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Research Article

Antifungal activity of *Bacillus subtilis* subsp. *spizizenii* (MM19) for the management of *Alternaria* leaf blight of marigold

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ABSTRACT: Biological control with bioagents is a cost effective alternate method for the management of crop diseases. The antagonistic bacterial strains were explored for the management of leaf blight of marigold which is caused by *Alternaria alternata*. The present study clearly proved that the mycelial growth of *A. alternata* was inhibited up to 83% by *Bacillus subtilis* subsp. *spizizenii* (MM19) *in vitro*. GC/MS analysis of partially purified extracts of *B. subtilis* subsp. *spizizenii* (MM19) revealed the presence of antifungal Phthalic acid esters which might be responsible for the inhibition of the pathogen. Foliar application of *B. subtilis* subsp. *spizizenii* (MM19) under field conditions suppressed leaf blight by 77%. This study highlighted the potential of *B. subtilis* subsp. *spizizenii* (MM19) for the management of *Alternaria* leaf blight.

KEY WORDS: *Alternaria*, *Bacillus subtilis* subsp. *spizizenii,* GCMS, PCR

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INTRODUCTION

Marigold is a seasonal flower and can be grown round the year. Marigold flowers gained popularity amongst gardeners and dealers on its easy cultivation and wide adaptability. Marigold as a cut flower and loose flower is extensively used in the social and religious function for internal decoration and garlands. The leaves and flowers are known to possess high phenolic and antioxidant properties can be exploited in pharmaceutical industry (Khalil *et al.*, 2007). Marigold is used as the trap crop in the borders to attract the insects attacking the main crop (Kolambkar *et al.,* 2013). Marigold is infected with fungal, viral and bacterial diseases. Among the diseases, inflorescence blight caused by *Alternaria zinnia* Ellis is the most common disease in marigold, the infection causes considerable damage to the ornamental crops (Karlatti and Hiremath, 1989). In 2014, Aktar and Shamsi reported the leaf, bud and flower blight of *Tagetes erecta* and *T. patula*. was caused by *A. alternata.* In 1966, Shome and Mustafee reported that the leaf spot and flower blight was a serious disease of marigold in northern Madhya Pradesh. The leaf spot in marigold initially appear as necrotic spot, latter it progresses as leaf blight and completely coalesce leads to drying of entire leaf. This disease emerged as major constraint in exploitation of high yielding marigold varieties in the country. Excessive use of fungicide leads to the resistance in pathogen and also it affects the human and environment directly or indirectly resulting in ecological imbalance (Waghmare *et al.,* 2011). Exploitation of biocontrol agents for the management of this disease is an alternative. Effective inhibition of mycelial growth of *A. solani* causing leaf blight of tomato by *Bacillus subtilis* has been reported (Babu *et al*., 2000; Mishra *et al.,* 2013). It was also found that *Bacillus* have strong antifungal activity both *in vitro* as well as *in vivo* conditions against *A. solani* isolated from tomato (Zhao *et al*., 2008). *Bacillus* species are capable of producing a wide variety of secondary metabolites that are diverse in structure and function. The production of antimicrobial metabolites determines their ability to control plant diseases (Silo-suh *et al*., 1994). Hence, the present investigation was carried out to find alternate methods for controlling the leaf blight of marigold by evaluating the bioefficacy of bacterial bioagents for the management of marigold leaf blight.

MATERIALS AND METHODS

Survey and collection of samples

Survey for the occurrence of leaf blight in marigold was conducted in Krishnagiri, Tharmapuri, Madurai and Sakthiyamangalam district of Tamil Nadu during 2015-2016. The fields were having marigold hybrids like US45,

Benz tall, Gold colour, Yellow. Plants showing characteristic blighting symptom were collected. The per cent disease index for each hybrids/variety was observed and they were classified using the disease score as described by Manojkumar *et al*., (2013).

$$\text{Per cent disease index} = \frac{\text{Sum of individual rating of infected leaves}}{\text{Number of leaves observed} \times \text{Maximum disease score}} \times 100$$

Isolation and identification of pathogen

Small segments of diseased tissue along with some healthy portion (5 × 5 mm^2) from the infected leaf samples were cut by sterile razor and surface sterilized in 0.5% sodium hypochlorite (NaOCl) for 1 minute. Surface sterilized plant tissue were rinsed twice by sterilized distilled water for removing the traces of Sodium hypochlorite, dried on filter paper and plated on Petri plates containing Potato Dextrose Agar medium (PDA) amended with 100µg/ml of streptomycin sulphate. The plates were incubated at room temperature for 48 to 72 hrs. Later the plates were observed for growth of fungus. The fungal pathogen was purified by single spore isolation method on plain agar medium (Tutte, 1969). Morphological characters of all the strains were studied on PDA as described by Simmons (2007).

Molecular characterization

The fungus was cultured on potato dextrose broth at room temperature for two weeks. Then the mycelium was collected, dried and powdered by freezing in liquid nitrogen. The genomic DNA was extracted by Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Chakraborty *et al*. (2010). The extracted genomic DNA was subjected to PCR with ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') primer pair (White *et al*., 1990). The PCR product was analyzed on 1.2 % agarose gel, stained with ethidium bromide and viewed under transilluminator. The amplified fragments were sequenced and confirmed using NCBI database.

Pathogenicity

Spore suspension was prepared with 10^5 conidia per ml. Tween 20 was added to the suspension at a final concentration of 0.05%. The spore suspension containing 10000 conidia/ml in phosphate buffer (pH 7) was sprayed over healthy plants using atomiser. Inoculated plants were covered in polythene bags and incubated at room temperature for one week. Simultaneously, a healthy control was also maintained. The experiment was replicated thrice with three plants per replication. The fungus was reisolated from the plants expressing the typical symptoms to confirm Koch's postulates.

In vitro* screening of antagonistic bacterial strains of *Bacillus* spp. against *Alternaria alternata

Antagonistic activity of twenty antagonistic bacterial strains of *Bacillus* spp (*B. cereus* -BSC5; *B. megaterium* -BmTNAU5; *B. subtilis* subsp. *spizizenii* -MM19; *B. amyloliquefaciens*- MM 12; *B. subtilis* - VB3 and *B. licheniformis* -B1TNAU2) against the pathogen *Alternaria alternata* was evaluated *in vitro* by standard protocol given by Matar *et al*. (2009) with slight modifications. Agar wells were created in four corners of the Petri plates at equal distance from the periphery using sterile cork borer. Later, 10 µl of 48 h old, bacterial suspension (OD Value @ 600 nm) was transferred into the wells. Then, mycelia disc (9 mm dia) was placed at the centre of the plate under sterile condition. Simultaneously, control plate without antagonists was also maintained. The cultures were incubated at $15^0 \pm 2^0$C for 3–5 days and diameter of the mycelial growth was measured. Per cent Inhibition (PI) was calculated using the formula

$$PI = \frac{C - T}{C} \times 100$$

Where,

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.

Extraction and detection of nonvolatile metabolites

The effective bacterial strain (*Bacillus subtilis* subsp. *spizizenii* -MM19) was cultured in Nutrient Broth (NB) and incubated at 28°C for 3 days. The culture filtrates along with the bacterial cells were centrifuged at 5000 rpm for 15 minutes at 4°C. The bacterial cells were discarded. Then supernatant was adjusted to acidic pH 2.0 with 1N HCl. Antifungal compounds in culture broth were extracted by adding equal volume of ethyl acetate and shaked for 2 h in an orbital shaker at 200 rpm. Culture broth was extracted twice with ethyl acetate for complete extraction. The solvent fraction with antifungal compounds were combined and concentrated by evaporation in the rotary flask evaporator maintained at 60°C at 80 rpm. The concentrated crude metabolites of the extracellular antifungal compounds obtained from the culture broth were dissolved in 1 ml HPLC grade methanol and used for GC/MS analysis (Dheepa *et al.,* 2016).

Evaluating the bio efficacy of *Bacillus* spp. against *Alternaria* Blight under open field conditions

Field experiment was conducted using Completely Randomized Block Design during 2018 at Paruvachi, Erode to assess the efficacy of *Bacillus* spp. for the management of leaf blight of marigold (variety-Benz tall). The plants were sprayed with respective bacterial suspensions (1% FS at 10^8CFU/ml) after disease initiation at weekly intervals for four weeks. An untreated control was also maintained. All the treatments were replicated thrice. Fifty plants in each treatment were selected at random and tagged to record observations on various traits and assessed for leaf blight incidence.

Treatment details	
T_1	Foliar spray with *Bacillus cereus* (BSC5) (1%) @ 10^8 CFU/ml
T_2	Foliar spray with *B. megaterium* (BmTNAU5) - (1%) @ 10^8 CFU/ml
T_3	Foliar spray with *B. subtilis* subsp. *spizizenii*(MM19) -(1%) @ 10^8 CFU/ml
T_4	Foliar spray with *B. amyloliquefaciens* (MM 12) - (1%) @ 10^8 CFU/ml
T_5	Foliar spray with *B. subtilis* (VB3) (1%) @ 10^8 CFU/ml
T_6	Foliar spray with *B. licheniformis* (B1TNAU2) -(1%) @ 10^8 CFU/ml
T_7	Foliar spray with *Bacillus sp.* (MM 16) - (1%) @ 10^8 CFU/ml
T_8	Foliar spray with *Pseudomonas flourescens* (Pf1) -(1%) at 10^8 CFU/ml
T_9	Control

Statistical analysis

Means differences of the treatment were evaluated using Duncan's Multiple Range-Test at 5% significance (Gomez and Gomez 1984). All the data were statistically analyzed with IRRISTAT (version. 3/93, Biometrics unit, International Rice Research Institute) and interpreted.

RESULTS AND DISCUSSION

The leaf blight pathogen was isolated from 15 different infected leaf samples of different varieties and cultured on PDA. The pathogen produced greyish black to black coloured colonies and was appressed, cottony and fluffy in nature. Mycelium was septate, branched, gray-brown to olivaceous with or without zonation. The morphological observation revealed that the leaf blight was caused by two different pathogens. Some strains produced obclavate or ovate or obpyriform to ellipsoidal conidia in chain, brown to golden brown with 3–8 transverse and 1–2 longitudinal septations representing *Alternaria* sp. and others produced dark colored 3–4 septate conidia with third cell is curved and larger than rest of the cells indicating *Curvularia* sp. Dhiman and Arora (1990) reported the occurrence of leaf spot and flower blight diseases of marigold in India and it was caused by *A. tagetica*. The pathogen associated was confirmed as *A. alternata* by Aktar and Shamsi (2014). The fungus produced black velvety colonies and muriform conidia. Nagrale *et al.* (2013) studied the morphological characters of *A. alternata.* They reported that the fungus initially produces hyaline mycelium that turned to grey-brownish, multicelled, septate and irregularly branched.

Molecular confirmation of 15 isolates corresponding to 18s rRNA gene fragment amplified the genomic product of 560 bp (Figure 1). The genomic products were sequenced and among the 15 isolates 8 isolates were found to be *Alternaria* spp. and the nucleotide sequences of the *Alternaria* strains had 94–99% similarity with the deposited strains at NCBI. The sequences were deposited in the NCBI database and were assigned with the accession numbers (Table 1). The Internal Transcribed Spacer (ITS) of rRNA gene were used for the molecular confirmation of *Alternaria* sp. using the universal primer pair ITS1 and ITS4 yielded an amplicon of 560 bp (Guo-yin *et al.,* 2013).

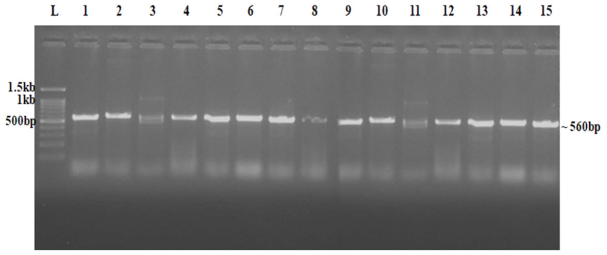

Fig. 1. PCR amplification of 18s-28sr RNA gene.
Lane L- 100bp Ladder; Lane 1- MG1; Lane 2- MG2; Lane 3- MG3; Lane 4- MG4; Lane 5- MG5; Lane 6- MG6; Lane 7- MG7; Lane 8- MG8; Lane 9- MG9; Lane 10- MG10; Lane 11- MG11; Lane 12- MG12; Lane 13- MG13; Lane 14- MG14; Lane 15- MG1**5**

Table 1. Identification of leaf blight pathogen of marigold by partial sequencing

S. No	Code of sequenced Isolate	Organism Identified	Identity of sequenced isolates (%)	Acession No.
1	MG1	*Alternaria* sp.	96	MH290559
2	MG2	*Curvularia* sp.	99	MH290510
3	MG3	*Alternaria alternata*	94	MH290511
4	MG4	*Alternaria alternata*	98	MH290561
5	MG5	*Alternaria alternata*	94	MH302507
6	MG6	*Alternaria* sp.	98	MH290564
7	MG7	*Setosperia rostrata*	99	MH290745
8	MG8	*Curvularia* sp.	99	MH290723
9	MG9	*Alternaria tenuissima*	90	-
10	MG10	*Alternaria tenuissima*	99	MH290766
11	MG11	*Alternaria porri*	99	MH291093
12	MG12	*Setosperia* sp.	99	MH302508
13	MG13	*Curvularia papendrofii*	98	MH292317
14	MG14	*Curvularia* sp.	99	MH292817

The leaves artificially inoculated with the conidial suspension of leaf blight pathogens *Alternaria* exhibited typical symptoms of leaf blight like small dark brown to black spots eight days after incubation. The pathogen was reisolated from the symptom expressed leaf and the morphological character was confirmed with the original isolates.

Among the twenty bacterial strains tested for its efficacy against *Alternaria alternata* through dual culture technique, fourteen strains recorded effective. The bacterial strain *Bacillus subtilis* subsp. *Spizizenii* (MM19) inhibited *A. aternata* to an extent of 83.99% over control and was followed by *B. amyloliquefaciens* strain MM12 (80.05%) and *B. subtilis* VB3 (73.23%). Among the twenty *Ochrobactrum intermedium* MM11 recorded the minimum inhibition of 54.56% (Table 2, Figure 2). Dragana *et al*., (2012) observed that *Bacillus* strain Q3 isolate caused a high percent of inhibition (61.75) on *A alternata* growth. Sid *et al*. (2003) has documented antagonistic activity of *B. subtilis* (HS93) against *A. alternata*. Hou *et al*. (2006) reported that *B. subtilis* strain LEV 006 was antagonistic to four major fungal pathogens of canola including *A. brassicae*.

Table 2. *In vitro* efficacy of bacterial biocontrol agents in the suppression of *Alternaria alternata*

S. No	Strains	Mycelial growth (Sq.cm)	Percent Inhibition over control
1	*Bacillus subtilis* subsp. *spizizenii* (MM19) - MG645182	10.16	83.99
2	*B. amyloliquefaciens* (MM12) - MG645177	12.66	80.05
3	*B. subtilis* (VB3) - KJ603238	17.00	73.23
4	*B. megaterium* (BmTNAU5) - KC540825	17.75	72.05
5	*B. cereus* (BSC5) - JX036520	18.42	71.00
6	*Ochrobactrum ciceri*(MM6) - MG645171	19.60	69.16
7	*B. licheniformis* (B1TNAU2) - KC540818	22.67	64.30
8	*O. ciceri*(MM3) - MG645169	24.33	61.68
9	*B. amyloliquefaciens* (VB2) - KJ603230	26.51	58.24
10	*B. wiedmanni* (MM15) - MG645178	27.75	56.30
11	*B. subtilis* (BS4) - JN873300	32.50	48.82
12	*B. amyloliquefaciens* (VB5) - KJ603232	42.75	32.68
13	*B. subtilis* (VB9) - KJ603236	27.50	56.69
14	*O. intermedium* (MM11) - MG645175	28.91	54.46
15	*O. pseudogrignonse* (MM10) - MG645176	63.5	0.0
16	*Brevundimonas naejangsanensis* (MM7) - MG645173	63.5	0.0
17	*O. intermedium* (MM4) - MG645170	63.5	0.0

18	*O. ciceri* (MM1) - MG601224	63.5	0.0
19	*O. daejeonense* (MM8) - MG645172	63.5	0.0
20	*O. intermedium* (MM13) - MG645186	63.5	0.0
21	Control	63.5	0.0
CD (0.05)		6.04	8.29
SED		2.99	4.11

Data is presented as Sq. cm (mycelial growth) and percentage (Inhibition in mycelial growth over control). Error bars indicate standard error obtained from three replicates

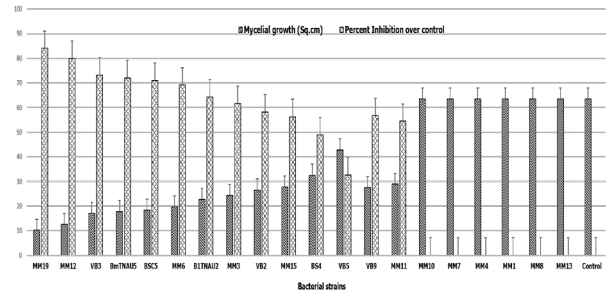

Fig. 2. Antifungal efficacy of bacterial strains against the mycelial growth of *Alternaria alternata in vitro*.

per treatment.

The nonvolatile compounds produced by the antagonistic bacteria *Bacillus subtilis* subsp. *spizizenii* (MM19) were Phthalic acid, isobutyl 2-pentyl ester; Dibutyl phthalate and Phthalic acid, butyl 2-pentyl ester (Figure 3, Table 3). Phthalic acid esters are reported to possess antifungal activity (Srinivasan *et al.,* 2009). Khatiwora *et al.* (2012) proved the antibacterial nature of dibutyl phthalate against *Klebseilla pneumonia, Proteus mirabilis, Pseudomonas aureginosa* and *P. aeruginosa.* The compounds Phenol, 2,4-bis(1,1-dimethylethyl)- and 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester possess antimicrobial nature (Santhi *et al.,* 2013). Similarly, nonvolatile metabolites like octadecenoic acid, hexadecenoic acid, heptadecenoic acid, pyrrolo, chloroxylenol and pentadecenoic acid produced by *B. amyloliquefaciens* VB7 effectively suppressed stem rot of carnation incited by *Sclerotinia sclerotiorum* under protected conditions (Vinodkumar *et al.,* 2017). Besides, Dheepa *et al.* (2016) demonstrated the antifungal nature of nonvolatile compounds like benzene, 1-Dodecene, Benzaldehyde, à-Dodecene, Nonadecene, Octadecanoic acid and n-Tetradec-1-ene produced by *B. subtilis* (BS2) against *Puccinia horiana* in chrysanthemum.

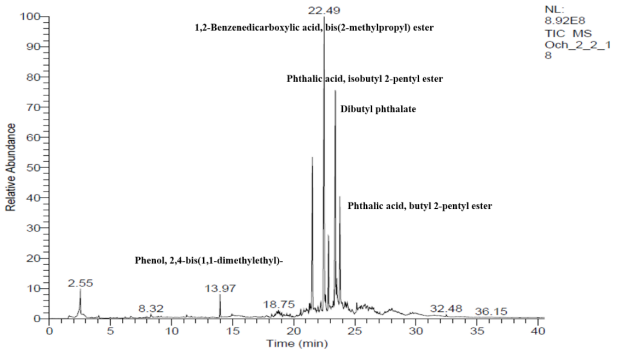

Fig. 3. Total ions chromatogram of Non volatiles compounds produced by *Bacillus subtilis* subsp. *spizizenii* (MM19).

GC/MS analysis was performed at Dept. of Nanotechnology, TNAU, Coimbatore.

Table 3. Nonvolatiles compound profile of *Bacillus subtilis* subsp. *spizizenii* (MM19)

S. No	RT	Name of the compound	Realative Abundance	Function	Reference
1	13.97	Phenol, 2,4-bis(1,1-dimethyl-ethyl)-	10	Antimicrobial Anti inflammatory Antioxidant	Santhi *et al.*, 2013
2	22.47	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	95	Antimicrobial	Santhi *et al.,* 2013
3	22.85	Phthalic acid, isobutyl 2-pentyl ester	25	Antibacterial Antifungal	Srinivasan *et al.,* 2009

4	23.39	Dibutyl phthalate	70	Antibacterial	Khatiwora *et al.,* 2012
5	23.78	Phthalic acid, butyl 2-pentyl ester	35	Antibacterial Antifungal	Srinivasan *et al.,* 2009

The effective six different antagonistic bacterial strains under *in vitro* were assessed for the management of leaf blight of marigold under field conditions. The values represent the mean of two season trails conducted during 2017 and 2018. Results indicated that, foliar spray of *Bacillus subtilis* subsp. *spizizenii* (MM19) - 1% (10ml/L at 10^8 CFU/ml) had the minimum leaf blight incidence of 8.15 PDI. It differed significantly from *B. amyloliquefaciens* (MM12) treated plants which had 11.48 PDI, followed by foliar spray with *B. megaterium* (BmTNAU5) -(1%) @ 108 CFU/ml with an incidence of 14.82. The maximum incidence of 34.14 was observed in the plants treated with *Bacillus* sp. (MM16). However, the mean incidence of 56.05 PDI of leaf blight was noticed in the untreated control (Figure 4, Table 4). Liuchienhui *et al.,* (1997) reported that *B. megaterium* MBS4, *B. breveis* and *B. subtilis* var. *globigii* CBS10 were antagonistic to *A. solani.* Wu *et al.* (2007) reported the efficacy of *B. amyloliquefaciens* against *A. cosmosa* and *A. patula* seed borne pathogen of *Cosmos sulfurous* and *T patula*. Sundaramoorthy and Balabaskar (2014) reported the antagonistic efficacy of *P. fluorescens* strains (Pf1, Py15 and Fp7) and *B. Subtilis* strains (EPCO 16 and EPC5) against *A. solani*. Mandhare and Suryawanshi (2003) reported that *Bacillus thermophillus* was found to be eaffective against *A. porri* (purple blotch of onion) and *A. alternata* (leaf spot of pomegranate).

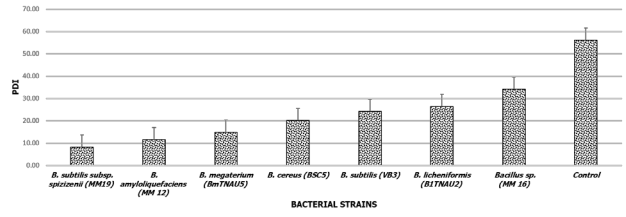

Fig. 4. Efficacy of bacterial strains on percentage leaf blight index of marigold.

The data represents mean value for two season trails conducted (2017 and 2018). Error bars indicate standard error obtained from three replicates per treatment.

Table 4. Efficacy of bacterial strains on percentage leaf blight index of marigold

Treatments	PDI*
Foliar spray with *Bacillus subtilis* subsp. *spizizenii* (MM19) -(1%) @ 10^8 CFU/ml	8.15
Foliar spray with *B. megaterium* (BmTNAU5) - (1%) @ 10^8 CFU/ml	11.48
Foliar spray with *B. amyloliquefaciens* (MM 12) - (1%) @ 10^8 CFU/ml	14.82
Foliar spray with *B. cereus* (BSC5) (1%) @ 10^8 CFU/ml	20.10
Foliar spray with *B. subtilis* (VB3) (1%) @ 10^8 CFU/ml	24.21
Foliar spray with *B. licheniformis* (B1TNAU2) - (1%) @ 10^8 CFU/ml	26.45
Foliar spray with *Bacillus sp.* (MM 16) - (1%) @ 10^8 CFU/ml	34.14
Foliar spray with *Pseudomonas flouresens* (Pf1) -(1%) at 10^8 CFU/ml	39.53
Control	56.05
CD (0.05)	2.37
SED	1.11

*Values are the pooled mean data for 2017and 2018 ($P \leq 0.001$)

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