increased flower diameter indicating the growth promotion and disease reduction (Table 7).

Origin of antimicrobial peptides (AMPs) from *Bacillus* spp. plays a vital role in biocontrol of plant pathogens responsible for foliar, soil and postharvest diseases[4 6]and in plant growth promotion[47]. The plant endogenous pool of phytohormones increase the presence of rhizobacteria[48]. In the present investigation, efficacy of *Bacillus* isolates (singly or in consortia) was tested based on the presence of different antibiotic genes. The increase in plant growth parameters may be due to the survival of the inoculated bacteria in the rhizosphere, which responded to the host endogenous pool of phytohormones as compared to non bacterized seedlings.

CONCLUSION

The present study was undertaken to meet eco-friendly management of the wilt disease in *Gerbera* caused by FOG which is found to be

more prevalent in the Nilgiris and Salem districts of Tamil Nadu, India. The extensive study on pathogen, bacterial antagonists and identification of AMPs synthetic genes in the antagonists was done to design the field experiment, which resulted in the consortia of *Bacillus* spp. isolates (BSD5+ PSB5+ BmTNAU1) @ 5ml/lit as the effective management of the wilt disease in *Gerbera*.

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