

ORIGINAL ARTICLE

Antifungal and plant growth promoting activity of *Trichoderma* spp. against *Fusarium oxysporum* f. sp. *lycopersici* colonizing tomato

Arshi Jamil 

Department of Plant Protection, Aligarh Muslim University, Aligarh, India

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*Corresponding address:
arshijamil.amu@gmail.comResponsible Editor:
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Abstract

Fusarium wilt, incited by *Fusarium oxysporum* f. sp. *lycopersici* (FOL), causes serious production losses of tomato (*Solanum lycopersicum* L.) plants. Biological control, using an antagonistic of *Trichoderma* species, is a bio-rationale and an alternative method to synthetic pesticides against most phytopathogens. The present study was undertaken to evaluate the effects of *T. harzianum* and/or *T. viride* in reducing Fusarium wilt and to determine the relationship between disease severity and plant growth promoting traits of these species. *Trichoderma viride* exhibited better phosphate solubilization and production of cellulases, ligninases, chitinases, proteases, hydrogen cyanide (HCN), siderophores and indole acetic acid (IAA) than *T. harzianum*. For field assessment, five treatments with three replicates were used. The field was inoculated with the wilt fungus (FOL). Both *Trichoderma* spp. used were applied as a seed treatment, mixed in the soil, and FOL inoculated soil served as the untreated control. During the two consecutive years, seed treatment with *T. viride* exhibited the least disease severity, the highest physiological activity, the highest biochemical and antioxidant contents, and tomato plants treated with it exhibited the best growth and yield. It was concluded that *Trichoderma viride* can potentially be used to reduce Fusarium wilt and promote plant growth and yield in commercial tomato production.

Keywords: antioxidants, biocontrol, physiological parameters, *Solanum lycopersicum*

Introduction

Tomato (*Solanum lycopersicum* L.) is frequently exposed to several fungal pathogens including *Fusarium oxysporum*, *Alternaria solani*, *Phytophthora infestans*, *Verticillium dahliae* and *Sclerotium rolfsii* which affect plant growth and yield (Sanoubar and Barbanti 2017). Of these, *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen causes Fusarium wilt of tomato. It is a soil borne disease that destroys plant roots (Ahmed 2011), resulting in tomato yield losses (Huang *et al.* 2012; Harikrushana *et al.* 2014; McGovern 2015). The pathogen is host specific (Castano *et al.* 2013) and produces typical disease symptoms of stunting, yellowing, necrosis of older leaves, browning of vascular

tissue and loss of the plant's rigidity (Huang *et al.* 2012; Li *et al.* 2018).

Disease severity depends on the cultivar's resistance and the environmental conditions. Soil and air temperatures of 28°C favor the disease. The pathogen disseminates through contaminated seed, infested soils, infected transplants and aerial dispersal of the conidia. The capability of the pathogen to persist for a long time in soil as spores and emergence of new pathogenic races makes disease management challenging. The use of synthetic chemicals has effectively prevented Fusarium wilt (Jamil and Ashraf 2020), but persistence of synthetic chemicals in soil and their detrimental impact on

plants, the environment and the soil micro-biome is of concern (Wightwick *et al.* 2013). Biological control of plant diseases with micro-organisms, called biocontrol agents, may be an alternative to synthetic fungicides. It is a safe, bio-rational approach and cost effective for disease management that stimulates disease resistance in plants through induced systemic resistance, promotes plant growth and increases yield (Abdelrahman *et al.* 2016; Jamil *et al.* 2020).

Among fungal biocontrol agents, *Trichoderma* spp. have been extensively explored against plant pathogens. They are naturally occurring soil fungi able to stimulate plant growth through root colonization. *Trichoderma* spp. are used as seed, root or soil treatments, which reduce disease severity, improve nutrient uptake by plants, increase soil fertility, elevate production of growth-promoting substances, bioactive metabolites and defense-related enzymes (like chitinase and β -1,3-glucanase) in plants, and reduce pathogen populations (Shoresh *et al.* 2010; Jogaiah *et al.* 2013; Abd-El-Khair *et al.* 2019; Sallam *et al.* 2019; Elshahawy and El-Mohamedy 2019). Biocontrol mechanisms of *Trichoderma* spp. involve competition for nutrients and space, antibiosis, mycoparasitism and exploitation of potential infection courts (Benítez *et al.* 2004; Verma *et al.* 2017; Yadav *et al.* 2018). Isolates of several *Trichoderma* spp. efficiently decrease Fusarium wilt diseases (Srivastava *et al.* 2010; Marzano *et al.* 2013). This study was undertaken to evaluate the antifungal activity of *T. harzianum* and *T. viride* against *F. oxysporum* f. sp. *lycopersici* and their efficacy in promoting growth and yield, biochemical and antioxidant constituents and physiological activity of tomato under field conditions.

Materials and Methods

Pathogen culture and inoculum preparation

Tomato wilt pathogen (*FOL*) was isolated from tomato plants which showed typical symptoms (wilted plant having stunted growth, yellow necrotic leaves and vascular discoloration). The isolate was grown on sterilized Komada selective medium (Komada 1975) and purified using the technique of Riker and Riker (1936). For proper pathogen identification, the Soesanto *et al.* (2011) key was followed and the fungal isolate compared with a standard culture of *F. oxysporum* f. sp. *lycopersici* procured from the Indian Type Culture Collection, Indian Agricultural Research Institute (ITCC, IARI), Pusa, New Delhi, India. For further confirmation of the pathogen, two pots having a 2 kg mixture of sterilized soil and compost at 3 : 1 (w/w) ratio were taken. The isolated pathogen was added to one pot while in another pot pathogen culture procured from ITCC was added. Two tomato seedlings were transplanted

into each pot and after 15 days plants in both pots produced similar disease symptoms. Pathogen isolation from both infected plants showed similar cultural and morphological characters thereby, confirming that the isolated pathogen was *F. oxysporum* f. sp. *lycopersici*.

Pathogen inoculum was developed on potato dextrose broth (PDB) medium. A 5 mm mycelial plug of *FOL*, removed from the center of a 7-day-old culture growing on a Petri-plate was transferred into 400 ml PDB medium in 1,000 ml Erlenmeyer flasks and incubated for a week at $27 \pm 2^\circ\text{C}$. Flasks were kept on a mechanical shaker. The liquid culture was filtered through sterile Whatman No. 1. filter paper and the conidial suspension was adjusted to 10^7 conidia \cdot ml⁻¹ using a haemocytometer.

Trichoderma isolates

Trichoderma harzianum (MTCC 9288) and *T. viride* (3180) were procured from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (MTCC, IMTECH), Chandigarh, India. The cultures were grown on potato dextrose agar (PDA) at $27 \pm 2^\circ\text{C}$ for 7 days. Periodic sub-culturing was done to maintain cultures.

Assessment of plant growth promoting and antifungal properties of *Trichoderma* spp.

For assessing the enzymatic degradation of cellulose and lignin compounds by *Trichoderma* spp., the method described by de Rodríguez *et al.* (2006) was followed. Five-mm mycelial discs, 7 days old, of *T. harzianum* and *T. viride* from colonies growing on PDA were removed and placed in Petri-plates containing PDA mixed with carboxymethyl cellulose and azure B that served as a carbon source (Kausar *et al.* 2011). Development of a transparent halo around the colonies, indicated the ability of biocontrol agents to degrade carbon sources. To compare the biocontrol agents, the circumference of the transparent halos was measured using the formula:

$$\text{Circumference} = 2\pi r,$$

where: π – 3.14 (constant) and r – radius of the halos.

Chitinase action of biocontrol fungi was assayed using chitin agar to which chitin powder was added as a carbon source (Hsu and Lockwood 1975). The development of distinct halos on chitin agar indicated the ability of chitinase production by *Trichoderma* spp. The phosphate-solubilizing ability of *Trichoderma* spp. was determined by mixing $\text{Ca}_3(\text{PO}_4)_2$, FePO_4 , $\text{Mg}_3(\text{PO}_4)_2$ and K_3PO_4 in sterilized Pikovskaya's medium (Rao and Sinha 1963). The presence of halos around colonies indicated phosphate solubilization. The circumference of the halo was measured. Protease production by

the *Trichoderma* spp. was with PDA containing 50% skimmed milk (Nielsen and Sørensen 1997). The formation of transparent halos on agar indicated protease activity. Hydrogen cyanide (HCN) was produced by *Trichoderma* spp. using the method of Ng *et al.* (2015). Tryptic Soy Agar (TSA) slants, supplemented with $4.4 \text{ g} \cdot \text{l}^{-1}$ of glycine, were inoculated with a 5 mm mycelial disc of *Trichoderma* spp. The slants were incubated at $28 \pm 1^\circ\text{C}$. Filter paper strips dipped in 0.5% picric acid and 2% sodium carbonate (w/v) solution were hung above the test tubes. Changes in strip color from yellow to brown signified HCN production (Jangir *et al.* 2019). To analyze siderophore production by *Trichoderma* spp. a chrome azurol sulphonate (CAS)-agar plate assay was used (Schwyn and Neilands 1987). The type of siderophore produced was identified according to color changes of the CAS medium (Pérez-Miranda *et al.* 2007). According to this method color changes from blue to purple indicated the presence of catechol-type siderophore, orange indicated hydroxamate-type siderophore and yellow indicated carboxylate-type siderophores, respectively. The production of indole acetic acid (IAA) by *T. harzianum* and *T. viride* was evaluated following Li *et al.* (2017). A 0.5 ml conidial suspension of *Trichoderma* spp., $10^6 \text{ conidia} \cdot \text{ml}^{-1}$ was mixed in 4 ml potato dextrose broth augmented with $200 \mu\text{g L-tryptophan} \cdot \text{ml}^{-1}$. After 7 days, the culture was centrifuged at 10,000 g for 10 min. One-ml of the supernatant was placed in a test tube and 0.1 ml of ortho-phosphoric acid and 4 ml of Salkowski's reagent (Ehmann 1977) were added. The suspension was left for half an hour in the dark at room temperature (approximately 30°C) for color change. The development of pink indicated production of IAA. The level of IAA produced was determined at an absorbance of 530 nm using a spectrophotometer (model UV-2450, Shimadzu, Tokyo, Japan).

Seedling preparation and transplanting in the field

Seeds of the wilt susceptible tomato 'Pusa Ruby' were surface sterilized in 0.2% sodium hypochlorite (NaOCl) for 3 min and washed several times with double distilled water. Washed seeds were sown in $64 \times 48 \times 8 \text{ cm}^3$ size plastic trays containing 4 kg of a mix of sterilized soil and compost at a 3 : 1 (w/w) ratio. No fertilizer or pesticide was used. The trays were placed in a growth chamber at $27 \pm 2^\circ\text{C}$, 12-h light and 60 to 70% relative humidity and were watered daily. Twenty-five-day-old seedlings were removed from the trays and transplanted by hand to $2 \times 2 \text{ m}^2$ field plots (3 rows; 10 seedlings per row) having sandy clayey loam soil. Experiments were carried out for 2 consecutive years (December 2018 and December 2019). Each treatment was replicated three times.

Application of pathogen and biocontrol agent

For pathogen application in the field, 10 liters of conidial suspension having $10^7 \text{ conidia} \cdot \text{ml}^{-1}$ was further diluted by adding 35 liters of water. Three liters of this final suspension was then evenly sprinkled in each treatment plot 10 days before transplanting. Both *Trichoderma* spp. were applied as seed treatment ($4 \text{ g} \cdot \text{kg}^{-1}$ seed) and as soil application at the rate of $4 \text{ g} \cdot \text{kg}^{-1}$ soil. The method of *Trichoderma* application followed was similar to that of Jamil *et al.* (2020). To carry out seed treatment, 50 g tomato seeds of variety Pusa Ruby were dipped for a minute in 50 ml of 5% sucrose solution which acted as a sticker. The seeds were divided into two equal parts and each part was then transferred to a 100 ml flask. In one flask 10 mg macerated mycelium of *T. harzianum* was added while to the other flask 10 ml of *T. viride* was added. Both flasks were thoroughly shaken for proper and uniform coating of the two *Trichoderma* strains. For soil application of the biocontrol agents, top soil (up to 8–10 cm depth) from five randomly chosen plots ($2 \times 2 \text{ m}^2$) were collected, weighed and the average was approximately 19.5 kg. Thus, it was assumed that each experimental plot contained 19.5 kg soil and accordingly 78 g of either *T. harzianum* or *T. viride* was uniformly mixed into these plots 2 days prior to seedling transplanting to obtain $4 \text{ g} \cdot \text{kg}^{-1}$ soil of *Trichoderma*. Field plots having only *FOL* inoculation were considered controls. The treatments were: (i) T1 – *FOL* inoculated untreated control; (ii) T2 – seed treatment with *T. harzianum*; (iii) T3 – soil application of *T. harzianum*; (iv) T4 – seed treatment with *T. viride*, and (v) T5 – soil application of *T. viride*. After plants were established in the field, irrigation was done every 20 days (a total of six irrigations). No fertilizer or pesticide was added in the field.

Disease severity

Disease severity was determined by visual observation 15 days after transplanting until plants were 2 months old. Scoring was on a 0–5 scale where: 0 – no wilting, 1 – 1–20%, 2 – 21–40%, 3 – 41–60%, 4 – 61–80%, and 5 – 81% to complete wilting of the plant (dead plant).

Assessment of plants' physiological parameters

A portable photosynthetic system Infra-Red Gas Analyzer (LICOR 6400, Lincoln, NE) was used to assess effects of treatment on photosynthesis of tomato plants. Data were recorded after equilibration to a steady state (approximately 30 min). One plant from each plot in the treatments was randomly selected. Fully expanded

leaves of a 3-month-old plant were placed in the photosynthetic system and net photosynthetic rate (P_N), transpiration rate (E) and stomatal conductance (g_s) were recorded. Leaf temperature was maintained at 25°C, and photosynthetic photon flux density (PPFD) was 1,000 $\mu\text{mol photons m}^{-2} \cdot \text{s}^{-1}$ at 45% relative humidity.

Assessment of biochemical and antioxidant contents

Chlorophyll and total phenolic contents were estimated on 3-month-old plants. To estimate total leaf chlorophyll content 100 mg fresh leaves were added to 7 ml dimethyl sulphoxide (DMSO) in a test tube for chlorophyll extraction (Hiscox and Israelstam 1979). After an hour, more DMSO was added to the test tube and the net volume was raised to 10 ml. The sample was assayed and absorbance read at 645 and 663 nm. The Folin-Ciocalteu reagent colorimetric analysis method (Zieslin and Ben-Zaken 1993) was followed for estimating the total phenolic content. One g of leaf tissue was homogenized in 10 ml methanol (80%) and allowed to stand for 15 min at 70°C. One ml of this solution was added to 5 ml double distilled water and 250 μl of Folin-Ciocalteu reagent. The mix was left to stand for 5 min at 25°C. Absorbance was quantified at 725 nm. Proline concentration in tomato plants followed the method of Mona *et al.* (2017). Fresh leaves (0.5 g) were placed in 3% sulpho-salicylic acid and centrifuged at 10,000 rpm for 10 min. Two ml of supernatant was incubated in equal quantities of acid ninhydrin and glacial acetic acid and left to stand at 100°C for an hour. Samples were placed on an ice bath to terminate activity. Proline was separated using toluene. Absorbance was read at 520 nm.

Determination of total ascorbic acid (vitamin C) in tomato fruit followed the method of Kapur *et al.* (2012). Ten- μl of fruit extract was placed in a test tube and the total volume was raised to 2 ml with double distilled water. To this solution 2 ml 2,4-dinitro-phenyl-hydrazine (DNPH) and a drop of 10% thiourea was added. The mix was placed on a water bath with boiling water for 15 min. then cooled to room temperature. Five- μl H_2SO_4 (80%; v/v) was added to the mix and placed on an ice bath at 0°C. Absorbance was recorded at 521 nm against absolute ascorbic acid which was used as a standard. Lycopene estimation was according to Fish *et al.* (2002) and absorbance was measured at a wavelength of 503 nm. Briefly, 0.6 g of whole tomato puree was added to a 40 ml amber vial containing 5 ml acetone with 0.05% butylated hydroxytoluene, 5 ml ethanol and 10 ml hexane. The mix was shaken at 180 rpm for 15 min. on an orbital shaker. The vial was kept at room temperature for 5 min. to allow for phase separation and the upper phase hexane was sampled to record absorbance.

Effect of *Trichoderma* spp. on tomato growth

Trichoderma harzianum and *T. viride* were screened for their ability to stimulate tomato growth. From each plot three plants were randomly selected 3 months after transplanting. Plant fresh and dry weights and plant length (root plus shoot length) were recorded. Plant yield was determined per plot as well as the average mean yield of three harvests at 3, 3.5 and 4 months after transplanting to the field.

Statistical analysis

Data were subjected to analysis of variance in Minitab 11.0 (ver. 11.12, Minitab Inc., State College, PA). Where interactions were significant, they were used to explain results. Where interactions were not significant, means were separated with Tukey's test.

Results

In-vitro plant growth promoting and antifungal properties of *Trichoderma* spp.

Both *Trichoderma* spp. showed several plant growth promoting traits that aided in disease suppression (Table 1). *Trichoderma viride* stood superior over *T. harzianum*. It produced larger transparent halos indicating better cellulase, chitinase and protease production. *T. harzianum* did not show any ligninase production and displayed phosphate solubilization only on Pikovskaya's medium mixed with $\text{Ca}_3(\text{PO}_4)_2$ while *T. viride* could solubilize phosphate from both $\text{Ca}_3(\text{PO}_4)_2$ and FePO_4 . HCN production was recorded in *T. viride*. It produced two types of siderophores (hydroxamate and carboxylate) whereas *T. harzianum* only produced hydroxamate-type siderophore. The quantity of IAA produced by *T. viride* was 29.83 $\mu\text{g} \cdot \text{ml}^{-1}$ and by *T. harzianum* it was 17.52 $\mu\text{g} \cdot \text{ml}^{-1}$.

Effect of *Trichoderma* spp. on disease severity

Disease severity score of tomato plants recorded 2 months after transplanting has been compiled in Table 2. Plots having the untreated control (T1) exhibited the highest severity scores (3.5 and 3.8) throughout the 2 years of experimental work. Minimum disease severity with 1.3 and 1.5 wilting scores was observed when T4 was applied (significant at $p \leq 0.05$). Although T2 reduced disease severity to 1.8 and 2.0 during the 2 years, it was found to be non-significant. Treatments T5 and T3 also showed substantial decreases in severity scores and were found to be significantly effective when compared with the untreated control.

Table 1. Anti-pathogenic and plant growth promoting properties of *Trichoderma harzianum* and *T. viride*

Functional trait	<i>Trichoderma harzianum</i>	<i>Trichoderma viride</i>
Cellulase production	+ (94.2 mm)	+ (69.08 mm)
Ligninase production	-	+ (50.24 mm)
Chitinase production	+ (75.36 mm)	+ (75.36 mm)
Phosphate-solubilization		
Ca ₃ (PO ₄) ₂	+ (87.92 mm)	+ (94.2 mm)
FePO ₄	-	+ (62.8 mm)
Mg ₃ (PO ₄) ₂	-	-
K ₃ PO ₄	-	-
Protease production	+ (56.52 mm)	+ (78.5 mm)
HCN production	-	+
Siderophore production	+ (hydroxamate-type)	+ (hydroxamate-type and carboxylate-type)
IAA-production	+ (17.52 µg · ml ⁻¹)	+ (29.83 µg · ml ⁻¹)

(+) – tested positive; (-) – tested negative

Table 2. Effect of different treatments on disease severity and physiological parameters of tomato plants

Treatments	I Year					II Year				
	disease severity (0–5 scale)	P_N [$\mu\text{mol}(\text{CO}_2) \cdot \text{m}^{-2} \cdot \text{s}^{-1}$]	E [$\text{mmol}(\text{H}_2\text{O}) \cdot \text{m}^{-2} \cdot \text{s}^{-1}$]	g_s [$\text{mol}(\text{H}_2\text{O}) \cdot \text{m}^{-2} \cdot \text{s}^{-1}$]	E/g_s (10^{-3})	disease severity (0–5 scale)	P_N [$\mu\text{mol}(\text{CO}_2) \cdot \text{m}^{-2} \cdot \text{s}^{-1}$]	E [$\text{mmol}(\text{H}_2\text{O}) \cdot \text{m}^{-2} \cdot \text{s}^{-1}$]	g_s [$\text{mol}(\text{H}_2\text{O}) \cdot \text{m}^{-2} \cdot \text{s}^{-1}$]	E/g_s (10^{-3})
T1	3.5 a	7.22 e	3.19 e	0.58 d	5.5 a	3.8 a	6.93 e	3.43 e	0.74 e	4.63 a
T2	1.8 c, d	17.99 b	6.3 b	1.59 b	3.96 b	2.0 c, d	17.83 b	6.65 b	1.68 b	3.95 b
T3	2.4 b	16.65 c, d	5.37 d	1.45 c	3.7 d	2.7 b	15.67 c, d	5.32 d	1.43 c, d	3.72 c
T4	1.3 e	19.09 a	7.05 a	1.96 a	3.59 d, e	1.5 e	18.78 a	6.68 a	1.85 a	3.61 c, d
T5	1.9 c	16.86 c	5.97 c	1.51 b, c	3.95 b, c	2.1 c	16.32 c	5.77 c	1.51 c	3.82 b, c
LSD	0.24	0.86	0.12	0.21	0.19	0.38	0.91	0.23	0.14	0.15

Values within a column followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's test; P_N – net photosynthetic rate; E – transpiration rate; g_s – stomatal conductance; T1 – *FOL* inoculated untreated control; T2 – seed treatment with *Trichoderma harzianum*; T3 – soil application of *T. harzianum*; T4 – seed treatment with *T. viride*; T5 – soil application of *T. viride*

Effect of *Trichoderma* spp. on physiological parameters

The tomato wilt pathogen had a significant effect on physiological parameters of tomato plants. *Trichoderma* spp. considerably improved these parameters (Table 2). A maximum significant increase in plant net photosynthetic rate (P_N) was seen in treatment T4 where *T. viride* was employed as seed treatment. In the first year a 62.18% increase in photosynthetic activity was recorded as the net photosynthetic rate increased to 19.09 $\mu\text{mol}(\text{CO}_2) \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ in contrast to 7.22 $\mu\text{mol}(\text{CO}_2) \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ in the untreated control. In the second year this value increased by 63.10% as T4 treatment showed a net photosynthetic rate of 18.78 $\mu\text{mol}(\text{CO}_2) \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ over 6.93 $\mu\text{mol}(\text{CO}_2) \cdot \text{m}^{-2} \cdot \text{s}^{-1}$

recorded in plants grown in untreated plots. Similarly, transpiration rate (E) and stomatal conductance (g_s) were also found to be the highest in plots having T4 treatment. Transpiration rate was 7.05 and 6.68 $\text{mmol}(\text{H}_2\text{O}) \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ showing 54.75% and 48.65% over the untreated control while stomatal conductance was 1.96 and 1.85 $\text{mol}(\text{H}_2\text{O}) \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ which was 70.41% and 60.0% higher during the 2 consecutive years. Although T3 improved plant physiological parameters, it was found to be the least efficacious when compared to the other treatments. The ratio of transpiration rate to stomatal conductance (E/g_s) was markedly reduced in *Trichoderma* treated plants. It was minimum in T4 treated plants (3.59×10^{-3} and 3.61×10^{-3}) exhibiting 65.27% and 77.97% increase when compared to T1 as

the highest E/g_s ratio 5.5×10^{-3} and 4.63×10^{-3} was recorded in the untreated control.

Effect of *Trichoderma* spp. on plant biochemical and antioxidant components

Biochemical components

Tomato plants infected with wilt pathogen showed manifold increases in their biochemical contents when treated with *T. harzianum* and *T. viride*. The highest significant increase in chlorophyll content was 42.56% i.e., $3.36 \text{ mg} \cdot \text{g}^{-1}$ fresh leaves in the first year and 45.70% ($3.02 \text{ mg} \cdot \text{g}^{-1}$ fresh weight) in the consecutive year when T4 treatment was used (Table 3). Similarly, maximum phenolic and proline contents were seen in plants that received T4 treatment (Table 3). Phenolic content was 96.61 and 93.59 μg catechol $\cdot \text{g}^{-1}$ fresh leaves, showing 45.02% and 44.70% increases, while proline values increased by 18.22% and 23.2% ($p \leq 0.05$). The value was quite high in comparison to the untreated control, T1, as it could only produce 53.12 and 51.78 μg catechol $\cdot \text{g}^{-1}$ fresh leaves of phenols and 108.41 and 103.57 $\mu\text{g} \cdot \text{g}^{-1}$ fresh weight of proline during the 2 consecutive years of tomato field trials. T2 which used *T. harzianum* as seed treatment was the second best option. In the first year, it significantly improved chlorophyll to $2.86 \text{ mg} \cdot \text{g}^{-1}$ causing 35.52% increase, phenols to $92.17 \mu\text{g}$ catechol $\cdot \text{g}^{-1}$ fresh leaves exhibiting 42.37% enhancement and proline to $126.78 \mu\text{g} \cdot \text{g}^{-1}$ fresh weight showing 14.49% increase in its value. In the second year, chlorophyll ($2.74 \text{ mg} \cdot \text{g}^{-1}$ fresh leaves) and proline ($123.53 \mu\text{g} \cdot \text{g}^{-1}$ fresh weight) showed significant increases of 40.15% and 16.16% while a non-

significant increase of 43.2% was observed in phenolic content ($91.11 \mu\text{g}$ catechol $\cdot \text{g}^{-1}$ fresh leaves).

Antioxidants

Pathogen infestation reduced antioxidant quantity in tomato fruits. In the untreated control (T1), $25.43 \text{ mg} \cdot \text{kg}^{-1}$ lycopene and 7.13 vitamin C was recorded in the first year and $29.89 \text{ mg} \cdot \text{kg}^{-1}$ lycopene and 7.59 mg per 100 g fresh fruit in the subsequent year of tomato cultivation (Table 3). However, during both years, the highest lycopene contents (71.28 and $75.51 \text{ mg} \cdot \text{kg}^{-1}$ fresh fruit), which showed 62.89 and 60.42% increase and vitamin C content (12.97 and $13.26 \text{ mg} \cdot 100 \text{ g}^{-1}$ fresh fruit) exhibiting 45.03% and 42.76% increase was found in fruits harvested from plots where *T. viride* was applied as seed treatment, i.e., in treatment T4. *Trichoderma viride* as soil application (T5) was the second best treatment that significantly increased the antioxidant content in tomato and was followed by *T. harzianum* seed treatment (T2) and *T. harzianum* soil application (T3) (Table 3).

Effect of *Trichoderma* spp. on plant growth and yield

The results pertaining to the efficacy of *Trichoderma* spp. in disease suppression and plant growth enhancement against Fusarium wilt of tomato have been compiled in Table 4. In contrast to the untreated control (T1), the use of both *Trichoderma* spp. in either of the two forms significantly ($p \leq 0.05$) enhanced the plant growth and yield parameters. Among all the treatments, T4 showed maximum increment in enhancing

Table 3. Effect of different treatments on biochemical and antioxidant contents of tomato plants

Treatments	I Year					II Year				
	chlorophyll content [$\text{mg} \cdot \text{g}^{-1}$ fresh weight]	total phenols [μg catechol $\cdot \text{g}^{-1}$ fresh leaf]	proline content [$\mu\text{g} \cdot \text{g}^{-1}$ fresh weight]	lycopene content [$\text{mg} \cdot \text{kg}^{-1}$ fresh weight]	vitamin C content [mg per 100 g fresh weight]	chlorophyll content [$\text{mg} \cdot \text{g}^{-1}$ fresh weight]	total phenols [μg catechol $\cdot \text{g}^{-1}$ fresh leaf]	proline content [$\mu\text{g} \cdot \text{g}^{-1}$ fresh weight]	lycopene content [$\text{mg} \cdot \text{kg}^{-1}$ fresh weight]	vitamin C content [mg per 100 g fresh weight]
T1	1.93 e	53.12 e	108.41 e	25.43 e	7.13 e	1.64 e	51.78 d	103.57 e	29.89 e	7.59 e
T2	2.86 b	92.17 b	126.78 b	61.19 c	12.07 b, c	2.74 b	91.11 a	123.53 b	64.41 c	12.22 c
T3	2.54 c, d	78.54 d	112.45 d	58.32 c, d	10.93 d	2.57 d	78.65 c	111.26 d	60.01 d	11.48 d
T4	3.36 a	96.61 a	132.56 a	71.28 a	12.97 a	3.02 a	93.59 a	134.89 a	75.51 a	13.26 a
T5	2.64 c	85.33 c	121.12 c	67.15 b	12.41 b	2.71 b, c	84.38 b	118.35 b, c	70.23 b	12.88 b
LSD	0.21	4.16	4.37	3.14	0.4	0.27	5.71	5.49	4.21	0.29

Values within a column followed by the same letter are not significantly different at $p \leq 0.05$ according to Tukey's test; T1 – FOL inoculated untreated control; T2 – seed treatment with *Trichoderma harzianum*; T3 – soil application with *T. harzianum*; T4 – seed treatment with *T. viride*; T5 – soil application with *T. viride*

Table 4. Effect of different treatments on growth and yield parameters of tomato plants

Treatments	I Year				II Year			
	fresh weight [g]	dry weight [g]	plant length [cm]	plant yield/plot [kg]	fresh weight [g]	dry weight [g]	plant length [cm]	plant yield/plot [kg]
T1	47.85 e	21.98 e	40.51 e	3.83 d, e	51.64 e	24.54 e	37.87 e	4.25 e
T2	82.44 b	38.27 b	64.51 b	4.98 b, c	85.89 b	39.85 b	63.08 b	5.44 c
T3	68.15 d	34.02 d	54.01 d	4.24 c, d	69.57 d	31.02 d	51.65 d	5.07 d
T4	89.93 a	41.78 a	70.49 a	6.91 a	91.31 a	45.68 a	67.15 a	7.72 a
T5	75.28 c	38.76 b, c	60.65 c	5.74 b	78.23 b, c	35.87 c	58.21 c	6.57 b
LSD	6.87	1.48	3.27	1.06	7.32	1.73	3.32	0.87

Values within a column followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's test; T1 – *FOL* inoculated untreated control; T2 – seed treatment with *Trichoderma harzianum*; T3 – soil application of *T. harzianum*; T4 – seed treatment with *T. viride*; T5 – soil application of *T. viride*

plant fresh and dry weights, plant length and tomato yield. In the first year of the field trial, it increased plant fresh weight by 87.94% (up to 89.93 g), dry weight by 90.08% (up to 41.78 g), plant length by 77.85% (68.49 cm) and plant yield by 168% (up to 4.91 kg per plot) in contrast to 47.85 g fresh weight, 21.98 g dry weight, 38.51 cm plant length and 1.83 kg per plot yield in the untreated control (T1). In the next consecutive year, there was an 83.94% increase in plant fresh weight (91.31 g), a 93.79% increase in dry weight (43.68 g), a 77.32% increase in plant length (67.15 cm) and a 171.42% increase in yield (5.32 kg) per plot in plants grown in plots having T4 treatment. These results were significantly superior to other treatments. Treatment T2 was observed as the second best option that caused significant increases in plant growth. However, treatment T5 involving soil application of *T. harzianum* was much better in increasing plant yield per plot than T2.

Discussion

Fusarium wilt is one of the most prevalent soil borne, systemic and highly damaging diseases of tomato (McGovern 2015; Ramaiah and Gampalli 2015). Adopting biological control measures for managing plant diseases is a promising, economical and environmentally safer method. Plant growth promoting properties as well as direct and indirect mechanisms of antagonism possessed by biocontrol agents are accountable for their aggressiveness against plant pathogens. In the present study, two selected *Trichoderma* species, *T. harzianum* (MTCC 9288) and *T. viride* (MTCC 3180), were examined against tomato wilt pathogen, *F. oxysporum* f. sp. *lycopersici*. Both *Trichoderma* species increased growth and yield of tomato

by suppressing the pathogenicity of the wilt fungus. *In vitro* assays showed that *T. viride* provided better antagonism as it had more functional attributes viz., production of cellulases, ligninases, chitinases, proteases, HCN and siderophores than *T. harzianum*. Other researchers also reported production of similar functional traits by different *Trichoderma* spp. that reduced pathogen infection, increased plant growth by producing bio-stimulant and hormone-like compounds and enhanced nutrient uptake (Lopez-Mondejar *et al.* 2010; Harman *et al.* 2012; Li *et al.* 2017; Jangir *et al.* 2019). Cell wall degrading enzymes such as proteases and chitinases and secondary metabolites produced by *Trichoderma* species have antifungal activities acting as elicitors of plant defense systems and thus aids in combating plant diseases (Nicolás *et al.* 2014; Vinale *et al.* 2014; Contreras-Cornejo *et al.* 2015; Al-Ani 2018; Khare *et al.* 2018; Deng *et al.* 2019). Cell walls of *Fusarium oxysporum* have mainly chitin and β -glucan polysaccharides (Schoffemeer *et al.* 1999) which attract *Trichoderma* spp. that parasitizes *F. oxysporum* f. sp. *lycopersici* through chitinase production (Li *et al.* 2017). Secretion of hydrolytic enzymes by pathogens becomes inactivated due to proteases produced by *Trichoderma* (Harman 2006). HCN produced by *Trichoderma* acts as a defense regulator and inhibitor against phytopathogens (Blumer and Haas 2000; Noori and Saud 2012) and thereby influences antibiosis. In support of our research, phosphate solubilizing ability and IAA and siderophore production by *Trichoderma*, promoting plant health and growth, has also been reported by several researchers (Antoun and Kloepper 2001; Ahemad and Kibret 2014; Paramanandham *et al.* 2017; Li *et al.* 2018). Siderophores chelate soil iron and thus hamper normal metabolic activities of the pathogens. (Chet and Inbar 1994; Eisendle *et al.* 2004). Their phosphate solubilizing ability enhances nutrient

uptake of plants (Rudresh *et al.* 2005; Saba *et al.* 2012). IAA (López-Bucio *et al.* 2015; Mishra *et al.* 2018) and siderophore production (Lacava *et al.* 2008) and phosphate solubilization (Otieno *et al.* 2015) by biocontrol agents increase plant growth parameters.

Field results showed a significant reduction in disease severity with an increase in fresh and dry weights, plant length and plant yield when *Trichoderma* strains were used against the wilt pathogen. The findings concur with Zaim *et al.* (2018) who investigated the effectiveness of *T. harzianum* and *B. subtilis* and found that these BCAs suppressed the activity of *F. oxysporum* f. sp. *ciceri* in chickpea. Similarly, Jamil *et al.* (2020) and Macías-Rodríguez *et al.* (2018) observed better plant growth due to *Trichoderma* in chilli and tomato plants, respectively.

Tomato plants treated with *T. viride* and *T. harzianum* showed improved net photosynthetic rates (P_N), stomatal conductance (g_s) and transpiration rates (E) when compared to the untreated control. Hasan *et al.* (2020) found that *Trichoderma* spp. increased the net photosynthetic rate (P_N), stomatal conductance (g_s) and transpiration rates (E) in cherry tomato plants in comparison to *FOL* infested plants. An increase in net photosynthetic rate was due to enhanced chlorophyll content which has a direct impact on plants' photosynthetic activity. This was in conformity with Harish *et al.* (2008) who also observed that the application of *Trichoderma* increased plant growth by enhancing leaf chlorophyll level leading to a higher degree of photosynthetic activity. Mei *et al.* (2019) also recorded stimulation of metabolism and activities of stress resistance enzymes with *T. pseudokoningii*. They observed that plant chlorophyll content is an important indicator of photosynthetic activity and found a significant increase in chlorophyll content, nitric nitrogen content, root activity, total root absorption area and root specific surface area in *T. pseudokoningii* treated plants. Khoshmanzar *et al.* (2019) recorded that *T. longibrachiatum* increased stomatal conductance and leaf water potential of tomato plants. This increase in stomatal conductance is due to the fact that *Trichoderma* reduces the abscisic acid concentration (Qi and Zhao 2013; Contreras-Cornejo *et al.* 2015). Chaves-Gómez *et al.* (2019) in their research observed that treatment with *Trichoderma koningiopsis*, *Trichoderma virens* and *Bacillus velezensis* improved stomatal conductance, leaf water potential, growth parameters, total chlorophyll, carotenoid, proline and malondialdehyde contents in cape gooseberry plants.

During the study, both *Trichoderma* strains increased biochemical and antioxidant compounds of tomato. However, *T. viride* was found to be superior in elevating the levels of these compounds. Increases in the levels of these biochemicals and antioxidants indicate induced host resistance (Woo *et al.* 2014). Sharma

et al. (2012) and Mona *et al.* (2017) observed improved total phenolic content in tomato plants inoculated with biocontrol agents including *Trichoderma* spp. Reduction in wilt severity in tomato plants treated with *Trichoderma* was primarily due to an increase in phenolic content. Larger amounts of phenolics act as free radical scavengers and protect the diseased plant by aiding in the formation of cell walls and other defense structures (Ahanger *et al.* 2014; Hashem *et al.* 2016). They have antimicrobial properties and are precursors of structural polymers such as lignin. Furthermore, they act as signal molecules for expression of defense related genes (Madhavan *et al.* 2011). In accordance to our results, an increment in growth and biomass accumulation in crop plants by *Trichoderma* was reported by Fang-Fang *et al.* (2017) and Luo *et al.* (2016). Mei *et al.* (2019) reported that several *Trichoderma* species (*T. asperellum*, *T. harzianum* and *T. pseudokoningii*) improved growth, yield and quality of cucumber plants having *F. oxysporum* infection. Sawant and Sawant (2010) stated that an increase in crop yield is due to growth regulators produced by *Trichoderma* spp. which help in enhancing biochemical metabolism in plants.

FOL affected the chlorophyll content of tomato leaves by reducing it to a significantly low amount. Our findings agree with previous studies in which *Trichoderma* treated plants showed chlorophyll enhancement (Kotasthane *et al.* 2015; Mona *et al.* 2017; Zehra *et al.* 2017; Jamil *et al.* 2020). Zhang *et al.* (2016) found that *T. harzianum* enhanced chlorophyll and phenols in *Sclerotinia sclerotiorum* infected soybean plants. This increase in chlorophyll is due to the strong effect of phytohormones (like IAA) produced by *Trichoderma* (Mona *et al.* 2017). Phenols also affect plant antioxidants as they directly improve antioxidant activity in pathogen infected plants by eliminating reactive oxygen species (ROS), thus protecting it from oxidative stress (Surekha *et al.* 2014; Ahmad *et al.* 2015). This corresponds with our findings where tomato plants treated with *T. viride* and *T. harzianum* exhibited increased levels of proline. Similarly, Zehra *et al.* (2017) reported elevated proline content and both Molla *et al.* (2012) and Hasan *et al.* (2020) found increased lycopene content in *FOL* infected tomato plants treated with *T. harzianum*. An increase in vitamin C in tomato was also reported by Li *et al.* (2017)

Conclusions

In conclusion, treatment with both *T. viride* and *T. harzianum* showed promising effects in lessening disease severity and enhancing plant growth and yield through production of pathogen degrading enzymes and improved physiological activity. However, these effects

were more pronounced when *T. viride* was used as seed treatment prior to sowing of tomato seeds. Therefore, *T. viride* may be potentially integrated with other management strategies to reduce losses caused by this disease. However, further studies need to be carried out to explore local strains of *T. viride* to validate their performance in suppressing the activity of *F. oxysporum* f. sp. *lycopersici* in naturally infested agricultural land.

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