



Management of Fusarium wilt of watermelon (*Citrullus lanatus*) using *Bacillus subtilis* and *Bacillus cereus*

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ABSTRACT

Fusarium wilt of watermelon [*Citrullus lanatus* (Thunb.) Matsumura & Nakai] is caused by *Fusarium oxysporum* f. sp. *niveum* (FON), is the most severe soil-borne disease under the prevailing conditions of Punjab. Present study was carried out during 2018–19 and 2019–20 at the pot house and research farm of Punjab Agricultural University, Ludhiana, Punjab to evaluate the antagonistic activity of 15 isolates of *Bacillus* spp. against FON. These isolates were screened by using dual culture assay and among them, *Bacillus subtilis* (B₁) and *Bacillus cereus* (B₃) showed maximum inhibition percentage i.e. 57.00 and 58.22, respectively. Similarly, these isolates (B₁ and B₃) showed maximum activity of chitinase (2.31 and 2.16 unit/ml) and glucanase (1.72 and 1.79 unit/ml). Further, Talc-based bioformulations of B₁ and B₃ isolates were evaluated in pot house and research farm of Punjab Agricultural University, Ludhiana, Punjab. Amongst all the treatments, *Bacillus subtilis* (B₁) and *Bacillus cereus* (B₃) as seed + soil @15 g treatment showed maximum inhibition of FON. Therefore, based on our findings, *Bacillus subtilis* (B₁) and *Bacillus cereus* (B₃) used as seed + soil treatment was able to effectively manage the fusarium wilt in watermelon under pothouse and field conditions.

Keywords: *Bacillus* spp., *Fusarium oxysporum* f. sp. *niveum*, Watermelon, Wilt

Watermelon [*Citrullus lanatus* (Thunb.) Matsumura & Nakai] is an annual trailing creeper belongs to the family Cucurbitaceae. In India, major watermelon growing states are Rajasthan, Punjab and Uttar Pradesh (Dhaliwal 2012). The major limiting factors responsible for its low productivity are fungal, bacterial and viral diseases. Among all the diseases, Fusarium wilt caused by *Fusarium oxysporum* f. sp. *niveum* (FON) is considered as the most devastating disease. In this disease, pathogen attacks all the stages of crop. If watermelon is grown within the same affected field over successive seasons, FON inoculum in soil increases with time, resulting in yield losses of up to 100% (Everts and Himmelstein 2015).

Managing plant diseases with chemical pesticides or fungicides poses a potential threat to the environment. Use of resistant varieties was an effective approach against the pathogens but the new virulent races of *Fusarium* appear within a short period of time (Ling *et al.* 2010). Biocontrol through the application of various antagonistic microbes of plant pathogens can be considered as safest and favourable option (Shafi *et al.* 2017). Effective biocontrol of

Fusarium wilt has been described using *Trichoderma* spp., *Pseudomonas* spp., *Bacillus* spp. or non-pathogenic *F. oxysporum* (Cao *et al.* 2012). Among the bacterial biocontrol agents, *Bacillus* and *Pseudomonas* are widely used for disease management. Disease suppression ability has been reported in numbers of *Bacillus* spp. strains, viz. *B. subtilis*, *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. mycoides* and *B. pumilus* (Heidarzadeh *et al.* 2015, Singh *et al.* 2017). *Bacillus* is one of the safest biocontrol agent which can synthesize a vast array of beneficial substances that promote plant growth and enhance the resistance of plants against various pathogens (Yang *et al.* 2009). The present study aims to isolate *Bacillus* spp. from the rhizospheric soil of watermelon growing regions of Punjab and to evaluate bioefficacy of *Bacillus subtilis* and *Bacillus cereus* against Fusarium wilt of watermelon.

MATERIALS AND METHODS

The present study was carried out during 2018–19 and 2019–20 at the pot house and research farm of Punjab Agricultural University, Ludhiana, Punjab to confirm the bio-efficacy of *Bacillus* isolates.

Collection, isolation and identification of pathogenic microflora: *F. oxysporum* f. sp. *niveum* (FON) used in our study was obtained from the infected plants of watermelon

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which were collected from the different districts of Punjab during survey. The culture of FON was isolated and maintained on potato dextrose agar at $25 \pm 1^\circ\text{C}$ which was sub-cultured at regular interval of 15 days. Their virulence was tested by growing watermelon in the pot house in soil mixed with the cultures of *F. oxysporum* f. sp. *niveum* isolates. Most virulent isolate was selected for further investigation.

Collection and isolation of *Bacillus* spp.: 15 isolates of *Bacillus* spp. were isolated from the rhizospheric soils of watermelon growing districts of Punjab. Isolation of *Bacillus* was done on Mannitol yolk polymixin agar medium as described by Walker *et al.* (1998). The characterization of the *Bacillus* isolates was done (Sneath 1986) by using a standard protocol as described in Bergey's Manual of Systematic Bacteriology. The pure cultures were maintained on Nutrient Agar (NA) media slants at 4°C and were used whenever required.

Confrontation assay: Isolates of *Bacillus* spp. were evaluated against FON by a dual-culture assay using PDA media as described by Skidmore and Dickinson (1976). The inhibition zone in cm was measured after 7 days. Petri plates inoculated with fungal pathogen discs alone served as control.

Molecular identification of *Bacillus* spp.: Most potent isolates of *Bacillus* spp. were subjected to 16S rDNA gene sequencing. The cultures of the selected isolates (B_1 and B_3) were sent to Eurofins Genomics India (EGI) Pvt. Ltd. for sequencing. The final sequences obtained were searched in BLASTn tool to collate the results acquired with the sequences formerly submitted at NCBI GenBank database and accession numbers were obtained.

Estimation of chitinase enzyme activity of *Bacillus* spp. isolates

Qualitative estimation of chitinase: *Bacillus* isolates ability to degrade chitin was demonstrated using the spot bioassay method. In petri plates, four spots of inoculum were placed over the NA medium containing 1% colloidal chitin (w/v) and incubated at 28°C . The observation of a clearing zone functioned as an indicator of chitinolytic activity.

Quantitative estimation of chitinase: The chitinase production in *Bacillus* isolates was assessed in Nutrient Broth (NB) chitin medium by the method described by Lim *et al.* (1991). The reducing sugar formed was estimated by using a spectrophotometer at 575 nm (Miller 1959). One unit of chitinase activity is defined as the amount of enzyme producing $1 \mu\text{mol}$ of reducing sugars per minute.

Quantitative estimation of β -1,3-glucanase: β -1,3-glucanase activity was assessed as per the method described Rais *et al.* (2017) by using nutrient agar (NA) medium containing laminarin (0.2%). The reducing sugar formed was estimated by using a spectrophotometer at 550 nm (Miller 1959). The enzyme activity was expressed as a unit of glucose per ml of culture filtrate.

Efficacy of bioformulation of *Bacillus subtilis* (B_1) and *Bacillus cereus* (B_3) against *Fusarium* wilt of watermelon

under pot house and field conditions: The experiments were carried out in randomized block design (RBD) with 16 treatments and three replications. Talc based bioformulation was prepared with 600 ml of inoculated nutrient broth per kg of talc powder. The *Bacillus* bioformulation was applied on "sugar baby" variety of watermelon as seed treatment (15 gm/kg of seed for 4 h), soil treatment (1 kg of talc based bioformulation in 25 kg of FYM/acre primed for 72 h in shade) and seed + soil treatment. For comparison, standard chemical check as seed treatment with carbendazim 50% WP @1.5 g/kg seed and control treatment were also maintained. Seed germination percentage (%), disease incidence percentage (%), disease inhibition percentage (%) were recorded.

Statistical analysis: All data were replicated in triplicates and analysed by one-way ANOVA (analysis of variance), through the statistical software CPCS1 (Completely Randomized Design-Arcsine Transformation) and were determined by the significant magnitude of *f* value ($P \leq 0.05$).

RESULTS AND DISCUSSION

In vitro screening of *Bacillus* isolates against *Fusarium* wilt: 15 isolates of *Bacillus* were screened against FON and percentage inhibition was observed in the range of 40.44–58.22% (Table 1 and Fig. 1). Among these, *Bacillus* (B_3) isolate showed a maximum inhibition of 58.22%, with an inhibition zone of 2.26 cm, followed by B_1 isolate (57.00% inhibition with 2.33 cm zone of inhibition). The minimum inhibition was observed in the B_{12} (38.88%). Our results were in corroboration with study of Zhu *et al.* (2019) who also found that *Bacillus subtilis* strain IBFCBF-4 was highly antagonistic against FON with an inhibition of 66%. Similar results were also reported by Francisco *et al.* (2016) and Choudhary *et al.* (2019).

Screening of *Bacillus* isolates by chitinase and glucanase assay

Qualitative assay of chitinase: 15 isolates of *Bacillus* spp. were subjected to the chitin agar plates. All *Bacillus* isolates exhibited clear halo zone ranging between 6.00–11.33 mm (Table 1). The maximum clear zone was

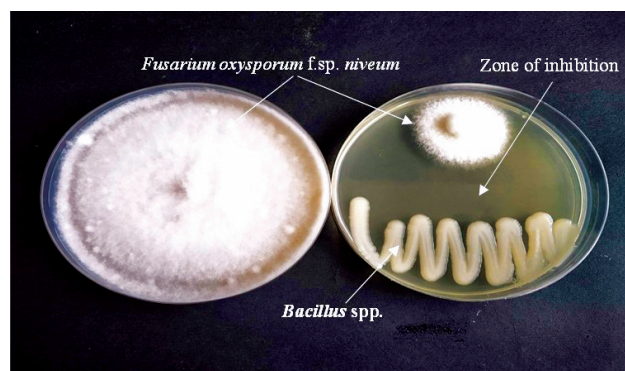


Fig. 1 Dual culture of *Bacillus* spp. against *Fusarium oxysporum* f.sp. *niveum* on PDA (right) and a control plate inoculated with *Fusarium oxysporum* f.sp. *niveum* alone (left).

Table 1 Inhibitory effect (dual culture), qualitative and quantitative estimation of chitinase activity, glucanase activity of *Bacillus* isolates against *F. oxysporum* f.sp. *niveum*

<i>Bacillus</i> isolates	Confrontation assay (% inhibition)			Quantitative clear zone (mm)* (Chitinase)	Quantitative	
	Growth of <i>F. oxysporum</i> f.sp. <i>niveum</i> (cm)*	Inhibition (%)	Zone of Inhibition (cm)*		Chitinase activity (unit/ml)*	β -1,3-glucanase activity (unit/ml) *
B ₁	3.87	57.00	2.33	11.33	2.31	1.82
B ₂	3.90	56.66	2.10	8.67	1.94	1.66
B ₃	3.76	58.22	2.26	10.67	2.16	1.79
B ₄	4.20	53.33	1.86	9.67	2.13	1.75
B ₅	4.30	52.22	2.07	9.00	1.96	1.57
B ₆	4.60	48.88	2.02	7.00	1.85	1.47
B ₇	4.76	47.11	1.86	6.00	1.73	1.38
B ₈	4.03	55.22	1.63	10.00	2.03	1.72
B ₉	4.20	53.33	2.16	7.33	1.90	1.64
B ₁₀	5.36	40.44	2.06	8.00	1.93	1.65
B ₁₁	5.20	42.22	1.96	8.67	1.99	1.73
B ₁₂	5.50	38.88	1.56	9.00	1.97	1.72
B ₁₃	5.36	40.44	1.90	10.33	2.06	1.77
B ₁₄	4.40	51.11	2.13	6.33	1.27	1.03
B ₁₅	5.10	43.33	2.00	7.67	1.93	1.69
Control	9.00	-	-	-	-	-
CD (<i>P</i> =0.05)	0.16	-	0.13	1.36	0.11	0.59

B₁–B₁₅, *Bacillus* isolates; *Mean of three replications.

found in B₁ (11.33 mm) followed by B₃ (10.67 mm).

Quantitative assay of chitinase and β -1,3-glucanase: The data in Table 1 illustrates the efficacy of *Bacillus* isolates for the production of chitinase and β -1,3-glucanase. The enzyme activity varied from 1.27 to 2.31 units/ml for chitinase and 1.03 to 1.82 units/ml for β -1,3-glucanase. The maximum chitinase and β -1,3-glucanase enzyme activities were expressed by the isolate B₁ with 2.31 units/ml and 1.82 units/ml, respectively, followed by B₃ (2.16 units/ml and 1.79 units/ml). Karunya *et al.* (2011) also found that chitinase activity of *B. subtilis* was indicated by the production of a clear zone of 7 mm diameter along with enzymatic activity (0.4 units/ml). Rais *et al.* (2017) demonstrated that *Bacillus* spp. decrease the infection of *Pyricularia oryzae* by production of β -1,3-glucanase (1.0–1.3 units/ml). Similarly, Suma *et al.* (2023) reported *P. fluorescens* (Pf 10) producing β -1,3- glucanase (1.52 units/ml) against by *Rhizoctonia solani*.

Molecular identification of *Bacillus* spp.: Based on 16S rDNA gene sequencing, isolate B₁ was identified as *Bacillus subtilis* (Accession numbers = ON564616.1) and isolate B₃ was identified as *Bacillus cereus* (Accession numbers = ON597604.1). Our findings were parallel with the observations of Dash *et al.* (2015) and Kim *et al.* (2012). They also used the universal primers of amplification of the 16S rDNA gene in *Bacillus* strains for their identification.

Bio-efficacy of *Bacillus* (B₁ and B₃) isolates against *Fusarium* wilt in watermelon under pot house conditions: Based on the findings of *in vitro* study, isolates B₁ and

B₃ were found to be most effective against FON. These isolates were tested over a period of two years (2018–19 and 2019–20) against watermelon wilt under pot house conditions. The maximum seed germination was observed in seed + soil @15 g/kg treatment of B₁ (90.00 and 93.33%) and B₃ (93.33 and 93.33%) which was statistically at par with the chemical treatment (90.00 and 93.33%). Minimum wilt incidence was observed in B₁, seed + soil @15 g/kg treatment (10.00 and 6.67%) and B₃, seed + soil @15 g/kg treatment (6.67 and 3.33%) and this was significantly at par with chemical treatment (3.33%) (Table 2). Our finding is corroborated with the results of Al-Mutar *et al.* (2023). They evaluated six isolates of *Bacillus* spp. and among them, *B. amyloliquefaciens* DHA55 was found to be most effective, achieving the highest *Fusarium* wilt suppression (74.9%) in watermelon under greenhouse conditions. Similarly, Valendia *et al.* (2019) found that *Bacillus* spp. significantly reduced the *Fusarium* wilt and enhanced the plant growth in cape gooseberry.

Bio-efficacy of *Bacillus* (B₁ and B₃) isolates against *Fusarium* wilt in watermelon under field conditions: Talc based bioformulations of B₁ and B₃ isolates were also evaluated under field conditions for two years (2018–19 and 2019–20) (Table 3). The maximum seed germination (95.83%) was recorded in both B₁ and B₃ seed + soil @15 g/kg treatments which was statistically at par with the chemical treatment (91.66%). The minimum wilt disease incidence was observed in seed + soil @15 g/kg treatment of B₁ (12.50 and 8.33%) and B₃ (8.33 and 8.33%) as compared to

Table 2 Effect of *Bacillus subtilis* (B₁) and *Bacillus cereus* (B₃) on germination and disease incidence of watermelon under pot house conditions

Treatment	2018–19			2019–20		
	Germination* (%)	Disease incidence* (%)	Decrease in wilt (%)	Germination* (%)	Disease incidence* (%)	Decrease in wilt (%)
B ₁ , Seed (5 g)	66.66(54.76)	33.33 (35.20)	37.50	73.33 (58.98)	30.00 (33.19)	30.76
B ₁ , Seed (10 g)	70.00(56.72)	30.00 (33.19)	43.74	76.66 (61.19)	26.66 (30.98)	38.47
B ₁ , Seed (15 g)	73.33(58.98)	26.66 (30.98)	50.00	80.00 (63.40)	23.33 (28.76)	46.15
B ₁ , Soil (15 g)	76.66(61.19)	23.33 (28.76)	56.25	80.00 (63.40)	20.00 (26.55)	53.84
B ₁ , Seed + soil (5 g)	80.00(63.04)	23.33 (28.76)	56.25	83.33 (66.11)	16.66 (23.84)	61.15
B ₁ , Seed + soil (10 g)	83.33(66.11)	20.00 (26.55)	62.49	83.33 (66.11)	16.66 (23.84)	61.15
B ₁ , Seed + soil (15 g)	90.00(71.53)	10.00 (18.42)	81.24	93.33 (77.67)	6.66 (12.28)	84.62
B ₃ , Seed (5 g)	73.33(58.98)	33.33 (35.20)	37.50	70.00 (56.76)	26.66 (30.98)	38.47
B ₃ , Seed (10 g)	73.33(58.98)	26.66 (30.98)	50.00	76.66 (61.19)	23.33 (28.76)	46.15
B ₃ , Seed (15 g)	76.00(61.19)	23.33 (28.76)	56.25	76.66 (61.19)	20.00 (26.55)	53.84
B ₃ , Soil (15 g)	80.00(63.04)	20.00 (26.55)	62.49	80.00 (63.40)	20.00 (26.55)	53.84
B ₃ , Seed + soil (5 g)	80.00(63.04)	16.66 (23.84)	68.76	83.33 (66.11)	13.33 (21.13)	69.23
B ₃ , Seed + soil (10 g)	83.33(66.11)	16.66 (23.84)	68.76	83.33 (66.11)	13.33 (21.13)	69.23
B ₃ , Seed + soil (15 g)	93.33(77.67)	6.66 (12.28)	87.51	93.33 (77.67)	3.33 (6.14)	92.31
Control	53.33(46.90)	53.33 (46.09)	-	50 (44.98)	43.33 (41.13)	-
Chemical (Carbendazim 50% WP)	90.0 (71.53)	3.33 (6.14)	93.75	93.33 (77.67)	3.33 (6.14)	92.31
CD (<i>P</i> =0.05)	5.98	8.36	-	9.22	9.59	-

B₁, *Bacillus subtilis*; B₃, *Bacillus cereus*; *Mean of three replications; *Figures in parentheses are arc sine transformed values.

Table 3 Effect of *Bacillus subtilis* (B₁) and *Bacillus cereus* (B₃) on germination and disease incidence of watermelon under field conditions

Treatment	2018–19			2019–20		
	Germination* (%)	Disease incidence* (%)	Decrease in wilt (%)	Germination* (%)	Disease incidence* (%)	Decrease in wilt (%)
B ₁ , Seed (5 g)	70.83 (57.38)	37.50 (37.40)	40.80	75.00 (59.97)	33.33 (35.16)	50.00
B ₁ , Seed (10 g)	75.00 (59.97)	33.33 (35.16)	46.67	79.16 (63.07)	29.16 (32.57)	56.25
B ₁ , Seed (15 g)	79.16 (63.07)	33.33 (35.16)	46.67	79.16 (63.07)	25.00 (29.98)	62.49
B ₁ , Soil (15 g)	79.16 (63.07)	29.16 (32.57)	53.34	79.16 (63.07)	25.00 (29.98)	62.49
B ₁ , Seed + soil (5 g)	83.33 (66.17)	25.00 (29.98)	60.00	83.33 (66.17)	20.83 (26.89)	68.75
B ₁ , Seed + soil (10 g)	83.33 (66.17)	25.00 (29.98)	60.00	83.33 (66.17)	20.83 (26.89)	68.75
B ₁ , Seed + soil (15 g)	95.83 (83.06)	12.50 (20.69)	80.00	95.83 (83.06)	8.33 (13.79)	87.50
B ₃ , Seed (5 g)	70.83 (57.38)	33.33 (35.16)	46.67	75.00 (60.48)	37.50 (37.57)	43.74
B ₃ , Seed (10 g)	75.00 (59.97)	29.16 (32.57)	53.34	79.16 (63.07)	29.16 (32.57)	56.25
B ₃ , Seed (15 g)	75.00 (59.97)	25.00 (29.98)	60.00	79.16 (63.07)	25.00 (29.98)	62.49
B ₃ , Soil (15 g)	79.16 (63.07)	25.00 (29.98)	60.00	79.16 (63.07)	25.00 (29.98)	62.49
B ₃ , Seed + soil (5 g)	83.33 (66.17)	16.66 (23.79)	73.34	83.33 (66.17)	20.83 (26.89)	68.75
B ₃ , Seed + soil (10 g)	83.33 (66.17)	16.66 (23.79)	73.34	83.33 (66.17)	20.83 (26.89)	68.75
B ₃ , Seed + soil (15 g)	95.83 (83.06)	8.33 (13.79)	86.67	95.83 (83.06)	8.33 (13.79)	87.50
Control	62.50 (69.26)	62.50 (52.39)	-	54.16 (47.39)	66.66 (54.97)	-
Chemical (Carbendazim 50% WP)	91.66 (76.16)	8.33(13.79)	86.67	91.66 (76.16)	8.33 (13.79)	87.50
CD (<i>P</i> =0.05)	6.49	8.64	-	12.10	11.52	-

B₁, *Bacillus subtilis*; B₃, *Bacillus cereus*; *Mean of three replications; *Figures in parentheses are arc sine transformed values.

control (62.50 and 66.66%). Our finding was corroborated with the results of Qiao *et al.* (2017). They noted biocontrol efficacy of *Bacillus subtilis* against *Fusarium oxysporum* and observed the enhanced growth in tomato. Similarly, Saman *et al.* (2019) evaluated the antifungal activity of two potent isolates of *Bacillus simplex* (PHYB1 and PHYB9) and concluded that they not only improved production but significantly reduced wilt of cumint.

Based on the above findings, it can be concluded that the *Bacillus subtilis* (B₁) and *Bacillus cereus* (B₃) as seed + soil @15 g treatment effectively suppressed *Fusarium oxysporum* f. sp. *niveum* in watermelon crop. Thus, the isolates B₁ and B₃ can be used as commercial bioagents for eco-friendly management of Fusarium wilt of watermelon as a substitute for chemical fungicides.

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