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Mycoparasitic nature of Egyptian *Trichoderma* isolates and their impact on suppression Fusarium wilt of tomato

A. M. Nofal[1], Mohamed Abd El-Rahman[1], T. M. Abdelghany[2*] and Mahmoud Abd El-Mongy[3]

Abstract

Background: Plant disease administration is difficult due to the soil-borne nature of the phytopathogens. Biological control of plant disease is a safe mode to avoid the problems related to fungal diseases that affect crops productivity.

Results: Twenty-three *Trichoderma* isolates were isolated from soil, surrounding healthy tomato roots from different regions in the Egyptian Governorate of Menoufia. Using a dual culture method to test the efficiency of *Trichoderma* isolates, the most effective isolate identified as *Trichoderma atrovirde* with percentage inhibition against *Fusarium oxysporum* f. sp. lycopersici (92.11%) and scanning electron microscope examination documented the mycoparasitic nature of *T. atrovirde* to *F. oxysporum*. Treatments with 10% filtrate *T. atrovirde* improved the growth aspects of tomato plants than the control plants or infected only, as well as the increase in phenol content (15.09 ug. g^{-1}dry weight) and decreased disease incidence percentage (8%) than the plants infected only (60%).

Conclusions: This study clearly demonstrated that *T. atrovirde* had a significant inhibition against *F. oxysporum*. Greenhouse assays displayed the protective role of *T. atrovirde* inoculation directly against pathogen or indirectly related to the defense mechanism in the plant. So, this study recommends using *T. atrovirde* for biological control of wilt disease in tomato plants.

Keywords: *Fusarium oxysporum*, *Trichoderma* spp., Biological control, Mycoparasitism, Tomato

Background

Tomato (*Solanum lycopersicum*) is a very economic vegetable crop cultivated in various regions of the world. *Fusarium oxysporum* is the causative agent of Fusarium wilt which has proved to be the most destructive disease affecting a wide variety of plants including weeds and commercially domesticated plants and crops. The disease results in a range of symptoms ranging from yellowing of the leaves, browning of the vascular tissue, sluggish growth, and even death of plants (Renu 2018). *F. oxysporum* is essential and a variety of pathogenic plant fungus (Anjul et al. 2017). *Fusarium oxysporum* f. sp. lycopersici was reported as a causative agent of tomato wilt in Egypt (Abo-Elyousr and Mohamed 2009). Wilt is one of the world's most economically important diseases (Fang et al. 2020). The toxicity of chemical fungicides for the soil and polluted environment was confirmed previously; therefore, biological control of fungal disease is considered a practicable alternative and safe friendly manner (Saad et al. 2019).

Biological control is an important manner of control the disease for phytopathogenic fungi, particularly for soil-borne pathogens. In recent years, the most abused biocontrol agents are the species of *Trichoderma* (Shrinkhala et al. 2019). Soil-borne plant pathogens controlled by using *Trichoderma* have been reported for biological control (Mohamed et al. 2020). With the application of various species of *Trichoderma*, remarkable decreases in

* Correspondence: tabdelghany.201@azhar.edu.eg; tabdelghany@yahoo.com
[2]Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt
Full list of author information is available at the end of the article

Fusarium wilted in several crops were recorded (Ramezani 2009).

The goal of the present work was to evaluate the efficiency of 23 *Trichoderma* spp. isolates in bio-control of *F. oxysporum* under in vitro and greenhouse conditions.

Methods

In vitro experiments

Isolation, purification, and identification of Fusarium oxysporum

Diseased tomato plants were collected from different regions of the Governorate of Elmenofya (Shebin El Kom, Kafer Dawood, and Bader), Egypt, showing various degrees of wilt symptoms. Infected roots were washed thoroughly with flowing tap water and cut into small pieces before plunging into sodium hypochlorite (0.5% chlorine) for 1 min. The surfaces were washed by distilled water, dried between 2 sterilized filter sheets, placed directly in Petri dishes on potato dextrose agar (PDA) medium, and incubated at 28°C for 3 days. Analysis was carried out when the fungal growth originated from the incubated materials. All the isolated fungi were extracted using single spore, or hyphal tip techniques were suggested by Dhingra and Sinclair (1985). The fungi were identified according to their morphological characteristics according to Booth (1985) and confirmed in Mycology center (MC), Assuit University, Egypt. The stock of isolates was stored on PDA slants and kept at 5–10°C in a refrigerator for further study.

Pathogenicity test of *F. oxysporum*

Pot and soil sterilization

Earthenware pots (20 cm in diameter) were sterilized by plunging them into a 5% formalin solution for 15 min and covered overnight with plastic sheets, then left to dry. Soil sterilization was carried out by autoclaving for 1 h at 121°C.

Nursery preparation

Susceptible marmand tomato cultivar obtained from the Research Institute of Horticulture, Agricultural Research Center (ARC), Giza, Egypt. Tomato seeds were sterilized by 1% sodium hypochlorite on the surface for 1 min, then washed in several changes of sterilized water, and left to dry. Sterilized seeds were sown on the surface in sterilized soil and irrigated with water for 30 days.

Inoculum preparation and soil infestation

Bottles containing the medium sand-barley were autoclaved and then the isolated fungus was inoculated after 2 weeks of incubation at 28°C. The fungus was thoroughly mixed with sterilized soil at a rate of 1gm inoculum/1 kg soil. Inoculated pots were watered regularly, 7 days before planting. The pots used as control were packed by a soil free-sand barley medium fungus (Shehata 2001).

Transferring of transplants and cultivation

Transplants were transferred to infested soil and planted under sterilized conditions. Five transplants were planted in sterilized un-infested soil. The wilting % after 30 days of transplant cultivation was recorded as follows:

$$\text{Wilting}\% = \frac{\text{Number of wilting plants}}{\text{Total number of plants}} \times 100 \quad (1)$$

Isolation of *Trichoderma* spp. from rhizospheric region

Rhizospheric soils were collected from different regions of Egypt. *Trichoderma* spp. were isolated from the rhizosphere soil samples by dilution plate technique, using PDA and Trichoderma selective medium (TSM). *Trichoderma* spp. were purified, using the hyphal tip technique (Tuite 1996). The isolated species (T1, T2, T3…T23) were identified dependent upon growth, mycelium structure, conidiophores, phialides, and conidia on colony characters.

Dual culture experiment

Antagonistic efficacy of *Trichoderma* spp. isolates was tested against the isolated pathogenic fungus by dual culture experiment (Dennis and Webster 1971). Disc of growth agar medium of *Trichoderma* was placed against component *F. oxysporum* and the 6-day incubation at 28±2°C monoculture plates served as controls of both, 7 days after incubated radial growth, measured for *F. oxysporum* and *Trichoderma* spp. The colony diameter of test fungus was observed and compared with control on a dual culture plate. The percentage of the inhibition of radial growth (% RGI) was determined using formula:

$$\text{RGI}\% = \frac{\text{C}-\text{T}}{\text{C}} \times 100 \quad (2)$$

where C is the growth of test pathogen with the absence of antagonist and T is the growth of test pathogen with of antagonist (Pandey and Vishwakarma 1998).

Identification of active Trichoderma isolates

The potent inhibitor of *Trichoderma* isolates against the tested phytopathogenic fungus was selected for identification up to colony character, growth, mycelium structure, conidiophores, phialides, and conidia (Kubicek and Harman 2002). Confirmed identification was done at MC.

Preparation of scanning electron microscopy (SEM)

Interaction among hyphae of pathogen *F. oxysporum* and bio-control agent *T. atroviride* was examined using

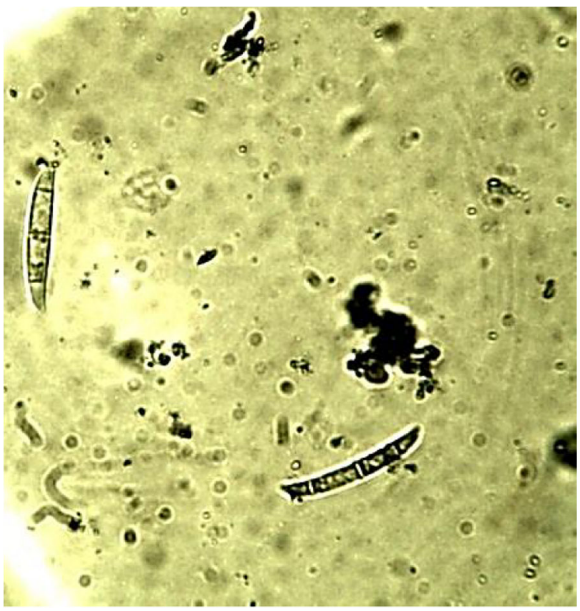

Fig. 1 Conidia of *F. oxysporum* (×400) light microscope

the scanner electron microscope (SEM). To obtain hyphae touch sites, PDA plate was inoculated with a mycelial disk (5 mm) cut from the front edge of the 2 at a constant distance from the edge of the Petri plate. From colonies *F. oxysporum* and *T. atroviride*, the 2 fungi grow up to each other, their hyphae mixed. After 4 days of incubation, the plate cultures were examined under a light microscope to check the early stage of touch. The contact sites were labeled, and blocks of 1 cm agar were removed for SEM preparation, were fixed with osmium oxide, and then were dehydrated using a serial dilution

Table 1 Pathogenicity test for different isolates of *F. oxysporum*

Isolate	Location	Total No. of plants	[a]Wilting	DI[a] (%)
F1	Shebin El kom	25	15	60
F2	Kafr dawood	25	12	48
F3	Bader	25	11	44

[a]Each value represents the mean values of 3 replicates

of the ethyl alcohol finally acetone. A critical point drier (EMS 850) was then used to dry the processed samples, coated with gold using a sputter coater (EMS 550), and then SEM (JEOL100CX-ASID-4D) was used to examine the samples at the Regional Center for Mycology and Biotechnology Center, Egypt.

In vivo experiments

Effect of T. atroviride on tomato plants infected with F. oxysporum

Planting, growth conditions, and treatments Susceptible tomato seeds (marmand) were sterilized on the surface for 1–3 min in 0.01% mercury chloride and washed with sterilized distilled water, then planted for 30 days in sterilized plastic pots containing sterilized soil before transplants were developed. The inoculum was prepared by introducing five discs of a 7-day-old culture of *F. oxysporum* grown on PDA medium into 500-ml size bottles containing sand-cornmeal medium (SCM), then incubated at 28°C for 14 days (Shehata 2001). The inoculum of *F. oxysporum* was added to sterilized pots (12 cm diameter × 20 cm height), containing 2.5kg sterilized soil at the rate of 10g inoculum/one kg soil, then watered whenever needed for 7 days. Healthy root systems of tomato transplants were soaked for 1–2 h

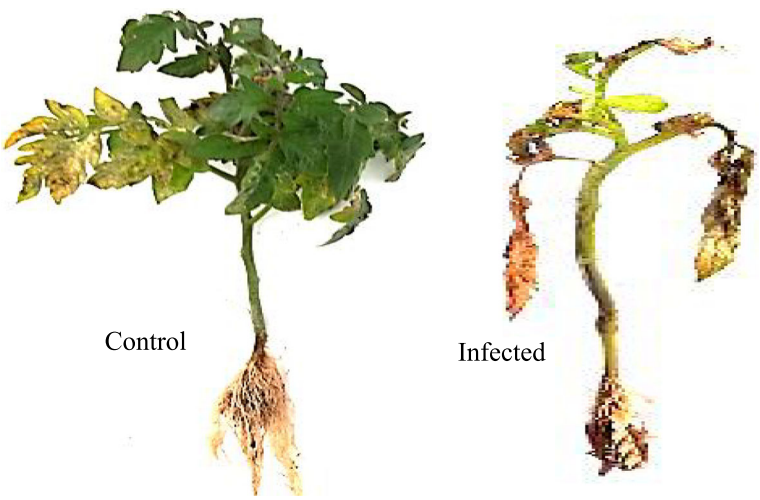

Fig. 2 Healthy tomato plants (control) and infected tomato plants (showed wilting symptoms)

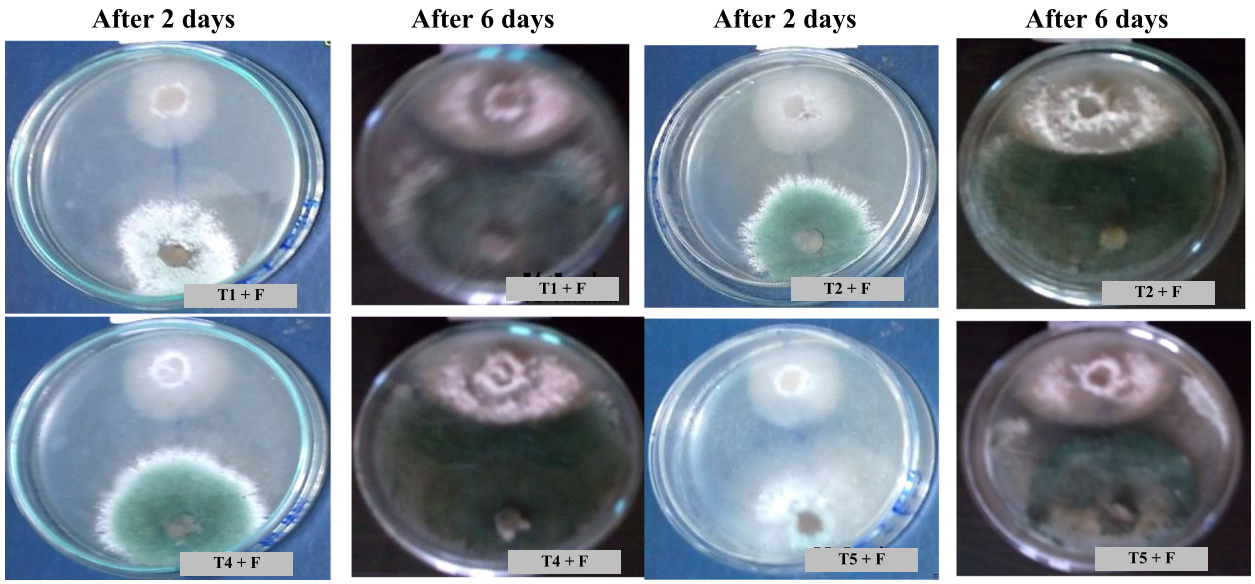

Fig. 3 Effect of *Trichoderma* antagonists on the mycelial growth of *F. oxysporum* (F) under in vitro conditions

in the 10% suspension of *T. atroviride*. Control roots were immersed in water only. Five pots, each containing 5 transplants, were used for each treatment. The fungicide Rizolex-T (Tolclofosm methyl+ Thiram) from Sumitomo Chemical Company Japan was used through this experiment for comparison at the recommended dosage (3 gm/l water) according to the Ministry of Agriculture, Egypt. The experimental design can be summarized as follows: C= (control), P= (pathogen), (*F. oxysporum*), P+F (pathogen + fungicide), and P+T (pathogen + *T.atroviride*). Data of this experiment were recorded 30 days after transferring. For each treatment, 3 plants were harvested and carefully washed by flowing water to dissolve debris from the soil. The following parameters were measured for the tomato plants: shoot length (cm), root length (cm), number of leaves, fresh and dry weight of shoot (gm), fresh and dry weight of root (gm), number of wilting plants, total number of plants, root surface area, and estimation of total phenol (ug. g^{-1} dry weight) and was performed in accordance with the method described by Folin Ciocalteau reagent (Malik and Singh 1980).

The disease incidence (DI) was measured as follows:

$$DI\% = \frac{\text{Number of wilting plants}}{\text{Total number of plants}} \times 100 \qquad (3)$$

Statistical analysis

All values were the sum of the triple determinations. Data was statistically analyzed using the SPSS (1999)

Table 2 Antagonists effect of *Trichoderma* isolates on the growth of *F. oxysporum* under in vitro conditions

Trichoderma isolates	*F. oxysporum*	
	[a]Growth (mm)	[a]Growth inhibition (%)
Control	85.0 a	0.00
T1	38.3bc	54.94
T2	6.7k	92.11
T3	35.7defc	58.00
T4	33.7efg	60.35
T5	39.7b	53.29
T6	37.3dbc	56.11
T7	35.3defc	58.47
T8	27.3kj	67.88
T9	35.5defc	58.23
T10	40.3b	52.58
T11	30.7ihg	63.88
T12	27.7ikj	67.41
T13	33.3efg	60.82
T14	30.0ihj	64.70
T15	35def	58.82
T16	32.7hfg	61.52
T17	30.0ihj	64.70
T18	34.0ef	60.00
T19	36.3dec	57.29
T20	30.7ihg	63.88
T21	28.3ikj	66.70
T22	32.7hfg	61.52
T23	37.3dbc	56.11
L. S. D.	3.03	

[a]Each value represents the mean values of 3 replicates. Means having the same alphabetical letter in column, within a comparable group of means, do not significantly differ, using Duncan's multiple range test procedure at p= 0.05 level of significance

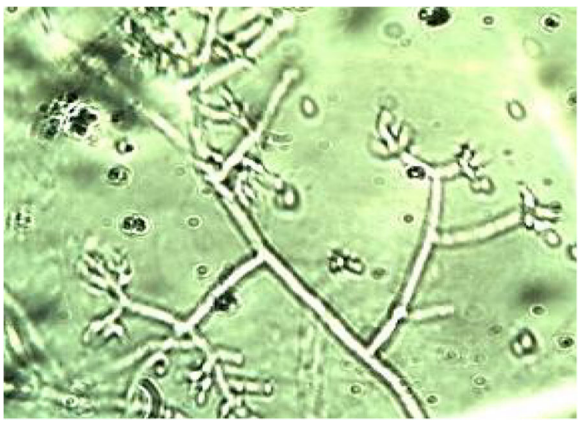

Fig. 4 *Trichoderma atroviride* Karsten (×400)

one-way variance analysis (ANOVA). The small difference was shortened as LSD and measured at $P \leq 0.05$.

Results

In vitro experiments

Phyto-pathological studies

Isolation, purification, and diagnosis of the causative agent of wilt disease The causative agent of wilt disease was isolated from diseased tomato roots collected from various regions of the governorate of Elmenofya (Shebin El Kom, Kafr Dawood, and Bader) and described according to the morphological and microscopic characteristics (mycelial production and spore formation). The isolate was identified as *F. oxysporum* (Fig. 1) and confirmed at MC as *F. oxysporum* 9704 AUMC.

Pathogenicity test of *F. oxysporum* All the isolates of *F. oxysporum* obtained from the infected tomato roots were examined for their pathogenicity on healthy susceptible tomato roots marmand to determine the most aggressive one. Control plants showed no symptoms, while the inoculated plants indicated that all isolates were pathogenic, resulting in typical wilt disease symptoms (Fig. 2) from isolates, Shebin El Kom (F1) isolate *F. oxysporum* reported the highest DI (60%) (Table 1). Therefore, this isolate was considered the most offensive and used for further research.

Table 3 Effect of *T. atroviride* on DI of tomato plants infected by *F. oxysporum*

Treatment	Total No. of plants	[a]Wilting	DI[a] (%)
Control	25	0	0d
F. oxysporum	25	15	60a
F. oxysporum + Rhizolex	25	2	8b
F. oxysporum + *T. atroviride*	25	2	8b
L. S. D.			2.71

[a]Each value represents the mean values of 3 replicates. Means having the same alphabetical letter in column, within a comparable group of means, do not significantly differ, using Duncan's multiple range test procedure at p= 0.05 level of significance

Isolation of *Trichoderma* isolates Obtained results reflected the growth of 23 isolates of *Trichoderma* from all collected rhizospheric soils. These soil samples from different regions of Egypt were considered as a source of *Trichoderma* isolates; therefore, the *Trichoderma* isolates were numbered (T1, T2, T3, …., T23) and applied for further studies.

Screening for antagonistic potential of *Trichoderma* isolates against *F. oxysporum* (dual culture experiments) On the PDA medium, the effectiveness of local *Trichoderma* isolates was determined to inhibit *F. oxysporum* myclial development in dual culture, and the inhibitory effect was remarkably at 6 days of incubation

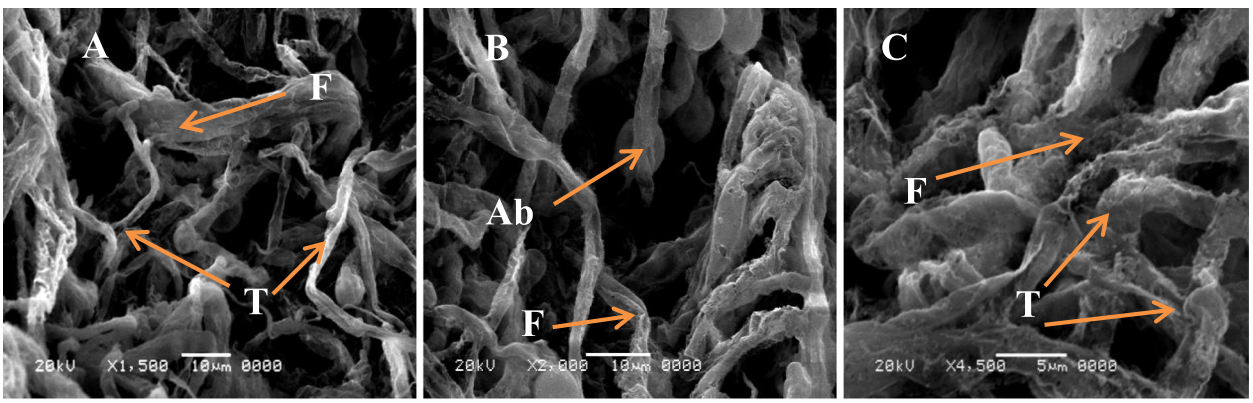

Fig. 5 Scanning electron microscopy observations of the mycoparasistic nature of *T. atroviride* on *F. oxysporum*. **A** Growing of *Trichoderma* hyphae (T) parallel to *Fusarium* hyphae (F) & coling of (T) around (F); **B** Sticking of of *Trichoderma* hyphae (T) with *Fusarium* hyphae (F) and formed of appressoria like structures, "Ab"; **C** Finally the *Fusarium* wall lysed

Table 4 Effect of *T. atroviride* on growth parameters of tomato plant infected by *F. oxysporum*

Treatment	[a]Growth parameters							
	RL (cm)	RFW (gm)	RDW (gm)	SL (cm)	SFW (gm)	SDW (gm)	Leaves NO	RSA (cm)
Control	14 b	3.87 ab	1.61 b	18 ab	12.83 ab	6.00 ab	9 ab	13.45 c
F. oxysporum	8 d	0.69 c	0.44 c	11 c	2.92 d	1.88 c	7 b	16.55 ab
F. oxysporum + Rhizolex	13 c	2.49 b	1.10 b	16 b	9.81 c	4.60 b	7 b	15.67 abc
F. oxysporum + *T. atroviride*	15 b	3.29 b	1.86 b	18 b	11.79 bc	6.10 ab	8 ab	15.09 cb
L. S. D.	3.27	1.45	0.84	3.50	2.74	2.33	2.71	3.15

[a]Each value represents the mean values of 3 replicates. Means having an alphabetical letter in column, within a comparable group of means, do not significantly differ, using Duncan's multiple range test procedure at p= 0.05 level of significance.
RL root length, *RFW* root fresh weight, *RDW* root dry weight, *SL* shoot length, *SFW* shoot fresh weight, *SDW* shoot dry weight, *RSA* root surface area

(Fig. 3). *F. oxysporum* radial development was substantially inhibited by *Trichoderma* isolates of different grades (Table 2 and Fig. 3). *Trichoderma* isolates were able to inhibit the mycelial growth of *F. oxysporum* by percentage inhibition (52.58–92.11%). The highest inhibition (92.11%) was recorded with isolate T2, followed by T8 (67.88%) and T12 (67.41%), while the lowest inhibition (52.58%) was with isolate T10. Therefore, the isolate T2, caused the highest antagonistic effect against tested phytopathogenic fungus, was selected for identification of its species level (Fig. 4). The identification was confirmed by MC (AUMC No.10639).

Scanning electron microscopy observations of the mycoparasistic nature of *T. atroviride* on *F. oxysporum* The mycoparasistic nature and events of *T. atroviride* on *F. oxysporum* as dual culture was examined by SEM (Fig. 5a–c). *T. atroviride* hyphae grew alongside hyphae of *F. oxysporum*, followed by quick and excessive coiling (plate a), and the formation on the surface of appressor-like structures on the surface of *F. oxysporum* hyphae (plate b). Then, *F. oxysporum* eventually lysis was observed (plate c).

In vivo effect of *T. atroviride* on tomato plants infected with *F. oxysporum*

Disease incidence

Obtained data represented in Table 3 showed that the DI was very high (60%) in tomato plants infected with *F. oxysporum* than the control plants. While treatment with *T. atroviride* decreased DI to 8%, the occurrence of the DI was substantially reduced by treating infected tomato plants, using *T. atroviride* alone or the Rhizolex fungicide.

Growth parameters

F. oxysporum infection of tomato plants significantly decreased all growth parameters (root length (8 cm), root fresh weight (0.69), root dry weight (0.44gm), shoot length (11 cm), and shoot fresh weight (2.92gm), while treatment with *T. atroviride* significantly enhanced the development of these parameters than the control plants (Table 4 and Fig. 6). The results showed that the plant infected only with *F. oxysporum* had a very low root surface area (0.5cm) as compared with the control (3cm), while sharply increment root surface area (5cm) was observed in the infected plant, which treated with *T. atroviride*.

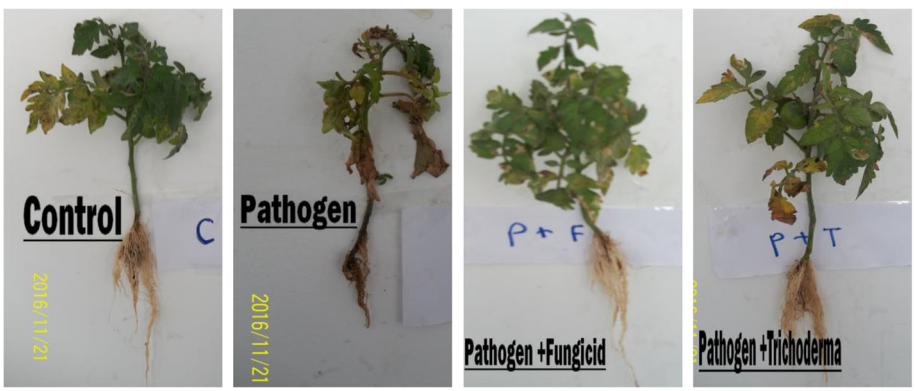

Fig. 6 Effect of *T. atroviride* on growth parameters of tomato plant infected with *F. oxysporum*

Discussion

Fungal diseases are the major problems associated with plant development. Biological control is a safe mode to avoid lethal chemical fungicides inhibiting pathogens. In dual in vitro research, antagonistic ability of *Trichoderma* isolates showed inhibitory effect on *F. oxysporum* growth, the causal pathogen of tomato wilt disease, ranging from 52.58 to 92.11%. The present study showed that *T. atroviride* was more successful in inhibition of *F. oxysporum* growth in vitro (Sallam et al. 2019). Dual culture approach was applied previously to determine the potential of *T. harzianum* to prevent the growth of *F. oxysporum* (Nwankiti and Gwa 2018). The antagonistic mechanisms of *Trichoderma* spp. against phytopathogenic fungi were described in numerous studies, for example, Shrinkhala et al. (2019) mentioned that *Trichoderma* spp. grew much faster leading to limit nutrients and space and therefore suppressed *F. oxysporum* which was responsible for the tomato wilting. SEM study on the antagonism between *T. atroviride* and *F. oxysporum* reported *T. atroviride* as mycoparasitic on *F. oxysporum*. Antagonism and increment of defensive mechanisms of plants are known to be mediated by a multiple of compounds produced by biocontrol agents, such as enzymes, toxic molecules, and volatile metabolites. The hydrolysis of the phytopathogenic fungi cell wall may take place by these enzymes. *Trichoderma* has been antagonized by antibiotics production against *F. oxysporum* and by mycoparasitism (El-Sobky et al. 2019).

Obtained results showed also that *T. atroviride* significantly reduced the DI of wilting on tomatoes with a reduction in disease ranging from 60 to 8% over time. Wilting in some leguminous crops caused by *Fusarium* spp. was suppressed by *Trichoderma* spp. and with the growth of the treatment plant root system (Raats 2012). *T. atrorviride* can be used to control a large number of soil-borne fungi *F.* solani and *Pythium* spp. (Ngo et al. 2006). In the present study, the plant height was also increased in plants treated with *T. atroviride*, and this may be due to suppress the infection, encouragement of plant resistance, high nutrient uptake, and promotion of plant development. Present findings were in accordance with (Rinu et al. 2014) by applying *T. gamsi* against numerous plant pathogenic fungi including *Fusarium oxysporum*, *F. solani*, *F. pallidoroseum*, *Alternaria alternate*, *Pythium afertile*, and *Phomopsis archeri.*

Conclusions

The present results revealed that the *T. atroviride* isolated from healthy tomato rhizosphere soil can be used effectively to manage tomato wilt disease. The study showed antagonistic potentials of *T. atroviride*, as a biological control agent against *F. oxysporum*, in vitro and under greenhouse experiment. *T. atroviride* could limit disease incidence and severity by inhibition of *F. oxysporum* growth through many mechanisms such as mycoparasitism, and antifungal metabolite synthesis.

Abbreviations

DI: Disease incidence; PDA: Potato dextrose agar; SEM: Scanner electron microscope; TSM: Trichoderma selective medium; P: Pathogen; F: Fungicide; T: *T. atroviride*

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Authors' contributions

NAM performed the experiments, analyzed and interpreted the data, and wrote the paper.
MAE performed some of the experiments, analyzed and interpreted the data. ATM conceived and designed the experiments, analyzed and interpreted the data, and wrote the paper. M A analyzed and interpreted some data, and wrote the paper. The authors have read and approved the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Sustainable Development Department, Environmental Studies and Research Institute, University of Sadat City, Sadat City, Egypt. [2]Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt. [3]Microbial Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City, Egypt.

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