Trop. plant pathol. (2016) 41:277–287
DOI 10.1007/s40858-016-0098-0

ORIGINAL ARTICLE

Trichoderma asperellum strains confer tomato protection and induce its defense-related genes against the Fusarium wilt pathogen

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Received: 8 December 2015 / Accepted: 19 July 2016 / Published online: 26 September 2016

Abstract Fusarium wilt of tomato (FW) caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL) is a major challenge for tomato production worldwide. For sustainable management of FW, the potential of five strains of *Trichoderma asperellum* was evaluated under greenhouse conditions. The results indicated that FOL infected plants treated with *T. asperellum* strains significantly reduced disease incidence and severity compared with FOL-only infected plants. The reduction of wilt disease on plants treated with *T. asperellum* strains was accompanied by a significant reduction in FOL populations in tomato stems and rhizosphere. Moreover, the application of *T. asperellum* promoted tomato plant growth irrespective of the presence or absence of FOL. Two strains of *T. asperellum* (TS-12 and TS-39) that showed the best performance in minimizing disease development and increases in plant growth parameters were selected for elucidating their ability in triggering tomato defense mechanisms. The expression levels of defense-related genes, chitinase (*SlChi3*), β-1,3-glucanase (*SlGluA*) and PR-1 (*SlPR-1a*) were significantly increased in the stems and roots of *Trichoderma* treated, FOL infected plants, compared with FOL-only infected ones. These results indicate that the application of *T. asperellum* strains TS-12 and TS-39 can be used as an alternative strategy to manage FW through their antagonistic activities and abilities to induce systemic resistance.

Keywords *Solanum lycopersicon* · *Trichoderma asperellum* · Defense genes · Induced resistance · Quantitative real-time PCR

Section Editor: Trazilbo J. de Paula Jr.

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Introduction

Fusarium oxysporum f. sp. *lycopersici* (Sacc.) W. C. Snyder & H. N. Hans. (FOL) is a soil-borne fungal plant pathogen that causes vascular wilt of tomato (*Solanum lycopersicon* L.). This systemic disease occurs worldwide (including Saudi Arabia) and causes severe tomato yield losses in both greenhouses and open fields (Abu Yaman and Abu Blan 1972; Jones et al. 1991; Larkin and Fravel 1998). The pathogen survives in the soil for long periods of time through the production of chlamydospores (Beckman 1987). There are many management strategies for tomato Fusarium wilt (FW), including the use of resistant cultivars, soil fumigation, and fungicides. However, while the use of Fusarium-resistant tomato cultivars provides some degree of control, the onset of new strains of the pathogen that overcome host resistance is a challenge (Larkin and Fravel 1998). Although chemical control is available for tomato FW, there is a great concern over the rise of new fungicide-resistant pathogen strains (Dekker 1981). Additionally, there is considerable concern regarding the risk of non-target effects of pesticides and the damage caused to the environment. For these reasons, alternative strategies for managing FW are needed.

The use of biological control agents that interfere with plant pathogens is an ecological and sustainable alternative disease management strategy to overcome the problems caused by other control strategies (Chet and Inbar 1994; Papavizas 1985). Species of the genus *Trichoderma* (Ascomycota, Hypocreales) are promising fungal agents for the biological control of many plant diseases, including tomato FW (Harman 2000, 2004, Harman et al. 2004; Kubicek et al. 2001; Papavizas 1985). These soil-borne, free-living fungi are non-pathogenic, ubiquitous, relatively easy to isolate, and grow quickly in the soil and plant rhizosphere. The beneficial effects of *Trichoderma* are based on a complex of different mechanisms, including direct mycoparasitism, competition for nutrients and space, and the production of antibiotic and volatile metabolites (Benítez et al. 2004; Howell 2003; Kubicek et al. 2001). Additionally, *Trichoderma* strains stimulate plant growth and induce systemic resistance in plants (Benítez et al. 2004; Howell 2003).

While numerous *Trichoderma* strains have been reported to be successful antagonists at the laboratory experimental level, few of them have been shown to be effective against plant pathogens under greenhouse and field conditions (Avis et al. 2001; Larkin and Fravel 1998; Lorito and Woo 2015; Meyer and Roberts 2002; Nicolás et al. 2014). Understanding the genetic basis of plant responses to inoculation with these biocontrol agents is essential for developing efficient management strategies against target pathogens (Mukherjee et al. 2012).

In this study, the potential use of five strains of *T. asperellum* (Samuels Lieckf. & Nirenberg) previously selected on the basis of their in vitro antagonism against FOL strains (El_Komy et al. 2015) was evaluated to manage tomato FW disease under greenhouse conditions. Subsequently, the two most efficient strains were investigated at the molecular level to elucidate their function in triggering tomato defense reactions.

Materials and methods

Tomato plants

Tomato seeds (*S. lycopersicon* cv. Farah, susceptible to FOL, a common greenhouse cultivar in Saudi Arabia) were surface sterilized for 30 s in 1 % sodium hypochlorite and then rinsed three times with sterile distilled water (Khan et al. 2004). The surface-sterilized seeds were pre-germinated for 3 days in Petri dishes containing sterile distilled water at 28°C. The germinated seeds were planted in 9-cm pots containing autoclaved potting mixture of soil, peat moss and perlite (2:1:1, v/v/v). Plants were grown in a growth chamber with a 16 h day (24°C) and 8 h night (20 °C) cycle at 70 % relative humidity. The seedlings were irrigated as needed and fertilized twice a week with a 20-20-20 (N-P-K) soluble fertilizer (1 g/L). Subsequent experiments were performed on 3-week-old tomato plants carrying three to five fully expanded leaves (Benhamou and Bélanger 1998).

Fungal strains and inoculum preparation

One aggressive strain of *F. oxysporum* f.sp. *lycopersici* (FOL-04) isolated in 2010 from wilted tomato plants grown under commercial greenhouse conditions in Riyadh, Saudi Arabia was used in this study. The single-spored FOL-04 strain was identified morphologically and microscopically according to the criteria of Leslie and Summerell (2006) (El_Komy et al. 2015). To confirm the identity of the FOL-04 strain, the internal transcribed spacers ITS-1 and ITS-2 of nuclear rDNA (ITS-rDNA) and a fragment of the translation elongation 1 alpha factor gene (*TEF-1*α) were PCR-amplified and sequenced. The obtained DNA sequences were searched against the GenBank database using BLAST. Koch's postulates were fulfilled for the FOL-04 strain through pathogenicity experiments using tomato cv. Farah, and the pathogen was re-isolated from the diseased tissues.

Five strains of *T. asperellum* were used (TS-9, TS-12, TS-36, TS-39 and TS-42) to assess their biocontrol efficacy against the FOL-04 strain. These isolates were recovered from soil samples of agricultural fields in the Riyadh region, Saudi Arabia, from 2009 to 2010 using dilution plate methods on *Trichoderma* selective medium (TSM) (Elad and Chet 1983). *Trichoderma* strains were identified on the basis of their cultural, morphological and microscopic characteristics (Gams and Bissett 1998; Rifai 1969). Molecular identification of these strains was performed by adapting the same techniques used for FOL-04 identification (data not published). The antagonistic potential of *T. asperellum* strains against FOL was previously demonstrated by El_Komy et al. (2015). For long-term preservation, fungal strains were maintained in 15 % glycerol and frozen at -80 °C.

Conidial suspensions were prepared from 7-day-old cultures grown on PDA at 28 °C. The cultures were carefully scraped with a sterile glass rod without disturbing the agar to dislodge spores into sterile distilled water. After filtering the resulting spore suspensions through two layers of sterile muslin cloth, the concentrations of the conidial suspensions were adjusted to 1×10^7 conidia/mL using a counting chamber (Webson Lancing) (Miranda et al. 2006). The quantity of the inoculum to be used was calculated to achieve a final concentration of 10^3 conidia/g soil for FOL-04 and 10^6 conidia/g of soil for the *T. asperellum* strains (Barakat and Al-Masri 2009).

Greenhouse evaluation of antagonists against vascular wilt

Pot experiments were performed in the greenhouse of the Plant Protection Department, College of Food and Agricultural Sciences, King Saud University in a completely randomized design to evaluate the effect of the *T. asperellum* strains against FOL-04. Plastic pots (16-cm in diameter) were filled with non-autoclaved sandy clay soil (1:1 v/v). The soil was infested with the pathogen inoculum (10^3 conidia/g soil) and left for one week for pathogen establishment. Then, the inoculum of the antagonistic fungi was applied (10^6 conidia/g of soil) in the FOL-04 infested soil and left for one more week for *Trichoderma* establishment. Seedlings were transplanted, one per pot and 20 replicates (pots) were used for each treatment. The treatments were: (a) healthy control (no pathogen); (b) soil infested with FOL-04; (c) soil infested with *T. asperellum* strains; and (d) soil infested with both FOL-04 and *T. asperellum* strains.

Wilt disease assessment

Disease incidence (%DI) was recorded at 7-day intervals using the formula: DI = (number of diseased plants/total number of plants) × 100. Disease severity based on foliar symptoms was scored at the end of the experiments using the following scoring system: 1, no infection (healthy plant and all leaves were green); 2, slight infection at approximately 25 % of full scale infection (one or two yellow leaves); 3, moderate infection (three or more yellow leaves); 4, extensive infection (dead lower leaves, some wilted upper leaves and growth inhibition); and 5, complete infection (100 % of leaves wilted and plant death) (Hibar et al. 2006). The basal stems were cut to evaluate vascular browning, which was rated based on the following scale: 0, healthy (no vascular discoloration); 1, ≤ 33 % (midpoint = 16.5 %); 2, ≥33–67 % (midpoint 50 %); and 3, ≥67–100 % (midpoint = 83.5 %) (Horinouchi et al. 2008). Disease scores were converted to disease severity (DS) using the following formula: DS = [(A × 1) + (B × 2) + (C × 3) + (D × 4) + (E × 5)]/(total number of plants) × 100, where A, B, C, D and E are the number of plants corresponding to scores of 1, 2, 3, 4 and 5, respectively.

Plant growth measurements

At the end of the experiments, three plant growth measurements were determined: dry weight (gm) and length (cm) of both root and shoot systems, and root volume (cm^3).

Monitoring of FOL-04 in tomato stems

FOL-04 populations in tomato stems were estimated at 15, 30 and 45 days after transplantation (dat) to the pathogen-infested soil. Four plants per time interval per treatment were randomly selected. The stem segments (10-cm long) were weighed and washed separately with sterile water and then homogenized in sterile distilled water (1:10 w/v) using a blender at 8000 rpm for 10 min. Two-hundred microliter aliquots of serial dilutions were spread evenly onto six replicate Petri dishes of Komada's selective medium (Komada 1975) per time interval. The plates were incubated at 23°C for 7 days and examined for the presence of *Fusarium* colonies. The number of colony-forming units (cfu) of FOL-04 per gram fresh weight of the stems was recorded.

Monitoring FOL-04 and *T. asperellum* strains in the tomato rhizosphere

Rhizosphere populations of FOL-04 and *T. asperellum* strains were estimated in pathogen-infested soil at 15, 30 and 45 dat. One gram of the soil sample (four replicates per time interval for each treatment) was suspended in 9 ml of sterile distilled water. The soil suspension was shaken for 1 minute on a vortex, and dilutions and cultures were prepared as described above, using Komada's selective medium for *Fusarium* and *Trichoderma* selective medium for *T. asperellum* as described by Elad and Chet (1983).

Systemic effects in split root experiments

The two most promising *T. asperellum* strains based on the greenhouse experiments (T-12 and T-39) were selected to follow-up their ability to induce resistance against FW disease in the split root system. Five-week old seedlings grown on sterilized vermiculite were carefully removed from the substrate. The root system was gently divided into approximately equal portions and transplanted as split root plants into 400-ml pots filled with an autoclaved mixture of potting soil and sand (Khan et al. 2004; Zang et al. 1996). Six days after transplantation, the pot on one side of all of the split root seedlings was inoculated with the *Trichoderma* strains at a final density of 10^6 conidia/g of soil. Four days later, the other part was inoculated with the pathogen at a final density of 10^6 conidia/g of soil. Control plants were treated with distilled water. The plants were kept at 24°C, 70 % relative humidity and a 16 hour photoperiod and watered twice a week with sterilized tap water. Disease severity was determined as described above. Root materials from three plants from each treatment were sampled at 2, 3, 6, 10, 15, 17 and 21 days after inoculation (dai). The sampled roots were washed, briefly dried, immediately frozen in liquid nitrogen, stored separately at -80°C and analyzed separately to represent three biological replications.

RNA extraction and cDNA synthesis

Root tissues were ground with a mortar and pestle in liquid nitrogen, and total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Extracted RNA previously treated with RNase-free DNase was examined using agarose gel electrophoresis and quantified spectrophotometrically using the 260/280 nm OD ratio (Jenway spectrophotometer, Bibby Scientific Limited). The RNA concentration was adjusted to 50 ng/μL. Subsequently, cDNA was synthesized using oligo-dT primers with the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs) according to the manufacturer's protocol.

Quantitative PCR (qPCR)

The expression profiles of pathogenesis-related genes, chitinase (*SlChi3*), β-1,3-glucanase (*SlGluA*) and PR-1 (*SlPR-1a*), were evaluated using quantitative real-time PCR (qPCR). The forward and reverse primers for the tested genes are shown in Table 1 (Aimé et al. 2008). The qPCR reaction mixture was composed of 25 ng of cDNA, 1 μL of each primer (10 μM), 10 μL of SYBR green master mix (Quanti Tech SYBR Green kit, Qiagen) and RNase-free water in a final volume of 20 μL. RNase-free water was used as the negative control. Quantitative real-time PCR was performed using the GeneAmp 7900HT Sequence Detection System (Applied Biosystems) with the following program conditions: 15 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 30 s at 58°C and extension for 30 s at 72°C. The qPCR reactions were performed twice. The actin gene was used as a reference gene, and the relative expression of gene of interest was calculated according to the method of Pfaffi (2001).

Data analysis

The experiments were arranged in a completely randomized design, and the statistical analyses of all experiments were performed using the SAS software system (SAS Institute Inc., 2003). All experiments were conducted twice. Levene's test was used to check the data for homogeneity of variance and indicated that the data of the two replications could be combined for the final analysis. Data expressed as percentages were subjected to arcsine transformation. Fungal cfu counts were transformed using square-root [sqrt $(x+0.5)$] prior to analysis to obtain homogeneity of variance. Analyses of variance (ANOVA) were performed for all data sets. The least significant difference (LSD) at $P<0.05$ was applied to detect differences among different treatments (Gomez and Gomez 1984). For polynomial regression analyses, IBM SPSS software was used (version 22; IBM SPSS Statistics, IBM Corp., USA).

Table 1 Primers used in real-time quantitative RT-PCR analyses of tomato defense-related genes

Primer	Sequence	Target gene
Actin-f	5′-AGGCACACAGGTGTTATGGT-3′	*Actin*
Actin-r	5′-AGCAACTCGAAGCTCATTGT-3′	*Actin*
LECHI3-F	5′-TGCAGGAACATTCACTGGAG-3′	*SlChi3*
LECHI3-R	5′-TAACGTTGTGGCATGATGGT-3′	*SlChi3*
LEGLUCA-F	5′-GGTCTCAACCGCGACATATT-3′	*SlGluA*
LEGLUCA-R	5′-CACAAGGGCATCGAAAAGAT-3′	*SlGluA*
LEPR1A-F	5′-TCTTGTGAGGCCCAAAATTC-3′	*SlPR-1a*
LEPR1A-R	5′-ATAGTCTGGCCTCTCGGACA-3′	*SlPR-1a*

Results

Incidence and severity of Fusarium wilt disease

The results presented in Table 2 indicate that the application of *T. asperellum* strains significantly decreased the disease incidence caused by the wilt pathogen compared with the infected control plants under greenhouse conditions. Moreover, *T. asperellum* strains decreased the internal stem discoloration and severity of foliar symptoms on tomato plants. Inoculation with FOL-04 only resulted in a significant reduction in the length of the root and fewer root tips compared to plants treated with the *T. asperellum* strains with or without pathogen inoculation.

The highest reductions in disease incidence, severe foliar symptoms and internal stem discoloration were gained with TS-12 (39.2, 47 % and 40.7, respectively) (Table 2). The next most effective biocontrol strain was TS-39 (29.6, 45 % and 37.3, respectively) followed by TS-42 strain (24.2, 35 % and 16.8, respectively). The lowest reductions were obtained with TS-9 and TS-36 strains (Table 2). These results indicated that treatment of tomato plants with the *T. asperellum* strains led to reduction in disease severity compared to the control plants. Moreover, TS-12 and TS-39 strains can be used as effective biocontrol agents to reduce the impact of Fusarium wilt on tomatoes.

Plant growth measurements

The application of *T. asperellum* strains to healthy tomato plants significantly increased plant growth in terms of root and shoot dry weight, height, root length and root volume relative to the healthy untreated control (Table 3). In pathogen infested soil, tomato plants treated with T-12 and T-39 had the highest increase in root/shoot dry weight (64.5/58.3 and 68.2/43.6 %, respectively) compared to the infected control plants.

Table 2 Efficacy of different *Trichoderma asperellum* strains on wilt incidence, foliar symptoms and discoloration severity caused by *Fusarium oxysporum* f.sp. *lycopersici* (FOL) strain FOL-04 on tomato plants under greenhouse condition

Treatments	Wilt incidence (%)		Foliar symptoms (%)		Discoloration severity (%)	
	Mean*	Protection (%)**	Mean	Protection (%)	Mean	Protection (%)
FOL-04	87.2 (69.5) a	–	71.3 (57.6) a	–	60.0 (50.8) a	–
FOL-04 + TS-9	74.8 (59.9) b	14.2	62.6 (52.3) b	12.2	48.7 (44.2) b	18.8
FOL-04 + TS-12	53.9 (47.2) e	39.2	42.3 (40.6) e	40.7	31.8 (34.3) d	47.0
FOL-04 + TS-36	67.1 (55.1) c	23.0	59.6 (50.5) c	16.4	49.0 (44.4) b	18.3
FOL-04 + TS-39	61.4 (51.6) d	29.6	44.7 (41.9) d	37.3	33.0 (35.0) d	45.0
FOL-04 + TS-42	66.1 (54.4) cd	24.2	59.3 (50.3) c	16.8	39.0 (38.6) c	35.0

*Mean of ten replications with two plants per replication; numbers in parentheses are arcsine transformed values; analysis based only on the transformed data; mean values in each column followed by the same letter are not significantly different at $P < 0.05$ according to the LSD test.

**Calculated according to the following formula: [C – T/C] × 100, where C is the disease measurements of control plants and T is the disease measurements of plants treated with *Trichoderma* strains

Generally, there was significant plant growth enhancement for FOL-04 infected plants treated with *T. asperellum* strains compared with FOL-04-only infected (control) plants. The highest values of plant height, root length and root volume of FOL-04 infected plants treated with *Trichoderma* were obtained by T-12 and T-39 strains; 46.9, 48.0, 35.0 % and 35.6, 33.9, 26.9 %, respectively (Table 3). These results indicated that the application of *Trichoderma* strains had a positive impact on tomato plant growth parameters irrespective of the presence or absence of FOL-04 (Table 3).

Monitoring FOL-04 in tomato stems

Populations of FOL-04 were estimated in tomato stems at 15, 30 and 45 dat of seedlings into FOL infested soil. Figure 1 (panel A) shows the polynomial regression relationship between FOL-04 population densities (cfu/g fresh weight stem) in tomato stems and the three different sampling times after transplantation into pathogen-infested soil and treated with different *T. asperellum* strains. All FOL-04 infected tomato plants treated with biocontrol strains showed significantly

Table 3 Effect of different *Trichoderma asperellum* strains on the root and shoot lengths of tomato plants irrespective of the presence or absence of the Fusarium wilt pathogen under greenhouse conditions

Treatments	Root dry weight (g)		Shoot dry weight (g)		Root length (cm)		Shoot length (cm)		Root volume (cm^3)	
	Mean*	Differences ** (%)	Mean	Differences (%)	Mean	Differences (%)	Mean	Differences (%)	Mean	Differences (%)
Control	0.95 bc	–	4.43 c	–	7.60 bc	–	21.6 d	–	6.96 d	–
TS-9	1.00 b	+05.3	4.30 c	–02.9	9.25 a	21.7	23.9 c	10.7	8.35 b	19.9
TS-12	1.34 a	+ 39.0	6.24 a	+ 40.9	8.30 ab	09.2	28.2 a	30.6	9.77 a	40.4
TS-36	1.25 a	+ 31.6	5.50 b	+ 24.2	8.40 ab	10.5	26.4 b	22.2	7.54 c	8.30
TS-39	1.37 a	+ 44.2	5.50 b	+ 24.2	9.58 a	26.3	24.3 c	12.5	8.44 b	21.3
TS-42	1.22 a	+ 28.4	5.41 b	+ 22.1	8.65 ab	13.8	26.7 b	23.6	7.97 cb	14.5
FOL-04	0.48 f	–	1.95 f	–	4.60 f	–	11.8 g	–	4.19 h	–
FOL-04 + TS-9	0.68 de	**+ 41.7**	2.50 ef	**28.2**	5.04 f	**09.6**	14.5 f	**22.9**	4.86 g	**15.9**
FOL-04 + TS-12	0.79 cd	**+ 64.5**	3.28 d	**68.2**	6.76 cde	**46.9**	16.0 e	**35.6**	5.66 e	**35.0**
FOL-04 + TS-36	0.59 ef	**+ 22.9**	2.38 ef	**22.0**	5.30 ef	**15.2**	14.6 f	**23.7**	4.74 g	**13.1**
FOL-04 + TS-39	0.76 de	**+ 58.3**	2.80 de	**43.6**	6.81 cd	**48.0**	15.8 ef	**33.9**	5.32 ef	**26.9**
FOL-04 + TS-42	0.60 ef	**+ 25.0**	2.80 de	**43.6**	5.64 ef	**22.6**	15.4 ef	**30.5**	4.93 g	**17.7**

*Each value represents the mean of ten replications with two plants per replication; values in each column followed by the same letter are not significantly different at $P < 0.05$ according to the LSD test

**Effect of the different *Trichoderma* strains on tomato growth parameters irrespective of the absence (values in normal font) or the presence of the Fusarium wilt (FW) pathogen (values in bold font) compared with the healthy control or FW-only infected control

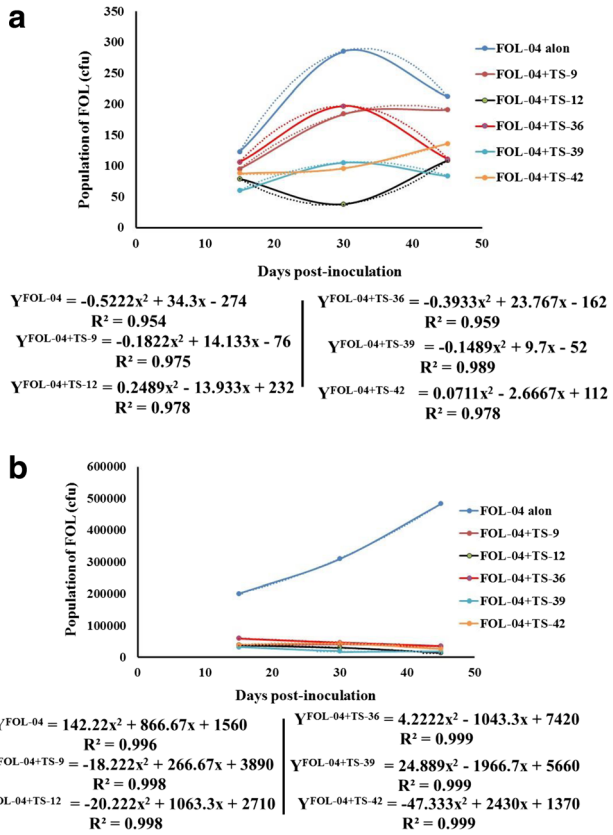

Fig. 1 Polynomial regression relationship between *Fusarium oxysporum* f.sp. *lycopersici* (FOL) strain FOL-04 population densities in either tomato stems (cfu/g fresh weight of stem) (**a**) or its rhizosphere soil (cfu/g fresh weight of soil) (**b**) and different time points after transplantation into pathogen-infested soil irrespective of the presence or absence of different *Trichoderma asperellum* strains under greenhouse conditions

lower FOL-04 population densities in the stems compared with FOL-only infected (control) plants (Table 4, Fig. 1a). The highest reduction in FOL-04 population was achieved in tomato stems treated with *T. asperellum* TS-12 strain, whereas the lowest FOL-04 population reduction was achieved by TS-9 strain (Table 4).

Monitoring FOL-04 and *Trichoderma* in tomato rhizosphere

The populations of *F. oxysporum* and *T. asperellum* (cfu/g rhizosphere soil) were evaluated in the treated soils at 15, 30 and 45 dat. Figure 1 (panel B) shows the polynomial regression relationships between FOL-04 population densities in tomato rhizosphere soil (cfu/g fresh weight soil) and the three different sampling times after transplantation into pathogen infested soil and treated with different *T. asperellum* strains. The FOL-04 population rapidly increased over time in the control treatment (Table 5, Fig. 1b). However, FOL-04 populations decreased in soils treated with *T. asperellum* strains compared to the soil treated with FOL only (Table 5, Fig. 1b). The average reductions in FOL-04 populations in tomato rhizosphere soil treated with TS-36, TS-9, TS-42, TS-12 and TS-39 were 85.8, 88.7, 88.9, 91.7 and 92.9 %, respectively (Table 5). Figure 2 shows the regression relationship between population densities of different *T. asperellum* strains in tomato plant rhizosphere soil and the three different sampling times after transplantation into pathogen infested soil. Generally, *Trichoderma* populations greatly increased over time in all treated soils (Table 6, Fig. 2). These results suggested that the reduction in disease incidence and development can be due to the reduction in FOL-04 population and the increase of *Trichoderma* populations.

Systemic effects in split root experiments

Based on the greenhouse evaluations, the *T. asperellum* strains T-12 and T-39 showed the best performance in minimizing wilt incidence and severity, in addition to remarkable increases in plant growth parameters. These strains were

Table 4 Effect of different *Trichoderma asperellum* strains on the reduction of population density of *Fusarium oxysporum* f.sp. *lycopersici* (FOL) strain FOL-04 in tomato stems (cfu/g fresh weight of stem) at different time points after transplantation into pathogen-infested soil

Treatments	Population of FOL-04 (cfu)				
	15 D[a]	30 D	45 D	Average	Control %
FOL-04 only	1.23×10^2 (11.1) cd	2.85×10^2 (16.9) a	2.12×10^2 (14.6) b	2.07×10^2	–
FOL-04 + TS-9	0.95×10^2 (9.77) efg	1.84×10^2 (13.6) b	1.91×10^2 (13.8) b	1.57×10^2	24.0
FOL-04 + TS-12	0.79×10^2 (8.94) gh	0.38×10^2 (6.20) i	1.09×10^2 (10.4) de	0.75×10^2	63.7
FOL-04 + TS-36	1.06×10^2 (10.2) def	1.97×10^2 (14.0) b	1.11×10^2 (10.5) cde	1.38×10^2	33.3
FOL-04 + TS-39	0.60×10^2 (7.79) h	1.05×10^2 (10.2) def	0.83×10^2 (9.13) fg	0.83×10^2	59.9
FOL-04 + TS-42	0.88×10^2 (9.36) efg	0.96×10^2 (9.82) efg	1.36×10^2 (11.7) c	1.07×10^2	48.3

[a] Data were recorded 15, 30 and 45 days after transplantation of tomato seedlings into pathogen-infested soil; data are the means of four replications per time point; numbers in parentheses are square-root transformed values; analysis based only on the transformed data; values followed by different letters indicate significant differences at $P < 0.05$ according to the LSD test

Table 5 Effect of different *Trichoderma asperellum* strains on the reduction of the population density of *Fusarium oxysporum* f.sp. *lycopersici* (FOL) strain FOL-04 in tomato plant rhizosphere soil (cfu/g fresh weight of soil) at different time points after transplantation into pathogen-infested soil

Treatments	Population of FOL-04 (cfu)				
	15 D[a]	30 D	45 D	Average	Control %
FOL-04 only	2.01×10^5 (1446.1) c	3.10×10^5 (1758.6) b	4.83×10^5 (2193.9) a	3.34×10^5	–
FOL-04 + TS-9	3.85×10^4 (196.2) d-g	4.08×10^4 (201.8) def	3.40×10^4 (184.4) d-g	3.78×10^4	88.7
FOL-04 + TS-12	3.88×10^4 (196.2) d-g	3.05×10^4 (174.5) e-h	1.40×10^4 (118.2) h	2.78×10^4	91.7
FOL-04 + TS-36	5.95×10^4 (243.9) d	4.67×10^4 (216.2) de	3.58×10^4 (189.1) d-g	4.73×10^4	85.8
FOL-04 + TS-39	3.27×10^4 (180.8) d-h	2.00×10^4 (141.2) fgh	1.85×10^4 (135.8) gh	2.37×10^4	92.9
FOL-04 + TS-42	3.95×10^4 (198.7) d-g	4.40×10^4 (209.7) de	2.72×10^4 (164.8) e-h	3.69×10^4	88.9

[a] Data were recorded 15, 30 and 45 days after transplantation of tomato seedlings into pathogen-infested soil; data are the means of four replications per time point; numbers in parentheses are square-root transformed values; analysis based only on the transformed data. Values followed by different letters indicate significant differences at $P < 0.05$ according to the LSD test

selected and screened for their ability to induce tomato systemic resistance against Fusarium wilt disease. The defense-related genes encoding chitinase (*SlChi3*), β-1,3-glucanase (*SlGluA*) and PR-1 (*SlPR-1a*) were studied. Expression levels of these genes were determined in tomato plants at seven time intervals between 2 and 21 dpi with FOL-04. The disease severity of the seedlings was also assessed.

As shown in Fig. 3, there was no accumulation of *SlChi3* transcripts in root and stem tissues of the control plants, whereas in plants inoculated with FOL-04 only, the *SlChi3* transcript increased gradually over time. However, in plants inoculated with FOL-04 and treated with *T. asperellum* strains T-12 and T-39, *SlChi3* expression was significantly higher compared to that of the plants inoculated with FOL-04 only, at all time points investigated (Fig. 3). For *SlGluA*, early expression was detected in FOL-04 inoculated plants and treated

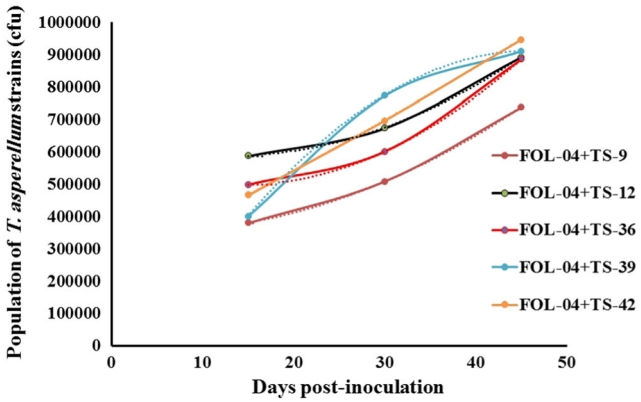

Fig. 2 Polynomial regression relationship between population densities of different *Trichoderma asperellum* strains in tomato plant rhizosphere soil (cfu/g fresh weight of soil) and different time points after transplantation into pathogen-infested soil under greenhouse conditions

with both the T-12 and T-39 strains in either the root (6 dpi) or stem (10 dpi) tissues (Fig. 4), and *SlGluA* expression levels continued to increase over time. In contrast, no *SlGluA* expression was detected in the control healthy plants at all sampling time points. However, the FOL-04-only inoculated plants showed a weak accumulation of *SlGluA* transcripts at 10 and 21 dpi (Fig. 4). The expression of *SlPR-1a* was observed only in root and stem tissues of FOL-04 inoculated tomato plants that were treated with *Trichoderma* strains (Fig. 5). No *SlPR-1a* expression was detected in either the healthy control or FOL-04-only inoculated plants at all sampling time points (Fig. 5). The disease severity of tomato plants grown in FOL-04 infested soil and treated with the *T. asperellum* strains TS-12 and TS-39 was 14.7 and 12.8 %, respectively, which was significantly lower than the plants grown in FOL-04 infested soil (24.6 %) (data not published). Consequently, the suppression of FW in tomatoes by the biocontrol strains appears to be accompanied by the enhanced expression of several defense-related genes.

Discussion

Biological control of the vascular wilt disease caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL), a major limiting factor of tomato production worldwide, has been considered as a suitable management strategy (De Cal et al. 1995; Larkin and Fravel 1998). In the present study, the antagonistic effectiveness of five *T. asperellum* strains (TS-9, TS-12, TS-36, TS-39 and TS-42) previously selected on the basis of in vitro antagonism against FOL strains (El_Komy et al. 2015) were evaluated for their protective effects under greenhouse conditions to manage tomato FW disease. The results showed that the application of *Trichoderma* strains significantly decreased the disease incidence caused by FOL-04 compared with control plants. The biocontrol strains also decreased internal stem discoloration and the severity of foliar

Table 6 Population density of different *Trichoderma asperellum* strains in tomato plant rhizosphere soil (cfu/g fresh weight soil) at different time points after transplantation into pathogen-infested soil

Treatments	Population of *T. asperellum* strains (cfu)			
	15 D[a]	30 D	45 D	Average
FOL-04 + TS-9	3.80×10^5 (615.8) h	5.07×10^5 (711.8) g	7.37×10^5 (858.5) cd	5.41×10^5
FOL-04 + TS-12	5.87×10^5 (766.2) f	6.72×10^5 (819.8) e	8.90×10^5 (943.1) ab	7.16×10^5
FOL-04 + TS-36	4.97×10^5 (705.2) g	6.00×10^5 (774.4) f	8.85×10^5 (940.7) b	6.60×10^5
FOL-04 + TS-39	4.00×10^5 (632.3) h	7.73×10^5 (878.8) c	9.10×10^5 (953) ab	6.94×10^5
FOL-04 + TS-42	4.65×10^5 (681.9) g	6.95×10^5 (833.5) de	9.45×10^5 (972.1) a	7.02×10^5

[a] Data were recorded 15, 30 and 45 days after transplantation of tomato seedlings into pathogen-infested soil; data are the means of four replications per time point; numbers in parentheses are square-root transformed values; analysis based only on the transformed data. Values followed by different letters indicate significant differences at $P < 0.05$ according to the LSD test

symptoms on tomato plants. Furthermore, the *Trichoderma* strains improved the performance of FOL-04 inoculated plants compared with plants inoculated with FOL-04 only, which showed significantly shorter root systems with fewer root tips. These results are in agreement with previous studies that demonstrated the efficacy of *Trichoderma* strains used as bio-fungicides to control FW and other soil-borne plant diseases (Barakat and Al-Masri 2009; Cotrarrera et al., Dubey et al. 2007; Hibar et al. 2006; Moreno and Cotes 2007; Osuinde et al. 2002).

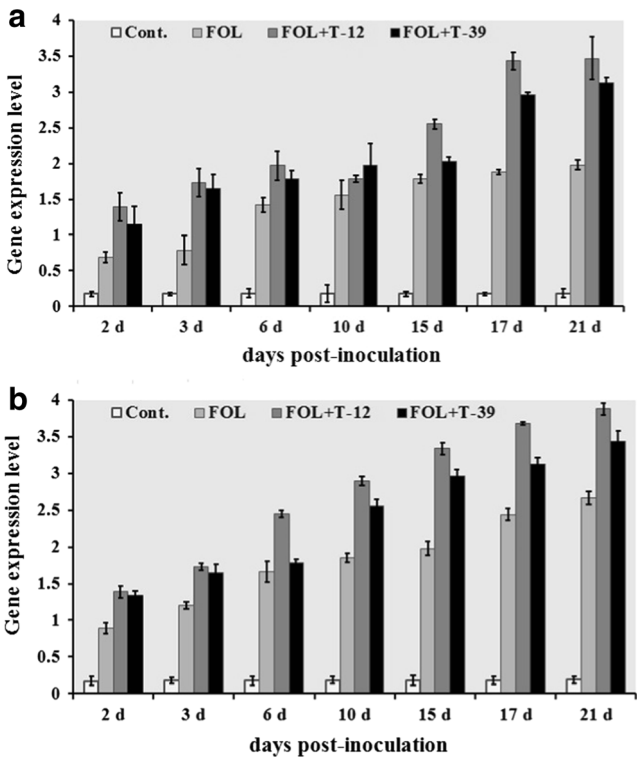

Fig. 3 Gene expression analysis of the defense-related gene encoding chitinase (*SlChi3*) in the stems (**a**) and roots (**b**) of tomato plants grown in soil treated with *Trichoderma asperellum* strains TS-12 and TS-39 at different time points after inoculation with the Fusarium wilt pathogen. Actin was used as the reference gene. *Each data point* represents the average for three independent biological replicates ± standard error

The mechanism of action of *Trichoderma* strains for disease control may be due to the promotion of plant growth and development (Avis et al. 2001; Harman et al. 2004, 2011, Harman 2011; Vinale et al. 2008). *Trichoderma* strains are considered as plant symbiont, opportunistic, avirulent organisms that are able to colonize plant roots by mechanisms similar to those of mycorrhizal fungi and produce compounds that stimulate growth and plant defense systems (Aleandri et al. 2015; Benítez et al. 2004; Harman 2006; Howell 2003; Huang et al. 2011; Mukherjee et al. 2012). The enhancement of plant growth might be associated with increased uptake of nutrients stimulated by growth-promoting factors such as IAA and GA3 and decreased level of ethylene owing to root colonization by *Trichoderma* (Benítez et al. 2004; Chen et al. 2007; Harman 2006, 2011; Gravel et al. 2007). The results obtained here show that the application of *Trichoderma* strains had a positive impact on plant growth in terms of root and shoot dry weight, height, root length and root volume irrespective of the presence or absence of the pathogen. Segarra et al. (2010) found that *T. asperellum* improved several plant physiological parameters, enhanced plant height and effectively managed tomato FW disease. Huang et al. (2011) reported that *T. harzianum* combined with bio-organic fertilizers could control *Rhizoctonia solani* damping-off disease in cucumber seedlings, mainly through mycoparasitism. Moreover, Zahoor et al. (2012) reported that Fusarium root rot of okra was effectively controlled of by *T. harzianum* and *T. viride* strains.

The strong capacity of *Trichoderma* strains to colonize the plant rhizosphere, mobilize and take up soil nutrients is usually part of a competition strategy involving antagonism, which explains their mode of action in controlling plant pathogens (Benítez et al. 2004; Harman et al. 2004; Hermosa et al. 2013; Lorito and Woo 2015; Vinale et al. 2008). In this study, tomato plants treated with the selected *Trichoderma* strains showed significantly lower FOL-04 population densities in the stems compared with the pathogen-only control. We also observed that the FOL-04 population rapidly increased over time in the tomato rhizosphere where no antagonistic

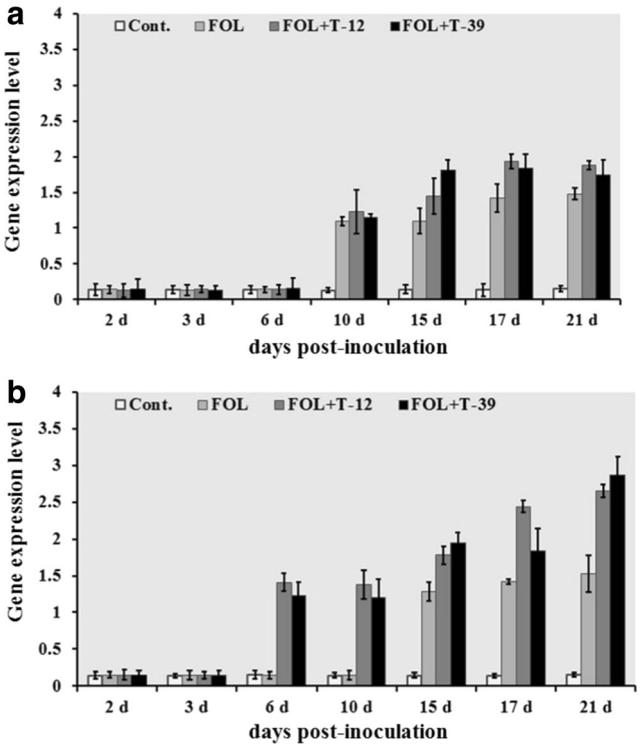

Fig. 4 Gene expression analysis of the defense-related gene encoding β-1,3-glucanase (*SlGluA*) in stems (**a**) and roots (**b**) of tomato plants grown in soil treated with *Trichoderma asperellum* strains TS-12 and TS-39 at different time points after inoculation with the Fusarium wilt pathogen. Actin was used as the reference gene. *Each data point* represents the average for three independent biological replicates ± standard error

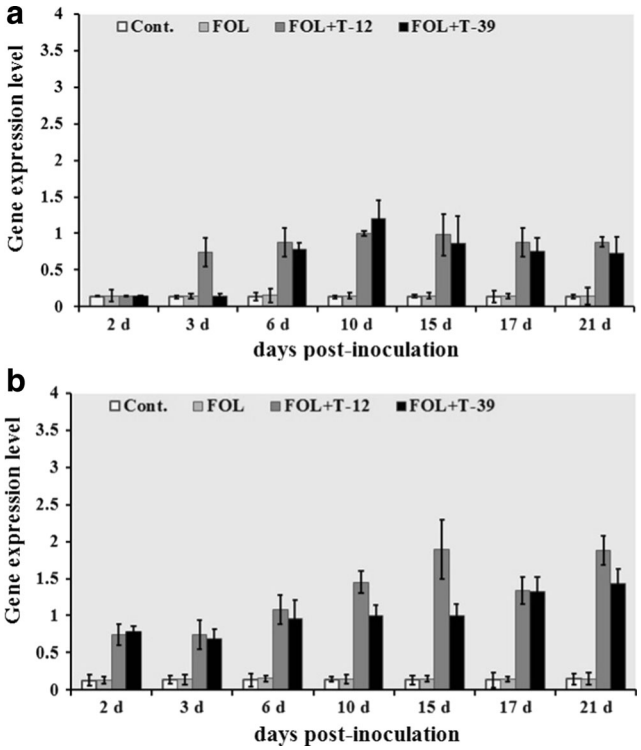

Fig. 5 Gene expression analysis of the defense-related gene encoding the PR1-protein (*SlPR-1a*) in the stems (**a**) and roots (**b**) of tomato plants grown in soil treated with *Trichoderma asperellum* strains TS-12 and TS-39 at different time points after inoculation with the Fusarium wilt pathogen. Actin was used as the reference gene. *Each data point* represents the average for three independent biological replicates ± standard error

organisms existed. However, FOL-04 population decreased in the soil treated with *Trichoderma* strains, whose populations greatly increased over time. These results showed that the reduction in disease incidence was accompanied by a reduction in the FOL-04 population and an increase in the *Trichoderma* population, probably due to competition for nutrients and space (Barakat et al. 2009).

Treating plants with biocontrol agents can provide systemic resistance against a broad spectrum of pathogens (Conrath et al. 2002; Kloepper et al. 2004; Pozo et al. 2005; Vinale et al. 2008). The induction of defense reactions was reported to be associated with increased lignification, stimulation of host-defense enzymes and synthesis of pathogenesis-related (PR) proteins (Hammerschmidt and Kuc 1995). PR proteins are produced in plants during pathogen invasion, and their synthesis and accumulation contribute to increased plant resistance against pathogen infection (Abdallah et al. 2010; Taheri et al. 2012). *Trichoderma* strains were reported to induce growth promotion and systemic resistance against many soil- and seed-borne foliar diseases of various vegetable crops, including tomatoes (Sid Ahmad et al. 2000; Kloepper et al. 2004; Papavizas 1985; De Palma et al. 2016; Sid Shoresh et al. 2005). We examined the ability of the *T. asperellum* strains TS-12 and TS-39 to induce defense responses in tomato plants. The results showed significant increases in the expression levels of *SlChi3*, *SlGluA* and *SlPR-1a* genes in both root and stem tissues of FOL-04 inoculated tomato plants and treated with *T. asperellum* strains compared with FOL-04-only controls. The expression of PR genes in *Trichoderma* treated, FOL-04 inoculated plants was induced earlier and stronger compared with FOL-04 only plants, resulting in high transcript accumulation. For instance, reduced disease development on the *Trichoderma* inoculated plants was detected compared with plants inoculated with the wilt pathogen alone. These results indicated that the biocontrol strains induced the expression of PR genes in tomatoes that increased their resistance to wilt pathogen infection. Our results are in agreement to those showing a good correlation between plant protection and increased levels of PR protein-related genes. For example, Shoresh et al. (2005) showed that the protective effect conferred by *T. harzianum* in cucumber plants against infection by the leaf pathogen *Pseudomonas syringae* pv. *lachrymans* was due to the activation of higher systemic expression of the *PR* genes encoding chitinase, β-1,3-glucanase and peroxidase. A study performed by Alizadeh et al. (2013) demonstrated that *T. harzianum* induced resistance in cucumbers against

F. oxysporum f. sp. *radicis* and significantly reduced the wilt severity via a mechanism associated with the primed expression of a set of defense-related genes. Similarly, Chowdappa et al. (2013) demonstrated that *T. harzianum* OTPB3 enhanced the levels of defense-related enzymes including peroxidase, polyphenol oxidase and superoxide dismutase in tomato plants.

Based on the present study, we conclude that the *T. asperellum* strains TS-12 and TS-39 can be used as effective biocontrol agents to control FW disease of tomatoes, offering an alternative strategy for disease management. Further studies are recommended to test the effectiveness of using the two *T. asperellum* strains either separately or in combination in open fields to develop long-term FW management strategies for tomato.

Acknowledgments This research was financially supported by the King Abdulaziz City for Science and Technology (KACST), Riyadh, Saudi Arabia.

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