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Management of cucumber wilt using the potential native biocontrol agents

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ABSTRACT

Cucumber wilt is a serious disease in cucumber (*Cucumis sativus* L.) cultivation which is caused by *Fusarium oxysporum* f. sp. *cucumerinum* (FOC). The field experiments were conducted during 2020–21 and 2021–22 at Punjab Agricultural University, Ludhiana, Punjab to evaluate the native potent isolates of *Pseudomonas* and *Trichoderma* against FOC. Among all the *Pseudomonas fluorescens,* Pf 6 showed maximum inhibition in dual culture (13.30 mm) assays against FOC. Similarly, in biochemical analysis*,* Pf 6 showed the highest chitinase activity and siderophore production, i.e. 11.56 unit/ml and 32.17 mm, respectively. Likewise, *Trichoderma asperellum* Thd emerged as the most effective BCA out of various *Trichoderma* isolates in the *in vitro.* The bio-efficacy bio formulations of *P. fluorescens* (Pf 6) and *T. asperellum* (Th d) were evaluated during 2020–2021 and 2021–2022. Among all the treatments, Pf 6 as seed + soil treatment showed minimum disease incidence (10.0% and 13.33%) along with enhanced plant growth and yield. Therefore, based on our findings, *P. fluorescens* (Pf 6) is an effective biocontrol agent and proved as an alternative approach for the effective management wilt of cucumber.

Keywords: Cucumber wilt, *Fusarium oxysporum* f.sp. *cucumerinum*, *Pseudomonas fluorescens*, *Trichoderma asperellum*

The cucumber (*Cucumis sativus* L.) is an important crop that belongs to the family Cucurbitaceae (Singh *et al.* 2016). Cucumber crop is attacked by various diseases such as downy and powdery mildew, Fusarium wilt, anthracnose, grey and white mold. Among all the diseases, Fusarium wilt caused by *Fusarium oxysporum* f.sp. *cucumerinum* (FOC) in cucumber which is considered as the most devastating disease. FOC has soil-borne nature so it invades the plant during any development stage of the crop plant and further it colonizes the vascular system of the plant thus leads to significant yield losses (Al-Tuwaijri 2015). Necrotic lesions, foliar yellowing and wilting followed by vascular tissue damage which leads to death of plant, are the visible symptoms of FOC infestation (Ahmed 2010). Till date, there is no effective control measure for FOC. There are couple of fungicides which are available in the market as an effective way against this pathogen but the concern for the environment and some constraints have limited their use while in crop production (Vethavalli and Sudha 2012). The use of resistant varieties of cucumber is an effective method but the new emerging highly virulent races of *Fusarium* appears within very short time (Ling *et al.* 2010). Soil fumigation with use of methyl bromide was promising method against wilt (Zhang *et al.* 2013). But, because of methyl bromide's detrimental effects on the stratospheric ozone layer, it has been banned during the end of 2004. In view of the new sustainable agriculture along with the organic farming, a need is there to search the effective biological control for managing the disease (Choudhary *et al.* 2021). Promising biocontrol of FOC has been described by using antagonistic *Trichoderma* spp., *Pseudomonas* spp. and *Bacillus* spp. (Cao *et al.* 2012, Geetika *et al.* 2024). According to Sallam *et al* (2019) biocontrol agents manage the various pathogens (soil-borne) by using different mechanisms like competition for nutrients, the release of various lytic enzymes (glucanase and cellulase) and defense response which ultimately increases the crop yield. Hence, the aim of this study was to evaluate the ability of the different isolates of *Pseudomonas* and *Trichoderma* against cucumber wilt.

MATERIALS AND METHODS

The field experiments were conducted during 2020–21 and 2021–22 at Punjab Agricultural University, Ludhiana, Punjab to evaluate the bio-efficacy of potent *Pseudomonas fluorescens* (Pf 6) and *Trichoderma asperellum* (Th d).

Collection and isolation of pathogenic microflora: FOC was isolated from the root samples of infected cucumber, which were collected from cucumber-grown regions of Punjab, i.e. Ludhiana [Malkpur Bet (30.9281°N, 75.7393°E)] and Bathinda district (Rampura phul

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(30.2701°N, 75.2398°E). The mycelial culture of FOC was isolated and then, for future use, it was maintained on PDA media at the temperature of 25 ± 1°C. The virulence of FOC was evaluated on cucumber plants grown under pot-house conditions. The experiment was conducted using a sterilized soil mixture (consisting of two parts soil and one part farmyard manure) in earthen pots, each containing 4 kg of soil. The soil was inoculated with different FOC isolate cultures at an average density of 4.5×10^5 colony-forming units per gram, ensuring uniform pathogen distribution in the 45-day plant growth period (Sneh *et al.* 1984). The most virulent isolate was selected for further investigation.

Isolating Pseudomonas *isolates*: Six isolates of *Pseudomonas* were isolated from rhizospheric soil by using the serial-dilution plating technique on King's B media (Choudhary *et al.* 2019). After sub-culturing, the pure cultures were maintained at 4°C. To characterize the *Pseudomonas* isolates Bergey's Manual of Systematic Bacteriology was followed (Sneath 1986) as a standard protocol.

Isolating the Trichoderma *isolates*: Total four *Trichoderma* isolates were isolated from rhizosphere soil by using the serial-dilution plating technique on the Trichoderma selected media (Elad *et al.* 1981). PDA was used for the purification of cultures and maintained at 4°C for further use.

Bioassay of Pseudomonas *and* Trichoderma *isolates against FOC*: The *Pseudomonas* and *Trichoderma* isolates were evaluated against FOC by a dual culture confrontation assay using King's B and PDA media (Dennis and Webster 1971). A control, inoculated with pathogen only, was also maintained. Radial growth of pathogen and inhibition zone were measured and percentage growth inhibition was calculated.

Qualitative estimation of chitinase enzyme production by Pseudomonas *isolates*: The capability of various isolates of *Pseudomonas* to release chitinase to degrade chitin amended in media was proved by using the spot bioassay method on King's B medium amended with 1.0% of colloidal chitin as by Viswanathan and Samiyappan (2001). Bacterial inoculum spots were placed over the medium at four places in Petri dishes and were kept at 28 ± 1ºC for seven days. Observation of a slight clear zone served as an indicator for chitinolytic activity.

Quantitative estimation of chitinase from Pseudomonas *isolates*: The chitinase production in *Pseudomonas* spp. was assessed in chitin peptone medium by Lim *et al.* (1991). The amount of reducing sugars produced was determined spectrophotometrically (575 nm).

Siderophores production by Pseudomonas *isolates*: Detection of siderophores as qualitatively was done by using CAS agar medium as by Schwyn and Neilands (1987). Various isolates of *Pseudomonas* were inoculated as spots on CAS and media plates were incubated (28˚C, 7 days). The formation of yellow-orange coloured zone in the dark blue media indicated the production of siderophores.

Quantitative estimation of chitinase from Trichoderma *isolates*: The chitinase production activity measurement was done by colorimetrically procedure in which colloidal chitin was used as substrate. The specific reducing sugars released were determined by a method involving the DNSA and NAGA (Khatri *et al.* 2017). The intensity of the developed colour in the media was measured spectrophotometrically (575 nm).

Molecular identification of Fusarium *isolate*: DNA from the *Fusarium* isolate was extracted by using the CTAB method by Murray and Thompson (1980). ITS (Internal transcribed spacer) region in the genome of fungi was amplified as described by White *et al.* (1990) by using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCC CCGCTTATTGATATGC-3').

Molecular characterization of Pseudomonas *fluorescens (Pf 6)*: *Pseudomonas* (Pf 6) DNA was isolated by using an extraction buffer and 16s rDNA region was amplified as described by Suma *et al.* (2023) by using 27F (5'-AGAGTTTGATCCTGGCTCAG- 3′) and 1492R (5′-TACCTTGTTACGACTT - 3′).

Molecular characterization of Trichoderma *(Th d)*: The CTAB method of Murray and Thompson (1980) was used for the extraction of genomic DNA of *Trichoderma* (Th d). PCR amplification of the Internal transcribed spacer (ITS) region was done by using ITS1 and ITS4 as described by White *et al.* (1990).

Analysis of DNA sequences: The amplified product of PCR was visualized using 1.5% Agarose Gel Electrophoresis at voltage of 5 V/cm for 60 min with a ladder (Promega) of 100 bp. The loaded PCR products were visualized by using the SYNGENE gel documentation system. Desired bands from FOC (510 bp), *Pseudomonas* (1450 bp) and *Trichoderma* (600 bp) were eluted and purified by using Nucleospin Gel and a PCR cleanup kit (Promega). Purified PCR products were sequenced by Bioserve Biotechnologies (India) Pvt. Ltd. Then, the final sequences obtained were searched in the BLASTn tool to collate the results acquired with the sequences formerly submitted at NCBI GenBank database and accession numbers were obtained.

Field evaluation of the talc bioformulations: The talc based bioformulations of selected strains of potent antagonists i.e. *Pseudomonas fluorescens* (Pf 6) and *Trichoderma asperellum* (Th d) were evaluated against Fusarium wilt of cucumber. The field experiment was conducted by randomized block design (RBD) with eight treatments and three replications during 2020–21 and 2021–22 at Punjab Agricultural University, Ludhiana, Punjab. The bio-formulations of *Pseudomonas fluorescens* (Pf 6) (6.1×10^9/g) and *Trichoderma asperellum* (Th d)(9.2×10^{11}/g) were applied as (a) seed treatment (15 g bioformulations/kg of seed); (b) soil treatment (2.5 kg bioformulations in 25 kg of well-rotted FYM/acre and (c) combination (seed treatment + soil treatment). A chemical fungicide, Carbendazim 50% WP (1.5 g/kg seed) was kept as a standard check. An untreated treatment was also maintained as a control. The percent disease incidence, plant growth promotion parameters and cucumber yield were recorded.

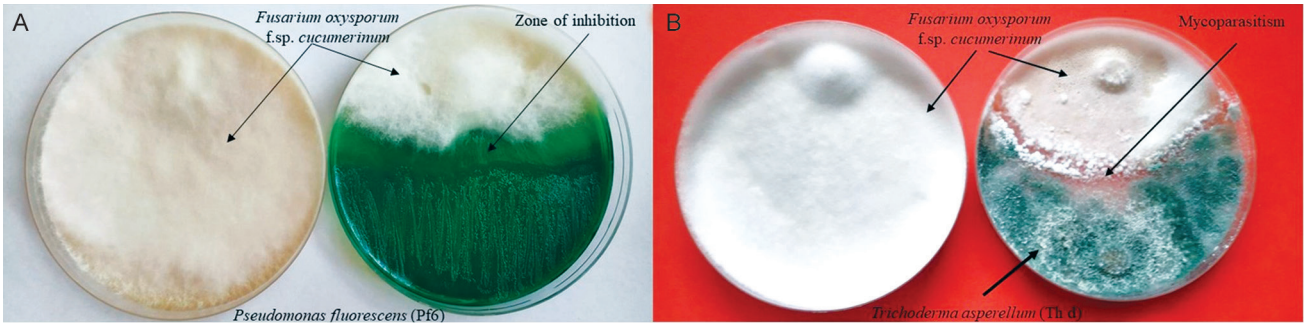

Fig. 1 (A) Dual culture of *Pseudomonas fluorescens* (Pf 6) and (B) *Trichoderma asperellum* (Th d) against *Fusarium oxysporum* f.sp. *cucumerinum* (FOC).

Statistical analysis: The parameter data in all experiments was in three replications and was represented as mean ± standard deviation. The results were analyzed by one-way ANOVA (analysis of variance), through the statistical software SPSS (26.0). The treatment means of various parameters were separated by Duncan's Multiple Range Test and determined by the magnitude of the F value ($p \leq 0.05$).

RESULTS AND DISCUSSION

Confrontation assay: All the isolates of *Pseudomonas* and *Trichoderma* significantly inhibited the mycelia growth of FOC. Among all the isolates of *Pseudomonas fluorescens* (Pf 6) showed a maximum inhibition zone of 13.30 mm (Table 1, Fig. 1A), whereas *Trichoderma asperellum* (Th d) showed the maximum percentage of inhibition, i.e. 61.44 (Table 2, Fig. 1B). Similar results were reported by Islam *et al.* (2018). They screened 35 isolates of *P. aeruginosa* against FOC and found that the BA5 isolate showed the highest antagonistic activity of 58.33% against FOC. Kumar *et al.* (2016) also found that *T. asperellum* reduced the mycelial growth of FOC by 85%.

Qualitative estimation of chitinase activity of Pseudomonas *isolates*: Chitinase activity of six isolates of *Pseudomonas* ranged from 7.07–17.60 mm. The maximum clear zone was produced by Pf 6 (17.60 mm) followed by Pf 5 (15.17 mm) (Table 1). Our findings are in line with study conducted by Suma *et al.* (2023) in which the qualitative chitinase activity of 15 isolates of *Pseudomonas* ranged from 6.67–15.33 mm.

Quantitative estimation of chitinase from Pseudomonas *and* Trichoderma *isolates*: The data in Table 1 shows the efficacy of isolates of *Pseudomonas* for chitinase production. The enzyme activity varied from 1.46–11.56 units/ml. The maximum chitinase activities were expressed by the isolate Pf 6 (11.56 unit/ml) followed by Pf 5 (9.34 unit/ml). The chitinase activity of the various *Trichoderma* isolates ranged from 3.43–10.37 units/ml. Th d (10.37 unit/ml) showed the maximum chitinase activity, followed by Th c (8.77 unit/ml) (Table 2). The study collates with the work of Suganthi *et al.* (2015), who found that the highest chitinase activity was produced by *P. fluorescens* (27.4 U/mL). Similarly, the chitinase activity of *Trichoderma* isolates ranged from 0.62–32.60 unit/ml in the study of Agarwal and Kotasthane (2012).

Estimation of siderophores production by Pseudomonas *isolates*: The siderophores production indicated by bright yellow orange colour zone varied from 17.00–32.17 mm (Table 1). The widest colour zone was produced by Pf 6 isolate (32.17 mm) followed by Pf 5 (28.33 mm). Similar

Table 1 Inhibitory effect, qualitative, quantitative chitinase activity and siderophore production of *Pseudomonas* isolates against FOC

Isolate	Dual Culture		Qualitative estimation clear zone (mm)*	Quantitative estimation chitinase activity (unit/ml)* (Mean ± SD)[1]	Siderophore coloured zone (mm)* (Mean ± SD)[1]
	Growth of FOC (mm)* (Mean ± SD)[1]	Zone of inhibition (mm)* (Mean)			
Pf 1	36.00 ± 0.36^{ab}	05.30	9.83 ± 0.76^{d}	2.67 ± 0.13^{e}	22.83 ± 1.76^{c}
Pf 2	31.00 ± 0.26^{b}	06.00	13.33 ± 0.71^{c}	5.89 ± 0.28^{d}	27.50 ± 1.32^{b}
Pf 3	35.00 ± 0.20^{ab}	08.00	14.00 ± 0.50^{bc}	8.82 ± 0.19^{c}	27.67 ± 3.06^{b}
Pf 4	37.00 ± 0.10^{a}	03.00	7.07 ± 0.60^{e}	1.46 ± 0.05^{f}	17.00 ± 1.32^{d}
Pf 5	34.70 ± 0.06^{ab}	10.30	15.17 ± 1.04^{b}	9.34 ± 0.12^{b}	28.33 ± 1.04^{b}
Pf 6	35.30 ± 0.10^{ab}	13.30	17.60 ± 0.66^{a}	11.56 ± 0.17^{a}	32.17 ± 1.26^{a}
Control	90.00	–	–	–	–
CD ($p \leq 0.05$)	3.37	–	1.61	0.31	3.13

*Mean of three replications; Pf1 to Pf 6 are *Pseudomonas* isolates. [1]Mean ± SD (standard deviation); values labelled with different letters are significantly different from the control level by Duncan test at 95.0% confidence.

Table 2 Inhibitory effect, quantitative chitinase activity of *Trichoderma* isolates against FOC

Isolate	Dual culture			Quantitative estimation of chitinase activity (unit/ml) * (Mean ± SD)1
	Growth of FOC (mm)* (Mean ± SD)[1]	Mycoparasitism (mm)*	Inhibition of mycelium (%)	
Th a	39.00 ± 0.10[a]	07.80 ± 0.32	56.67	3.43 ± 0.15[d]
Th b	38.22 ± 0.11[ab]	10.70 ± 0.41	57.52	5.30 ± 0.20[c]
Th c	37.69 ± 0.15[ab]	09.00 ± 0.18	58.15	8.77 ± 0.12[b]
Th d	34.66 ± 0.45[b]	12.70 ± 0.63	61.44	10.37 ± 0.25[a]
Control	90.00	–	0.0	–
CD ($p \leq 0.05$)	3.97	–	–	0.35

*Mean of three replications; Th a to Th d are *Trichoderma* isolates; [1]Mean ± SD (standard deviation); values labelled with different letters are significantly different from the control level by Duncan test at 95.0% confidence.

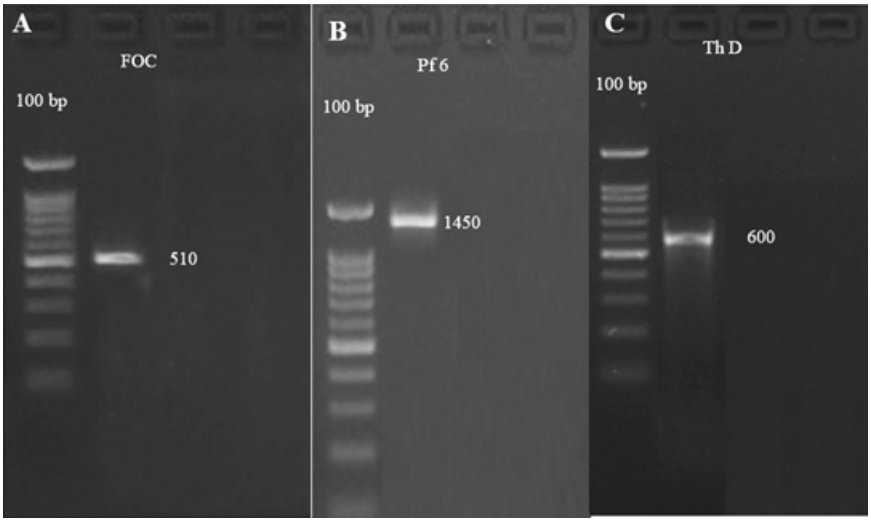

Fig. 2 A) DNA profile generated by ITS primers with *Fusarium* isolate (FOC); B) DNA profile generated by using universal primers with *Pseudomonas* isolate (Pf 6); C) DNA profile generated by ITS primers with *Trichoderma* isolate (Th d) (M= 100 bp marker).

observations were reported by Suma *et al.* (2023) that the siderophore production by *Pseudomonas fluorescens* was effective in controlling plant pathogenic fungi and observed a coloured zone ranging from 15.33–33.67 mm in 15 isolates.

Molecular identification of the FOC: Identification of the FOC was done by using ITS primes, which gave an amplified product of 551bp (Fig. 2A). The product was sequenced, and it showed 98% resemblance with *Fusarium oxysporum* f.sp. *cucumerinum* (accession number = DQ452450 with 544 bp). After that sequence was submitted to NCBI GenBank and the accession number obtained was OP002306. Our study corroborated with the research of Otieno *et al.* (2022), where they targeted the ITS by using universal primers for the molecular characterization of *Fusarium* species and obtained the amplicon of size (500–550 bp).

Molecular identification of the Pseudomonas *and* Trichoderma *isolates*: Pf 6 and Th d were found to be most potent among the *Pseudomonas* and *Trichoderma* isolates. They were identified by using universal bacterial and fungal primers and amplified regions of 1450 bp (Fig. 2B) and 600 bp (Fig. 2C) were obtained for Pf 6 and Th d, respectively. Based on sequencing, isolate Pf 6 was identified as *Pseudomonas fluorescens* (Accession number = OP002308) and isolate Th d was identified as *Trichoderma asperellum* (Accession number = OP012701). Similarly, Suma *et al*. (2023) used universal primers (27F and 1492R) for the molecular identification of the *Pseudomonas* strains. Choudhary *et al.* (2021) also identified the *Trichoderma* spp. by universal ITS primers by targeting 18S rDNA regions.

Bioefficacy of bioformulation of Pf 6 and Th d against cucumber wilt under open field conditions: Based on *in vitro* evaluation, the two potential isolates Pf 6 and Th d were selected and formulated individually in commercial talc powder and were evaluated against cucumber wilt under field conditions. Data presented in Table 3 shows that the minimum incidence of disease (10% and 13.3%) was observed in Pf 6 (seed + soil) treatment as compared to the untreated control (53.33% and 60%) during 2020–21 and 2021–22. Among all treatments, the maximum fruit yield was obtained with Pf 6 (seed + soil) treatment i.e. 66.36 q/acre and 61.14 q/acre. Similarly, the maximum total length (Table 4) of the cucumber plants was obtained in the (seed + soil) treatment of Pf 6 i.e. 383 and 362 cm as compared to the untreated control (216 and 210 cm). Our study is in agreement with the findings of Arya *et al.* (2018) who found that *P. fluorescens* strains synthesized siderophores and exhibited significant control of *F. oxysporum.* Lian *et al.* (2023) also demonstrated that treatment with *T. harzianum* significantly increased the fresh weight, dry weight, vigour index and chlorophyll content of seedlings which led to increased total mass of the plant. It was concluded from the study that *Pseudomonas fluorescens* (Pf 6) can be used as a commercial bioagent for eco-friendly management of cucumber wilt as a substitute for chemical fungicides.

Table 3 Effect of various isolates of selected antagonists applied as various treatments on germination, disease incidence and yield under field conditions

Treatments	2020–21			2021–22		
	Disease incidence (%) *	Decrease in wilt incidence (%)	Yield*(qt/acre) (Mean ± SD)[1]	Disease Incidence (%)*	Decrease in wilt incidence (%)	Yield*(qt/acre) (Mean ± SD)[1]
Th d seed	23.33 (28.87)^{e}	56.25	46.84 ± 2.47^{bc}	26.67 (31.07)^{f}	55.56	53.71 ± 2.84^{abc}
Th d soil	20.00 (26.54)^{d}	62.50	51.34 ± 8.14^{bc}	23.33 (28.85)^{e}	61.11	54.88 ± 8.98^{abc}
Th d seed + soil	16.67 (24.06)^{c}	68.75	58.67 ± 2.14^{ab}	20.00 (26.54)^{d}	66.67	50.76 ± 3.99^{bcd}
Pf 6 seed	16.67 (24.08)^{c}	68.75	55.57 ± 4.4^{abc}	16.67 (24.08)^{c}	72.22	53.58 ± 4.35^{abc}
Pf 6 Soil	13.33 (21.37)^{b}	75.00	64.57 ± 7.10^{a}	13.33 (21.39)^{b}	77.78	53.44 ± 4.56^{abc}
Pf 6 seed + soil	10.00 (18.42)^{a}	81.25	66.36 ±4.96^{a}	13.33 (21.39)^{b}	77.78	61.14 ± 5.58^{a}
Bavistin** (Carbendazim 50% WP)	10.00 (18.34)^{a}	81.25	59.56 ± 9.15^{ab}	10.00 (18.37)^{a}	83.33	58.54 ± 2.80^{ab}
Control	53.33 (46.89)^{f}	0.00	27.68 ± 10.13^{d}	60.00 (80.75)^{g}	0.00	25.65 ± 8.89^{e}
CD ($p \leq 0.05$)	2.59	–	11.27	2.28	–	8.41

*Mean of three replications; Th d, *Trichoderma asperellum*; Pf 6, *Pseudomonas fluorescens*; ** Standard check; [1]Mean ± SD (standard deviation); values labelled with different letters are significantly different from the control level by Duncan test at 95% confidence.

Table 4 Effect of different isolates of selected antagonists applied as seed, soil and seed + soil treatments on shoot and root length of cucumber under field conditions

Treatments	2020–21		2021–22		2020–21	2021–22
	Shoot length (cm)* (Mean ± SD)[1]	Root length (cm)* (Mean ± SD)[1]	Shoot length (cm)* (Mean ± SD)[1]	Root length (cm)* (Mean ± SD)[1]	Total length (cm)* (Mean ± SD)[1]	Total length (cm)* (Mean ± SD)[1]
Th d Seed	256.33 ± 18.23^{bc}	49.00 ± 3.61^{fgh}	230.00 ±13.45^{bcd}	52.67 ± 5.51^{efg}	309 ± 10.34^{d}	282 ± 12.22^{cd}
Th d Soil	282.67 ± 45.21^{ab}	57.33 ± 5.69^{cdef}	246.67 ± 36.25^{abcd}	57.67 ± 5.03^{cdef}	340 ± 33.19^{bcd}	304 ± 18.29^{bcd}
Th d Seed+Soil	293.00 ± 21.63^{ab}	62.33 ± 4.04^{bcd}	271.67 ± 06.03^{abc}	63.67 ± 5.69^{bc}	335 ± 4.11^{abc}	334 ± 19.77^{ab}
Pf 6 Seed	292.33 ± 07.77^{ab}	62.33 ± 9.87^{bcd}	268.33 ± 53.46^{abc}	63.00 ± 4.58^{bcd}	355 ± 47.15^{bc}	331 ± 11.02^{abc}
Pf 6 Soil	295.67 ± 39.55^{ab}	65.33 ± 5.86^{bc}	277.33 ± 18.56^{ab}	64.00 ± 4.48^{abc}	358 ± 11.44^{abc}	341 ± 30.50^{ab}
Pf 6 Seed+Soil	306.33 ± 18.15^{a}	77.67 ± 6.66^{a}	288.67 ± 30.09^{a}	73.67 ± 4.16^{a}	383 ± 26.95^{a}	362 ± 19.81^{a}
Bavistin** (Carbendazim 50% WP)	284.33 ± 12.34^{ab}	60.67 ± 5.13^{cde}	266.00 ± 22.91^{abc}	62.67 ± 4.73^{bcde}	345 ± 16.68^{bc}	328 ± 13.95^{abc}
Control	182.33 ± 11.00^{d}	34.67 ± 5.69^{j}	178.33 ± 23.25^{e}	32.47± 6.11^{h}	216 ± 19.65^{f}	210 ± 9.93^{e}
CD ($p \leq 0.05$)	34.47	13.49	43.75	9.18	37.68	50.83

*Mean of three replications. Th d, *Trichoderma asperellum*; Pf 6, *Pseudomonas fluorescens*; ** Standard check.

[1]Mean ± SD (standard deviation); values labelled with different letters are significantly different from the control level by Duncan test at 95.0% confidence..

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